

THE EVOLUTION OF NEUROSENSORY STRUCTURES: EVALUATING ECOLOGICAL  
DRIVERS OF BRAIN AND EYE SIZE VARIATION

By

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## Abstract

### THE EVOLUTION OF NEUROSENSORY STRUCTURES: EVALUATING ECOLOGICAL DRIVERS OF BRAIN AND EYE SIZE

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Neurosensory structures (i.e., brain and eye size) vary extensively across taxa. The evolution of large neurosensory structures has long been an area of interest for scientists, but focus has largely been placed on macroevolutionary patterns at or above the species level. Few studies have investigated the role contrasting ecological factors play in the evolution of such structures within species. My dissertation examines 1) how divergent ecological pressures, particularly predation and competition, drive evolutionary shifts in brain and eye size within species and across sexes, and 2) why such variation evolves, particularly if larger brains drive shifts in behavior and fitness. First, I further explore known differences in male brain size in Trinidadian killifish (*Anablepsoides hartii*) to determine if brain structures also evolve across divergent predation regimes and if brain size, brain structure, and eye size co-evolve. In my second chapter, I investigate female brain size and structure to determine if selection operates in a different manner on these traits between sexes and if brain size, structure, and eye size co-evolve in a sex-specific manner. In my third chapter, I evaluate behavioral correlates of increased brain size to determine if jumping behavior in killifish, which is a proxy for fitness, is connected to shifts in brain size and structure. In my fourth chapter, I test for selection on increased brain size and increased fitness by transplanting killifish into novel environments in a transplant mark-recapture experiment. Lastly, I use *Daphnia sp.* to test the influence of light

availability on the evolution of eye size and anti-predator behavior using mesocosm experiments.

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## CHAPTER 1:

### Coordinated evolution of brain size, structure, and eye size in Trinidadian killifish

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## ABSTRACT

Brain size, brain architecture, and eye size vary extensively in vertebrates. However, the extent to which the evolution of these components is intricately connected remains unclear. Trinidadian killifish, *Anablepsoides hartii*, are found in sites that differ in the presence and absence of large predatory fish. Decreased rates of predation are associated with evolutionary shifts in brain size; males from sites without predators have evolved a relatively larger brain and eye size than males from sites with predators. Here, we evaluated the extent to which the evolution of brain size, brain structure, and eye size covary in male killifish. We utilized wild-caught and common garden-reared specimens to determine whether specific components of the brain have evolved in response to differences in predation and to determine if there is covariation between the evolution of brain size, brain structure, and eye size. We observed consistent shifts in brain architecture in second generation common garden reared, but not wild caught preserved fish. Male killifish from sites that lack predators exhibited a significantly larger telencephalon, optic tectum, cerebellum, and dorsal medulla when compared with fish from sites with predators. We also found positive connections between the evolution of brain structure and eye size but not between overall brain size and eye size. These results provide evidence for evolutionary covariation between the components of the brain and eye size. Such results suggest that selection, directly or indirectly, acts upon specific regions of the brain, rather than overall brain size, to enhance visual capabilities.

## INTRODUCTION

Brain size and structure are well known to vary extensively across vertebrates (Andrew, 1962; Dunbar, 1998; Lefebvre et al., 2004; Striedter, 2005). A large body of research has shown that there is frequently a link between such variation and fitness. Increases in vertebrate brain size are positively correlated with a longer lifespan and increased survival (Amiel et al., 2011; González-Lagos et al., 2010; Sol et al., 2007, 2008; Sol & Lefebvre, 2000). Large brains have also been associated with shifts in mating behavior, predator avoidance, learning, and behavioral flexibility (Buechel et al., 2018; Herczeg et al., 2019; A. Kotrschal et al., 2015; Ratcliffe et al., 2006; van der Bijl et al., 2015). Similarly, research has shown that individual components of the brain are correlated with shifts in cognitive abilities that may alter fitness (Garamszegi & Eens, 2004; Hutcheon et al., 2002; Lefebvre et al., 1997; Ratcliffe et al., 2006; Safi & Dechmann, 2005). For example, Hutcheon et al. (2002) showed that various brain structure sizes (i.e., auditory nuclei, olfactory bulb, hippocampus) were connected to foraging ecology in bats.

