Diversity of intraspecific patterns of brain region size covariation in fish

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Abstract

Traits often do not evolve in isolation or vary independently of other traits. Instead, they can be affected by covariation, both within and across species. However, the importance of within species trait covariation and, critically, the degree to which it varies between species has yet to be thoroughly studied. Brain morphology is a trait of great ecological and behavioral importance, with regions that are hypothesized to vary in size based on behavioral and cognitive demands. Sizes of brain regions have also been shown to covary with each other across various taxa. Here we test the degree to which covariation in brain region sizes within species has been conserved across ten teleost fish species. These ten species span five orders, allowing us to examine how phylogenetic proximity influences similarities in intraspecific trait covariation. Our results showed a trend that similar patterns of brain region size covariation occur in more closely related species. Interestingly, there were certain brain region pairs that showed similar levels of covariation across all species regardless of phylogenetic distance, such as the telencephalon and optic tectum, while others, such as the olfactory bulb and the hypothalamus, varied more independently. Ultimately, the patterns of brain region covariation shown here suggest that evolutionary mechanisms or constraints can act on specific brain regions independently, and that these constraints can change over evolutionary time.

Introduction

Trait Covariation

Trait variation is ubiquitous between and within species, providing the substrate on which natural selection can act. However, traits rarely vary independently and instead covary with other traits due to functional, genetic, and developmental links (Armbruster et al., 2014; Peiman & Robinson, 2017). These effects can limit the degree of trait change because linked traits either need to change together, or selection needs to be strong enough to break those links, as supported by theory (Jones et al., 2014; Pigliucci, 2003), simulations (McGlothlin & Ketterson, 2008), and empirical observations (Ungar & Hlusko, 2016). For example, Unger and Hlusko (2016) showed that covariation in tooth morphology traits in early hominid species led to the evolution of suboptimal tooth structure in these species, with teeth unable to evolve to optimally match the diet of individual species.

Covariation between traits can be studied at multiple scales, including broad evolutionary to within population ones (Atwell et al., 2014). However, the degree to which within species trait covariation may be conserved or vary across species remains understudied (Peiman & Robinson, 2017). Similar patterns of trait covariation across species would indicate that the level of interdependence of traits may be evolutionarily conserved, while different patterns of trait covariation would suggest that these links are evolutionarily flexible. An example of this can be seen in the integration of marsupial skull morphology. Goswami (2007) showed that marsupial skulls show a phenotypic integration of bone morphology within species, resulting in constraints on the phenotypes in skull morphology that could evolve in these mammals. This paper also showed that closely related

species showed more similar levels of integrated changes in skull morphology across phylogenetic distance. Constraints to trait covariation across species can limit the ability of individual species to evolve optimal phenotypes in their local environments. Therefore, understanding the degree of these constraints within and across species is critical to our ability to link form and function in the evolution of organisms.

Brain region variation

Brain morphology is one trait that has been of interest to biologists for centuries~~,~~ and is critical to animal performance across environments. It can influence both cognitive and behavioral abilities of animals and thus affects animal performance and survival across environments (Axelrod et al., 2022; Benson-Amram et al., 2016; Buechel et al., 2018; A. Kotrschal et al., 2013; MacLean et al., 2014; Yang et al., 2024~~ n.d~~.). Brains are divided into distinct regions, which are associated with specific cognitive, perceptual, and behavioral functions (Healy & Rowe, 2007; Huber et al., 1997; K. Kotrschal et al., 1998; Park & Bell, 2010; Pollen et al., 2007; Schumacher & Carlson, 2022; Striedter, 2005; Sukhum et al., 2018). Variation in the size of brain regions within and between species can be indicative of differential selection due to environmental variation and other factors (Axelrod et al., 2021; Gonzalez-Voyer & Kolm, 2010; Laberge & Hara, 2001). However, the sizes of different regions are not independent of each other and can covary, as individuals with larger brains tend to also have similar proportioned larger brain regions both within and across species (Striedter, 2005). Here, we examine the degree to which variation in brain region size covaries within fish species, and how these patterns may differ across species.

The degree of covariation in brain regions has been of interest to evolutionary biologists for decades. The "concerted model" of brain evolution posits that the evolution of brain regions is highly constrained, with evolutionary change in the size of brain regions resulting primarily from shifts in overall brain size (Finlay & Darlington, 1995; Yopak et al., 2010). These studies found that the proportional size of brain regions was highly predictable based on the size of the whole brain, suggesting that the evolution of brain region size does not vary independently and is likely constrained by brain region covariation. On the other hand, the "mosaic model" of brain evolution suggests that specific brain regions are targeted to change in size, independent of other structures, due to their association with certain behavioral or cognitive abilities (Barton & Harvey, 2000). Since the development of these two alternative models, the evolution of brain morphology has been studied as reflecting a degree of covariation between brain region size and whole brain size, suggesting that the two hypotheses are not mutually exclusive. At broad evolutionary scales, brain region scaling appears to be conserved, with occasional "mosaic" shifts occurring in some clades (Hoops et al., 2017; Striedter, 2005; Sukhum et al., 2018; Yopak et al., 2010). For example, the evolution of electrosensory abilities in fish is linked to a mosaic shift in the sizes of the cerebellum and hindbrain (Schumacher & Carlson, 2022). However, at the intraspecific level, evidence of this degree of brain region independence is less well established (Hager et al., 2012; A. Kotrschal et al., 2017; Noreikiene et al., 2015).

Approach and Hypotheses

Here, we test how much intraspecific brain region covariation is conserved across 10 teleost fish species. These 10 species are paired across 5 orders of fish, allowing us to test how evolutionary

proximity might impact the similarity of brain region covariation patterns. Similarities we find in brain region covariation patterns across species would indicate that covariation of brain region sizes is conserved across the teleost phylogeny. If the degree of brain region covariation is very flexible evolutionarily, we would expect covariation patterns to differ across species, and not be linked to evolutionary relatedness. Finally, a midpoint between these extremes could indicate that the degree of independence of within brain region variation can evolve, but more closely related species would be more likely to share these patterns of covariation. In this case we would expect species within orders to have more similar patterns of brain region covariation, with differences emerging at more distant phylogenetic comparisons.

Methods

Specimen collection

To compare patterns of brain region covariation, we collected data from 10 teleost fish species spanning 5 orders (Perciformes, Scorpaeniformes, Cyprinodontiformes, Cypriniformes, and Osteoglossiformes) (figure 1). We collected the data for the Trinidadian guppy (*Poecilia reticulata*), Western mosquitofish (*Gambusia affinis*), Creek chub (*Semotilus atromaculatus*), Blacknose dace (*Rhinichthys atratulus*), African butterfly fish (*Pantodon buchholzi*), and Black baby whale fish (*Brienomyrus brachyistius*). Of these, the guppy fish were lab reared descendants of wild collected fish from Trinidad. *G. affinis*, *S. atromaculatus*, and *R. atratulus*~~, and *S. atromaculatus*~~ were wild collected samples. *P. buchholzi* and *B. brachyistius* were sourced from a tropical fish supplier. We sourced data for the Pumpkinseed sunfish (*Lepomis gibbosus*), Bluegill sunfish (*Lepomis macrochirus*), Threespine stickleback (*Gasterosteus aculeatus*), and Ninespine stickleback (*Pungitius*

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pungitius) from previously published studies that employed the same data collection method. All species contain sufficient variation in body size to allow measure region covariation. The sources of data, sample size, and coefficients of variation in body size for each species can be seen in tTables 1 and 2. After euthanasia, all fish samples were fixed by being completely submerged in 10% formalin, and stored until processing.

Brain region size estimation

To measure brain region size, we estimated the volumes of five regions of the brain: cerebellum, optic tectum, telencephalon, olfactory bulb, and hypothalamus. For all species, we (or the authors of the relevant studies) extracted brains via dissection, ensuring that all regions were extracted intact and that external nerves were trimmed consistently. We then took photographs of the brains from dorsal, ventral, and lateral orientations. From these photographs, we measured the length (L), width (W), and height (H) of each brain region in ImageJ, and then calculated region volumes (V) using the ellipsoid formula ($V=(LxWxH)\pi/6$) (White & Brown, 2015) (supplementary figure 1). We estimated each region's length as a straight line from its most rostral plane to its most caudal plan. Its width was perpendicular, or 90 degrees, to the length, at the widest point of that ovoid. We estimated the height of each region by generating a straight line perpendicular to the horizontal axis of the brain, at the widest part of that lobe from the lateral view. To estimate total volume for regions with bilaterally symmetrical lobes, we summed both sides together. For example, estimating the telencephalon involved measurements of two bilaterally symmetrical lobes, and the volumes of these lobes were added together to obtain the total telencephalon volume. One exception was the olfactory bulb of *S. atromaculatus*, for which we could not reliably measure

177 olfactory bulb height. As such, we calculated the area of this region in this species (A=L/2xW/2x π).
178 These data are still usable as area and volume of brain regions are highly correlated (C.A. personal
179 observation).
180
181 Since the cerebellum of *B. brachyistius* drastically varies morphologically from other teleost fish
182 species (Sukhum et al., 2018), we had to deviate from the volume estimation methods used in the
183 other species. Seven ovoid regions were estimated to encompass the shape and size of the
184 enlarged *B. brachyistius* cerebellum (supplementary figure 2). Two heights, for both the rostral and
185 caudal ovoids, were taken for the *B. brachyistius* cerebellum, corresponding to its rostral and
186 caudal ovoid lengths and widths, respectively. The volumes of each of the seven cerebellum ovoids
187 were summed together to estimate total cerebellum volume.
188
189 *Brain region principal component variation*
190 To determine how brain region volumes vary within species and observe trends in their covariation,
191 we ran separate principal component analyses in each species. Percentage of variation explained
192 by PC1 correlated with total brain volume, which is expected in allometric relationships
193 (Klingenberg, 1996). These percentages were compared across species, but all regions in all species
194 loaded in the same direction along PC1. Additionally, the direction of where the brain regions
195 loaded along PC2 were qualitatively compared across species. PCAs were performed using the R
196 package "FactoMineR" (Lê et al., 2008), which generated PC1-5 for each species. The majority of
197 the variation was explained by PC1 and PC2 (ranging from ~79-98% across the 10 species), so we
198 focused on those two axes for comparisons across species.

Within species brain region correlations

To establish the level of independence of brain region variation within each species, we calculated Pearson correlations of each pairwise comparison of brain regions in each species. We use this approach because Pearson correlations allow for differences in scale between regions being compared, which is important as regions differ in size within species. This approach also allowed for the area of the olfactory bulb in *S. atromaculatus* to be effectively compared to the volume of the other regions in that species. From each pairwise correlation of brain regions, we can establish a separate correlation matrix for each species. Lower levels of correlation between brain regions indicate higher levels of independent variation.

Species comparisons of brain region correlation patterns

To compare the patterns of within species brain region correlation we estimated the similarity of species correlation matrices. We used a Mantel test (Vegan R package; Oksanen et al. 2020) to estimate the pairwise correlation of each species' correlation matrix. This test examines the correlation of two matrices using a Pearson correlation method, and then employs a permutation approach using 10,000 permutations to estimate the significance of that correlation. We chose this approach as our goal was to get a broad sense of the similarity of within species brain region correlation patterns and how these vary between species, rather than to establish specific mechanisms of brain region covariation. Understanding the mechanisms of how and why brain regions covary and how these differ among species would require additional data including genetic data or the use of selection or breeding experiments, though this was not the focus of this study.

Phylogenetic distance comparison

Finally, to test whether more closely related species have more similar patterns of brain region correlation, we compared phylogenetic distance between species to the similarity of their correlation matrices. We used the correlations estimated from the Mantel test comparing individual species to construct a species matrix including all pairwise species comparisons (figure 4). We then generated a phylogenetic distance matrix showing time (in millions of years) since the most recent common ancestor for each pairwise species comparison (supplementary figure 3). Time since most recent common ancestor for each species comparison was gathered from Timetree.org. We then compared these two species level matrices using the same Mantel test approach as we used to compare brain region correlation matrices. In this case, if more closely related species share more similar brain region correlation patterns, we expect a negative correlation between the species level matrices.