A growing body of literature has provided links between ecologically divergent conditions and evolutionary shifts in brain size. Ecological conditions such as habitat (Axelrod et al., 2018; Gonda, Herczeg, & Merilä, 2009a, 2011; Keagy et al., 2018; Park & Bell, 2010) and predation (Samuk et al., 2018; Walsh et al., 2016) have been linked to phenotypic shifts in brain size and brain structure. For example, Keagy et al. (2018) found that stickleback that are adapted to forage on benthic invertebrates ('benthic sticklebacks') exhibited larger relative brain volumes than stickleback that forage in open water environments (i.e., 'limnetic sticklebacks'). This same study showed that benthic stickleback had larger relative optic tecta and smaller olfactory bulbs than limnetic fish (see also Park & Bell, 2010). Axelrod et al. (2018) found that brain size, but not structure, differed between sunfish from habitats that vary in structural complexity; sunfish from the more structurally complex, littoral habitats in a lake exhibited larger brains than those from open water. Samuk et al. (2018) performed a selection experiment in seminatural ponds

and found that increased predation led to the evolution of significantly smaller brains and brain structures, specifically smaller optic lobes and telencephala, in sticklebacks. This growing body of work provides clear connections between divergent ecological conditions and shifts in brain size and brain architecture. A number of these studies have also shown that this phenotypic variation in brain size is likely genetically based as differences were maintained after multiple generations of laboratory common garden rearing (Gonda et al., 2009a; Samuk et al., 2018; Walsh et al., 2016).

In addition to variation in brain size and brain architecture, organisms also exhibit extensive variation in eye size (Land & Fernald, 1992). Increases in vertebrate eye size are associated with improved vision (Caves et al., 2017; Møller & Erritzøe, 2010; Motani et al., 1999; Walls, 1942) and thus enhanced fitness via shifts in foraging, predatory, and mating behavior (Garamszegi et al., 2002; Hall & Ross, 2007; Huber et al., 1997; Møller & Erritzøe, 2014; Thomas et al., 2006). Evolutionary shifts in eye size have also been linked to changes in ecological conditions. For example, studies have quantified selection on eye size due to such factors as light availability (Hall, 2008; Hiller-Adams & Case, 1988; Kröger & Fernald, 1994; Veilleux & Lewis, 2011), predation (Glazier & Deptola, 2011; Møller & Erritzøe, 2010; Nilsson et al., 2012), and competition (Beston et al., 2017, 2019; Beston & Walsh, 2019). This growing body of work illustrating similar shifts in brain and eye size in response to ecologically mediated selection foreshadows the possibility that selection favors coordinated shifts in these neurosensory systems. However, the extent to which there are connections between the evolution of brain and eye size is unclear.

There are several reasons why the evolution of brain size, brain architecture, and eye size may be intricately linked. This is, in part, because there are well known connections between brain structures and organismal performance. For example, the telencephalon is linked to emotional learning, temporal and spatial memory, spatial cognition, and spatial behavior such as predator avoidance, foraging, and mating in teleost fish (Broglio et al., 2003; Portavella et al.,

2002). The cerebellum is involved in controlling the execution of motor activity and is therefore important to memory and learning (Broglia et al., 2003). The medulla is involved with auditory function and relaying information between the brain and spinal cord (Collin et al., 2015; Tomchik & Lu, 2005). More importantly, various brain structures are connected to aspects of vision. Specifically, the vertebrate telencephalon receives visual information from the retina (Cooper et al., 1989; Garamszegi et al., 2002; Luiten, 1981), while the optic tectum is linked to multisensory integration, coordinated eye and body movements, and processes visual stimulus information (Broglia et al., 2003). Few studies have investigated potential covariation between overall brain size and eye size (Burton, 2008; Corral-López et al., 2017; Garamszegi et al., 2002). These studies have found significant, positive associations between relative brain size and relative eye size across species of birds (Burton, 2008; Garamszegi et al., 2002). Artificial selection on brain size in guppies (*Poecilia reticulata*) showed that fish selected for larger brains also evolved larger eyes, but did not exhibit increased visual acuity (Corral-López et al., 2017). However, tests of this connection across divergent ecological environments within a single species have yet to transpire.