Results

Intraspecific brain region principal component variation

All brain regions covaried in the same direction along PC1, which reflected total brain volume (figure 2), but the percent variation explained by this axis varied across the 5 different fish orders (supplementary figure 4). The Cypriniformes had the highest amount of variation explained by PC1 (90.06% for *S. atromaculatus* and 95.03% for the *R. atratulus*), whereas the Scorpaeniformes and Osteoglossiformes had lower amounts of brain region variation explained by total brain volume

(~65-70% across the four species in these orders). The Perciformes and Cyprinodontiformes had more intermediate levels of variation explained by PC1 (~75-85%).

There was a noticeable difference in spread along the PC2 axis (for the five brain regions), indicating the differing strengths of independent contributions separate regions have on brain variation (figure 2). There was a split between species where the olfactory bulbs contributed disproportionately to most of the percent variation described by PC2, (~6-10% for Cyprinodontiformes, 14-20% for Scorpaeniformes, ~10-13% for Perciformes), and those where multiple regions contributed to the variation along PC2. These include the Cypriniformes, which had relatively little variation and spread explained by PC2 in any brain region (~2-4%), and the Osteoglossiformes, for which different brain regions contributed to varying degrees to PC2 in the two species studied (~13-14%). In *P. buchholzi*, the hypothalamus contributed the most to PC2, followed by telencephalon, optic tectum, olfactory bulb, and finally cerebellum. By contrast, the hypothalamus and olfactory bulb contributed the most to PC2 in *B. brachyistius*, followed by the optic tectum, cerebellum, and finally telencephalon. Interestingly, the cerebellum and optic tectum loaded in the same direction for all species except *B. brachyistius*.

Intraspecific brain region correlation

Across all species, brain region comparisons showed a positive correlation, though the strength of these correlations varied within and among species (figure 3). All these positive correlations were statistically significant, other than the olfactory bulb and cerebellum comparison, and the olfactory bulb and optic tectum comparison, in *P. pungitius*. To get a more general sense of within species

brain region independence, for every species we calculated the average correlation for each brain region with the other regions of the brain. ~~This revealed that species vary in their overall level of brain region covariation, with average correlations ranging from 0.56 in *G. aculeatus* and *P. pungitius* to 0.94 in *R. atratulus* (table 3). The Cypriniformes showed very high levels of region correlations, with all regions correlating with each other by at least 0.82 in *S. atromaculatus* and 0.88 in *R. atratulus*.~~ ~~Further, i~~In all fish species other than the two Osteoglossiform species (*P. buchholzi* and *B. brachyistius*), the olfactory bulb showed the lowest average correlation with the other brain regions, though in the Cypriniformes this was a very small difference (0.03 or less compared to other region averages) (tt~~t~~able 3). In both Osteoglossiform species, the hypothalamus showed the lowest average correlation with the rest of the brain regions (0.51 for *P. buchholzi* and 0.52 for *B. brachyistius*). The correlation between optic tectum size and telencephalon size showed the highest correlation of any region comparison in every species other than *P. buchholzi*, which showed the highest correlation between the optic tectum and cerebellum (tt~~t~~able 3).

Species comparison

Within species brain region covariation patterns correlated across certain fish species (figure 4). *P. reticulata*, *G. affinis*, *L. gibbosus, L. macrochirus*, *G. aculeatus*, and *P. pungitius* all showed relatively high correlations of their brain region covariation patterns (at least 0.751), with all pairwise correlations being significant other than *P. pungitius* with *P. reticulata*, *P. pungitius* with *L. macrochirus*, and *P. pungitius* with *G. aculeatus* (figure 4). This indicates that these groups all share a similar pattern of brain region correlations. *S. atromaculatus* and *R. atratulus* also share some similar patterns with this broad group, though the correlations were weaker (figure 4). *S.*

atromaculatus showed a significant correlation with the brain region covariation patterns of *P. reticulata* (r=0.784), *L. macrochirus* (r=0.681), and *G. aculeatus* (r=0.581). *R. atratulus* correlated with *P. reticulata* (r=0.773) and *L. macrochirus* (r=0.770). *S. atromaculatus* and *R. atratulus* also showed a strong significant correlation with each other, at 0.833. Both Osteoglossiform species showed divergence in their brain region covariation patterns, with the only significant correlation occurring between *B. brachyistius* and *R. atratulus* (0.839). The covariation pattern of *P. buchholzi* was not significantly correlated with any other species.

~~We note that differences in average brain region correlation levels among species observed here may be related to differences in body size variation, as greater body size variation could establish the possibility for stronger correlations. However, this effect would not impact our ability to compare the degree of similarity of correlation matrices, as the Mantel test comparison is not affected by differences in average matrix values. To support this assumption, we compared the similarity of the *S. atromaculatus* matrix and a correlation matrix calculated from a truncated *S. atromaculatus* data set with the largest (>20g) and smallest (<2g) individuals removed, resulting in a lower coefficient of body mass variation of 0.55 (supplementary figure 5). These matrices were highly correlated with each other (r=0.96, p=0.0083), indicating that differences in average levels of trait correlations do not impact the outcomes of our Mantel test. As such, we will only consider results related to differences related to comparisons among regions within species, and how these vary across species, and not discuss overall average levels of brain region correlation within species.~~

We note that differences in average brain region correlation levels among species observed here may be related to differences in body size variation (table 1), as greater body size variation could establish the possibility for stronger correlations. However, this effect would not impact our ability to compare the degree of similarity of correlation matrices, as the Mantel test comparison is not affected by differences in average matrix values. To support this assumption, we used an additional analysis of the species in our data set with the highest body mass coefficient of variation, *S. atromaculatus*. We compared the similarity of the *S. atromaculatus* matrix and a correlation matrix calculated from a truncated *S. atromaculatus* data set with the largest (>20g) and smallest (<2g) individuals removed, resulting in a lower coefficient of body mass variation of 0.55 (supplementary figure 5). These matrices were highly correlated with each other (r=0.96, p=0.0083), indicating that differences in average levels of trait correlations do not impact the outcomes of our Mantel test. ~~As such, we will only consider results related to differences related to comparisons among regions within species, and how these vary across species, and not discuss overall average levels of brain region correlation within species.~~

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Brain region covariation patterns appeared to be influenced by phylogenetic distance. We found that the similarity in brain region covariation patterns was inversely correlated with phylogenetic distance (figure 5). This trend was not significant (r=-0.671, p=0.999).

Discussion

We tested the degree of covariation of brain region sizes within 10 teleost fish species to examine how brain region covariation patterns vary across species. Our results showed that brain regions

varied in the strength of their covariation within and across species, with the telencephalon and optic tectum usually showing very high levels of covariation, and the olfactory bulb showing lower covariation with the rest of the brain. Further, we found an insignificant trend that patterns of brain region covariation are shared across more closely related species. Specifically, this trend indicates that the similarity of within species brain region correlation patterns decreases as phylogenetic distance between species increases.

Within species patterns of brain region covariation

Patterns of brain region volume covariation within species indicate the potential for independent change in brain region size. These patterns can arise through various, non-mutually exclusive mechanisms, including differences in the strength of selection on different brain regions, or from genetic and developmental constraints (Davidowitz et al., 2012; Peiman & Robinson, 2017).

Covariation between the sizes of brain regions can occur due to patterns of selection acting on brain regions independently. This pattern can occur when selection consistently acts in similar ways on different traits (Sinervo & Svensson, 2002). For example, we observed that the telencephalon and the optic tectum showed a high level of correlation in most species. If selection consistently acts in the same direction on these two regions, it could result in this pattern. Similarly, the high level of independence of the olfactory bulb in most of the species we studied could result from selection on this specific region acting in a different direction to other regions of the brain. We consider this explanation to be unlikely to explain most of the variation we see in brain region covariation for two reasons. First, we found similar patterns in brain region covariation across

several fish species. Selection would have needed to be consistent in its effect on all brain regions across all these species regardless of population or environment, which decreases its likelihood as an explanation. Second, prior research suggests that selection can act differently across brain regions, which would result in less covariation across regions and is unlikely to then lead to very consistent patterns in brain region covariation between species. For example, Gonzalez-Voyer & Kolm (2010) found that sexual selection in Lake Tanganyikan Cichlid species resulted in divergence in the size of the hypothalamus and cerebellum, with other regions of the brain diverging in the opposite direction. Further, Kotrschal et al. (2017) found that Trinidadian guppy brain regions differed in how they diverged in response to predation levels across populations. Although we consider other explanations of the patterns we observed to be more likely, we cannot rule out correlated patterns of selection. Further research on the consistency of how selection acts on different brain regions within and across species would be needed to fully evaluate this possibility.

The other possible mechanism that can generate covariation between traits is some type of constraint, including genetic constraints and developmental constraints. Genetic constraints occur through pleiotropy (a single gene being associated with multiple phenotypic traits) or gene linkage (genes for different traits occurring close together on the same chromosome), limiting the ability of traits to evolve independently of each other (Ott et al., 2015; Solovieff et al., 2013). Although the degree of pleiotropy in determining brain region size has not been specifically measured, prior work has suggested that aspects of brain connectivity and structure are influenced by pleiotropic or linked genes (Moreau et al., 2022; van der Meer et al., 2020; Zhao et al., 2021). It is therefore likely that the evolution of the size of brain regions may be similarly influenced by genetic architecture

373 constraints. The strength of these genetic links between brain regions could thus result in the
374 patterns of brain region covariation that we observed across species.
375
376 Developmental constraints are limitations to the variability of traits caused by the structure or
377 dynamics of organism ontogeny (Smith et al., 1985). This was the form of constraint proposed to
378 explain the covariation of brain regions among mammals under the concerted brain evolution
379 hypothesis, where the evolution of larger brain size was linked to increases in the relative size of
380 later developing brain regions (Finlay & Darlington, 1995). As fish have indeterminate growth and
381 maintain widespread neurogenesis of the brain into adulthood (Zupanc, 2006), this specific process
382 is unlikely to explain our results. However, a similar kind of developmental limitation may. The
383 growth of different brain regions may be influenced by overlapping developmental mechanisms
384 such as the ability to generate new neurons in specific areas of the brain (Ganz & Brand, 2016;
385 Kaslin et al., 2008). We are not able to determine specific mechanisms generating the covariation
386 patterns we observed, and future work examining the genetic architecture and developmental
387 processes of brain region evolution will be needed to fully elucidate these.
388
389 *Evolution of brain region covariation patterns*
390 We found a trend that more closely related species tend to share similar patterns of brain region
391 covariation, while these patterns diverge as phylogenetic distance increases. Our results indicate
392 that Perciformes and Cyprinodontiformes show strong evidence of a shared pattern of brain region
393 covariation. Scorpaeniformes also show a similar pattern to these orders, but with ~~higher overall~~
394 ~~levels of brain region independence and~~ less consistency, as *P. pungitius* appear to be less similar to

the rest of these species. These six species are closely related in evolutionary terms compared to the rest of the species we included, with a common ancestor ~112 million years ago, though they differ in terms of ecology, particularly between orders (Lowe-Mcconnell, 2012; Magalhaes et al., 2016). This similarity suggests that the constraints limiting independent brain region change are evolutionarily conserved across closely related fish species, even when ecology, and likely selection pressures, differ.

~~Our two Cypriniform species (*S. atromaculatus* and *R. atratulus*) show similar patterns to each other, particularly in terms of overall level of brain region independence, as both show very little independence in brain regions. The Cypriniform brain differs morphologically from other fish as they are characterized by extended and somewhat enlarged olfactory bulbs (Brandstätter & Kotrschal, 1990; Evans, 1952). Beyond this morphological difference, it is unclear why these species have such strong correlations between their brain regions. Broadly, this result indicates that selection would need to be very strong in order to shift individual brain regions in these species independent of the rest of the brain, as evolution would need to break this strong covariation pattern, potentially limiting the ability of these species to respond to selective pressures.~~

Our results indicate that patterns of intraspecific brain region covariation can evolve, and these patterns are more similar between more closely related species. The link between phylogenetic distance and within species brain region covariation is supported by two pieces of evidence here. First, qualitatively, our results support this conclusion as we found high similarity in brain region covariation patterns among closely related species, and the most distantly related species in our

sample, the two Osteoglossiform species, show very little similarity to each other and to the rest of the species in their brain region covariation patterns. This order diverged from the rest of the species in our samples approximately 263 million years ago. *B. brachyistius* and *P. buchholzi* diverged from each other approximately 200 million years ago, much greater than the divergence time between the species in the other orders we tested. Second, our quantitative comparison suggests that phylogenetic distance between the species is negatively correlated with the similarity of their brain region covariation patterns, though this pattern was not statistically significant. This is likely due to the limited variation in time since the most recent common ancestor, which restricts the statistical power of permutation tests. Permutation tests rely on variability in the data to allow for estimating a random distribution of potential results. Many species comparisons here share the same time since most recent common ancestor, resulting in extremely limited variation from which the permutation test can derive a results distribution. Our evidence of a negative correlation between brain region correlation patterns and phylogenetic relatedness is therefore limited in its reliability. Together these indicate that evolutionary time is required to break the constraints that cause these patterns of brain region covariation, potentially through selection acting strongly in an opposite direction on correlated regions. Understanding the specific selection forces that lead to divergence in brain region covariation patterns will require further study focusing on mechanisms and evolution of constraints in brain region correlation.

Beyond more ancient divergence times between each other and the remainder of the species, the two Osteoglossiform species possess phenotypic distinctiveness in comparison to the other teleosts studied here. For example, *P. buchholzi*, a surface-oriented fish, exhibits the slowest rate of

morphological divergence among other studied Osteoglossiformes, despite tens of millions of years of genetic divergence between populations (Lavoué et al., 2011). This high level of phenotypic stability over such a long timescale in this fish lineage is thought to surpass all other known examples of morphological stasis in extant vertebrates. The other osteoglossomorph we studied, *B. brachyistius*, is in the superfamily Mormyroidea, a group of African freshwater fishes that have electromotor and electrosensory systems (Carlson & Arnegard, 2011; Crampton, 2019). In addition, mormyroids also possess extremely large brains (Nilsson, 1996; Sukhum et al., 2016, 2018), a suite of diverse craniofacial morphologies (Peterson et al., 2022), large intra- and interspecific variety in communication signals and behaviors (Hopkins 1986), and are the only known vertebrates whose sperm lack flagella (Saunders & Gallant, 2024). These novel phenotypic features in both *B. brachyistius* and *P. buchholzi,* along with their ancient divergence times, could potentially be related to breaks in genetic and/or developmental constraints maintained within other teleost species. However, further research is needed comparing more Osteoglossiform species across a greater variety of phylogenetic distances to examine how covariation in brain region sizes has been maintained over time in this specific group.

Limitations

There are key limitations to our study that must be considered. First, due to logistical reasons the number of species we sampled here was low. Our focus was on building enough within species samples to confidently measure within species brain region covariation, which made sampling a larger number of species difficult. This lower representation of species particularly limited our ability to adequately test the within and across species trait covariation patterns in a

461 phylogenetically controlled way. Second, we built our database using a combination of pre-
462 collected data and fish samples and newly sampled species, resulting in variation in sampling
463 techniques, including fish that were reared in labs and wild collected fish. This benefits our study in
464 some ways, notably by increasing the possible variability between species, making our observation
465 of similar patterns across species stronger. However, by doing this we are unable to determine how
466 differences in selection or environmental variation may be impacting our results, limiting our ability
467 to elucidate the mechanisms generating the covariation patterns we see. Finally, we do not have
468 any genetic information about the patterns of covariation we observed. Previous work on
469 evolutionary constraints caused by the covariation of traits has focused on genetic covariation
470 (Davidowitz et al., 2012; Lande, 1984; Olson & Miller, 1958; Ott et al., 2015), with the goal of linking
471 trait covariation to the evolutionary mechanism of constraint. We also are unable to determine the
472 heritability of patterns of trait covariation we observed. The genetic data needed for this type of
473 analysis were not available to us. Future work should examine both the heritability of brain region
474 covariation patterns as well as the genetic architecture causing these patterns of covariation.
475
476 *Conclusion*
477 Here, using large, within-species data sets, we examined the degree of covariation of brain region
478 sizes within 10 fish species, and tested whether those patterns vary across species. Our results
479 indicate that brain regions vary in their level of covariation, and that these patterns are shared
480 between closely related species of fish. Broadly, these results could indicate that the size of
481 individual brain regions is constrained in its ability to evolve within species, and that it takes a great
482 deal of evolutionary time to break or change these constraints. Future work will be needed to

elucidate the specific nature of these constraints, as well as the evolutionary processes that can break these patterns.

Tables and Figures:

Table 1. List of fish species used, including the source of the data and the sample size.

Common Name	Scientific Name	Order	Source	N	Coefficient of variation in body mass
Pumpkinseed Sunfish	*Lepomis gibbosus*	Perciformes	Axelrod et al. 2021	113	0.59
Bluegill Sunfish	*Lepomis macrochirus*	Perciformes	Axelrod et al. 2021	94	0.52
Threespine Stickleback	*Gasterosteus aculeatus*	Scorpaeniformes	Herczeg et al. 2015	231	0.24
Ninespine Stickleback	*Pungitius pungitius*	Scorpaeniformes	Gonda et al. 2009	120	0.37
Trinidadian Guppy	*Poecilia reticulata*	Cyprinodontiformes	Authors	296	0.46
Mosquitofish	*Gambusia affinis*	Cyprinodontiformes	Authors	133	0.68
Creek Chub	*Semotilus atromaculatus*	Cypriniformes	Authors	194	0.91
Black Nose Dace	*Rhinichthys atratulus*	Cypriniformes	Authors	82	0.8
African Butterflyfish	*Pantodon buchholzi*	Osteoglossiformes	Authors	61	0.39
Black baby whale	*Brienomyrus brachyistius*	Osteoglossiformes	Authors	56	0.57

Table 2. Collection details for each fish species used.

Species	Collection Details
Lepomis gibbosus	Wild collected fish, collected via angling from the wild in summer 2016 and 2017 from four pelagic sites and four littoral sites of Ashby Lake, Ontario.
Lepomis macrochirus	Wild collected fish, collected via angling from the wild in summer 2017 from one pelagic site and one littoral site of Holcomb Lake, Michigan.
Gasterosteus aculeatus	F1 offspring of wild collected fish from the Baltic Sea in 2011. F1 fish were reared until adulthood in either an enriched environment or a simple environment.
Pungitius pungitius	F1 offspring of wild collected fish from four sites in the Baltic Sea in 2011. F1 fish were reared until adulthood either alone or in groups of 20 fish.
Poecilia reticulata	F2 offspring of wild collected fish from two populations in Trinidad, Aripo high predation (HP) and Aripo low predation (LP). F2 fish from both populations were reared until sexual maturity under two predator environments (pred+ and pred-) and three social environments (Solo, with HP conspecifics, and with LP conspecifics).
Gambusia affinis	Wild collected fish from 4 freshwater sites in southern Louisiana in the summer of 2022. Fish were collected with dip nets from one high salinity site and one low salinity site in each of two streams.
Semotilus atromaculatus	Wild collected via electrofishing in the summer of 2018 and 2019 from 17 freshwater stream representing a gradient of agricultural intensity within 6 major watersheds in Southwest Ontario, Canada.
Rhinichthys atratulus	Wild collected fish from Fall Creek, NY. Fish were collected in November of 2023 using seine nets.
Pantodon buchholzi	Sourced from tropical fish supplier
Brienomyrus brachyistius	Sourced from tropical fish supplier

Table 3. Average correlation coefficients for brain regions across species. Averages are calculated from the correlations of each region with every other region in each species.

Species	Cb	OT	Tel	OB	Hyp	Total
Poecilia reticulata	0.72	0.78	0.79	0.64	0.72	0.73
Gambusia affinis	0.73	0.78	0.80	0.67	0.74	0.75
Lepomis gibbosus	0.70	0.76	0.79	0.61	0.74	0.72
Lepomis macrochirus	0.72	0.76	0.75	0.53	0.66	0.68
Gasterosteus aculeatus	0.60	0.64	0.59	0.43	0.55	0.56
Pungitius pungitius	0.59	0.64	0.64	0.23	0.66	0.56
Semotilus atromaculatus	0.86	0.91	0.90	0.86	0.86	0.88
Rhinichthys atratulus	0.94	0.95	0.96	0.92	0.92	0.94
Pantodon buchholzi	0.65	0.69	0.62	0.66	0.51	0.63
Brienomyrus brachyistius	0.64	0.61	0.67	0.53	0.52	0.59

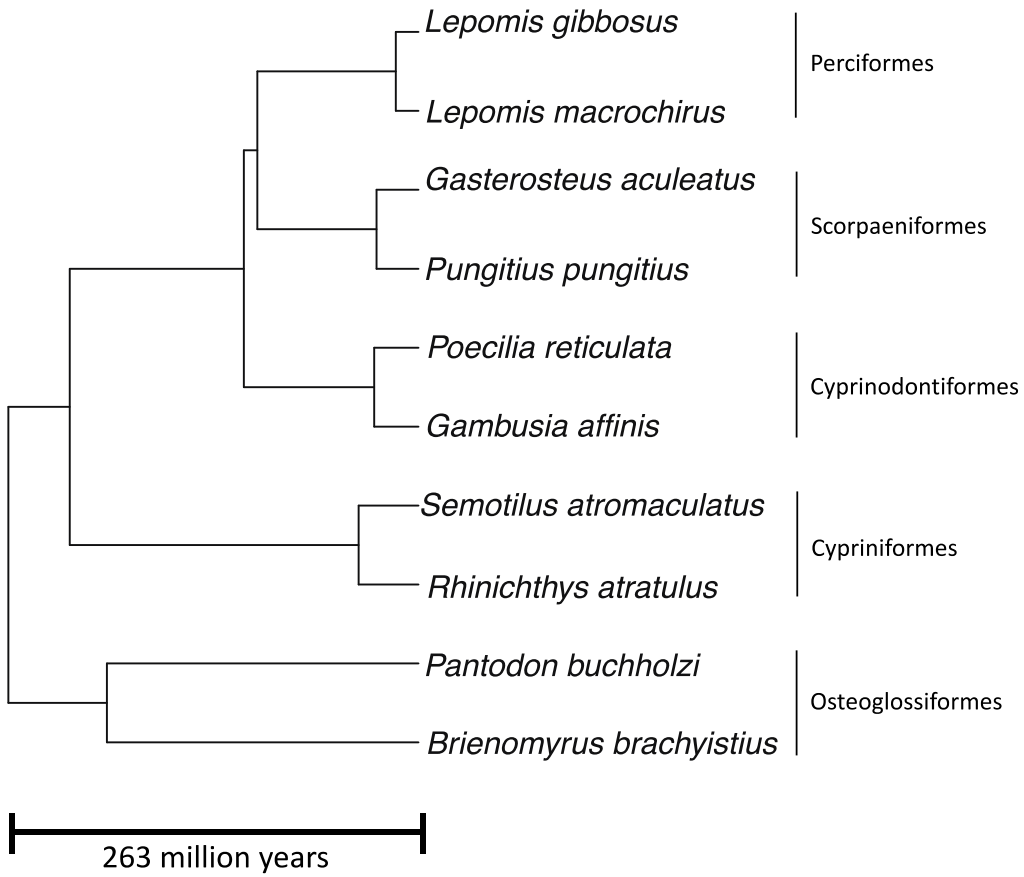

Figure 1. Phylogenetic relationships between the 10 teleost fish species in our study.

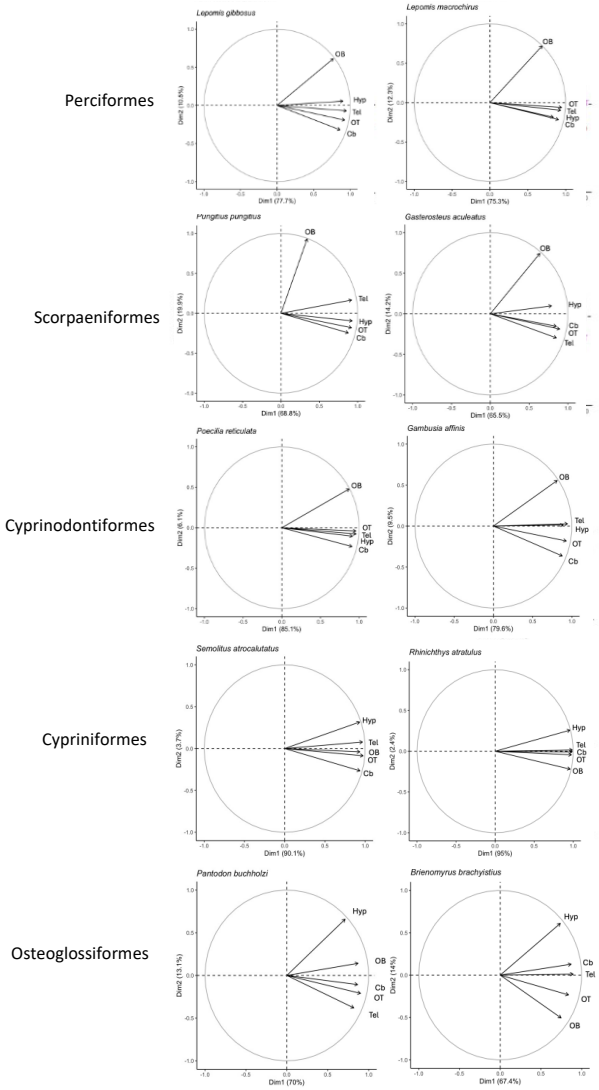

Figure 2: PC loading vectors of each brain region for each species, for PC1 (Dim1, correlated with total brain volume) and PC2 (Dim2, correlated with covariation in brain regions not related to total brain size). Cb, cerebellum; Hyp, hypothalamus; OB, olfactory bulbs; OT, optic tectum; Tel, telencephalon.

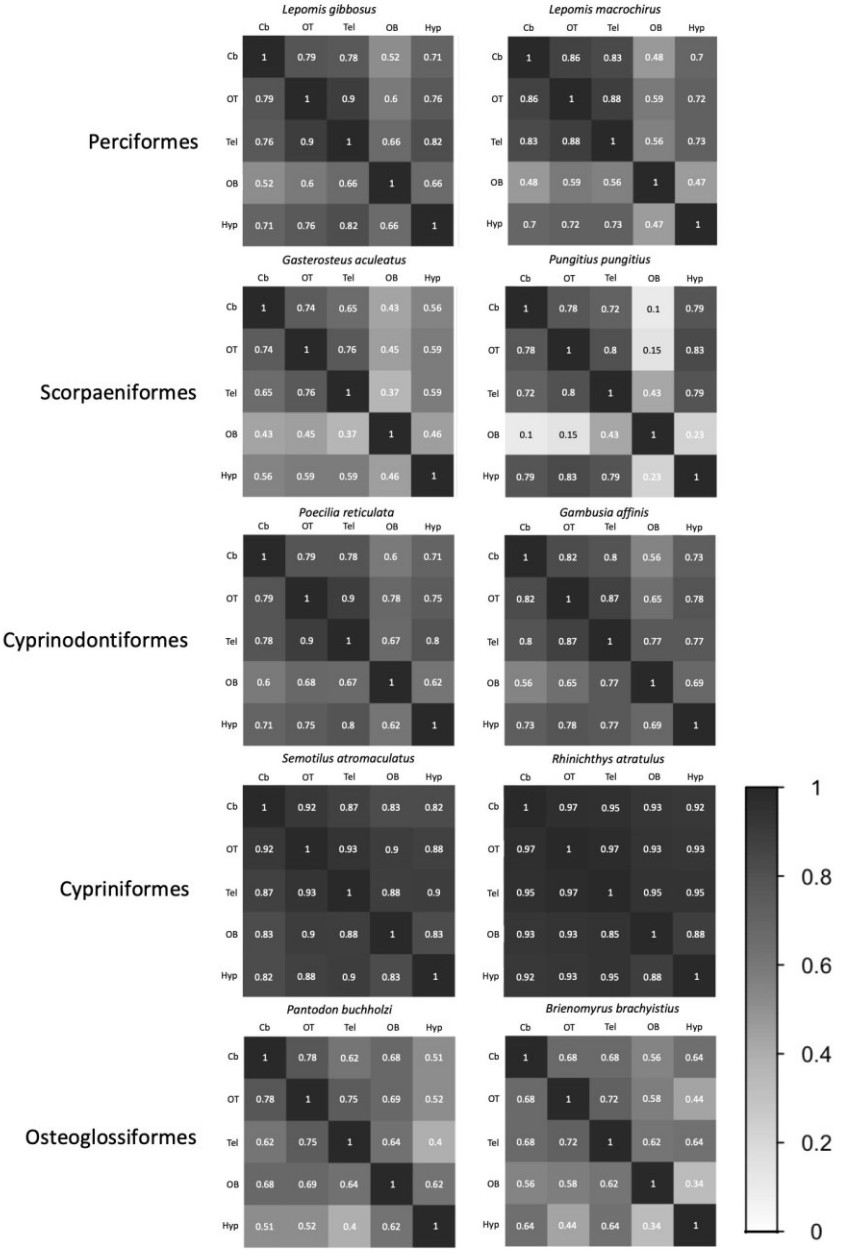
Perciformes
Scorpaeniformes
Cyprinodontiformes
Cypriniformes
Osteoglossiformes
Lepomis gibbosus
Lepomis macrochirus
Gasterosteus aculeatus
Pungitius pungitius
Poecilia reticulata
Gambusia affinis
Semotilus atromaculatus
Rhinichthys atratulus
Pantodon buchholzi
Brienomyrus brachyistius
Cb
OT
Tel
OB
Hyp
1
0.8
0.6
0.4
0.2
0

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Figure 3. Matrices showing each pairwise Pearson correlation among five brain regions for all 10 fish species: cerebellum (Cb), optic tectum (OT), telencephalon (Tel), olfactory bulb (OB), and hypothalamus (Hyp). Box color shows the strength of the correlations as indicated by the scale bar on the right, and numbers show the correlation coefficient. Statistically significant correlations are shown as white numbers, and non-significant correlations are shown as black numbers: all correlations are significant other than the Cb/OB and OT/OB correlations in *P. pungitius*.

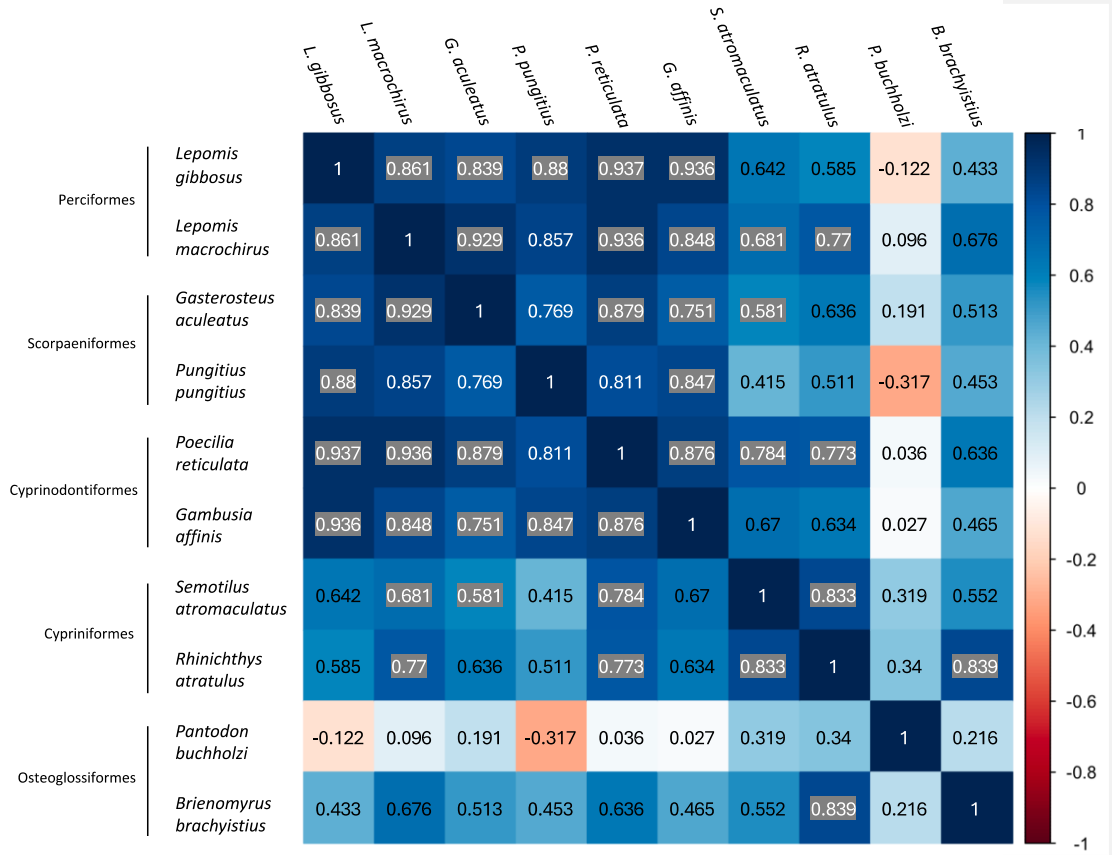

Figure 4. Matrix showing the pairwise Pearson correlations of within species brain region size correlation matrices for each of the 10 fish species in our study. Box color shows the strength of the correlations as indicated by the scale bar on the right. Numbers show the correlation coefficient (r) for each comparison. Gray boxes indicate significant correlations. Numbers are colored for optimal visibility.

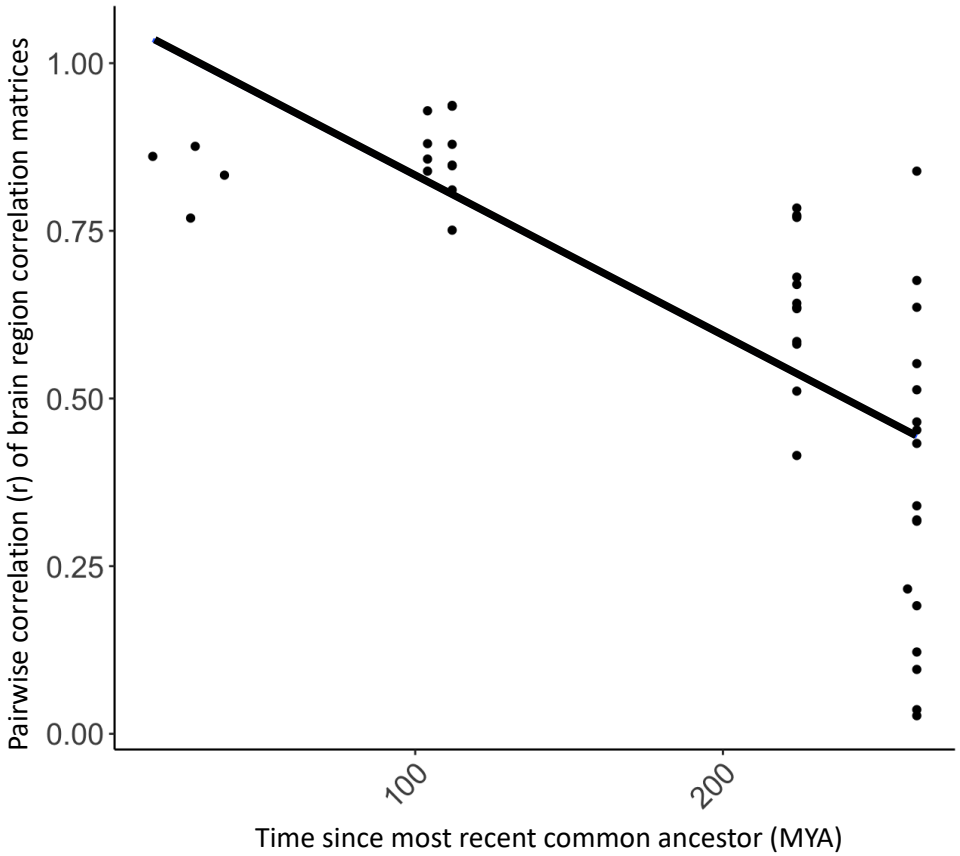

Figure 5. Linear relationship between time since most recent common ancestor and correlation of within species brain region size correlation matrices (see figure 4). Dots show individual species comparisons, and the line shows the linear regression. Mantel test r^2=-0671, p=0.999. Line of best fit equation: r=1.07-0.0024(MYA).

Conflict of interest: Authors declare no conflicts of interest.

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