Trinidadian killifish (historically '*Rivulus hartii*' but now *Anablepsoides hartii*) are found in sites that differ in predation intensity due to their ability to disperse and colonize novel upstream environments. This includes downstream, lowland 'high predation' (HP) sites where killifish co-occur with several species of piscivorous fish (e.g., *Crenicichla frenata*, *Hoplias malabaricus*), and upstream, '*Rivulus*-only' (RO) sites where killifish are the only species of fish that is present ('*Rivulus*-only' is used given historical precedent; Fraser et al., 1999; Gilliam et al., 1993; Walsh & Reznick, 2008). In HP sites, killifish experience increased mortality rates likely due to the presence of predators (Furness & Reznick, 2014). Several variables covary with the presence of predators that may also exert selection on the traits of killifish. HP sites exhibit a more open canopy cover (Reznick et al., 2001), the densities of killifish are lower, and per capita food availability is higher when compared to these same features in RO sites (Walsh & Reznick,

2008, 2009). Conversely, killifish are found at much higher densities in RO sites and thus experience strong intraspecific competition for limited resources (Gilliam et al., 1993; Walsh & Reznick, 2008). Research has shown that the ecological differences between HP and RO sites is associated with evolutionary divergence in life-history traits (Walsh et al., 2011; Walsh & Reznick, 2008, 2009, 2010, 2011). Recent work has also revealed an association between increased predation and evolutionary shifts in brain and eye size between HP and RO sites (Beston et al., 2017; Walsh et al., 2016). Males, but not females, from RO sites have evolved larger brains than males from sites with predators. These trends were maintained after two generations of common garden rearing, which indicates that these differences are likely genetically based (Walsh et al., 2016). Similarly, male killifish from RO sites have also evolved a larger relative eye size than males from high predation sites (Beston & Walsh, 2019; Beston et al., 2017). These evolved differences in brain and eye size between sites with and without predators present the opportunity to determine whether increased predation has also driven shifts in brain architecture and whether there is a coordinated pattern of evolution among brain size, brain structure, and eye size.

Here, we evaluated the extent to which the evolution of brain size, brain structure, and eye size covary in killifish. We utilized wild-caught and common garden-reared specimens of killifish to address three specific questions. First, is there evidence that specific components of the brain have evolved in response to differences in predation? Second, are the patterns of brain architecture evolution consistent between wild-caught and common garden-reared fish? Lastly, is there covariation between the evolution of brain size, brain structure, and eye size? We predict that the telencephalon and optic tectum will be the strongest predictors of eye size, given that these structures are likely the most intricately linked to vision. That is, we expect to observe positive relationships between these brain structures and eye size.

## MATERIALS AND METHODS

### *Wild caught specimens.*

We collected killifish from high predation and *Rivulus*-only sites from the Arima, Aripo, and Guanapo rivers during May–June 2016 using small seines. All captured fish were immediately euthanized with MS-222, preserved in 10% formalin, and then stored in 70% ethanol. Fish were measured for total length and photographed on their side for assessment of eye size using a Canon PowerShot ELPH180 or Nikon CoolPix S610 camera. Eyes were measured using the diameter of the eye cavity at the widest part for each photograph. All brains were then dissected during the Summer–Fall of 2016. Brains were dissected from each preserved specimen by cutting from the top of each gill slit and then removing the lower jaw and the tissue between the roof of the mouth and the braincase. All brains were stored in 70% ethanol until they were photographed for estimates of the volume of the brain structures in January 2019. To do so, we took separate images for the dorsal, lateral, and ventral views (Figure S1). All measurements were completed by individuals unaware of the population of origin. We then quantified the volume of the telencephalon, optic tecta, cerebellum, and dorsal medulla via the ellipsoid model:  $V = (L \times W \times H)\pi/6$ . This approach has been shown to provide a highly accurate estimate of the volume of fish brain structures (e.g. Huber et al., 1997; Pollen et al., 2007). Our total sample size included 143 male killifish (Arima high predation = 18 fish, Arima *Rivulus*-only = 21, Aripo high predation = 24, Aripo *Rivulus*-only = 13, Guanapo high predation = 25, Guanapo *Rivulus*-only = 42).

We evaluated killifish from HP and RO sites for differences in the volume of the telencephalon, optic tectum, cerebellum, and dorsal medulla via linear mixed models (SPSS v. 26 IBM Corporation) with fish community (high predation, *Rivulus*-only), river (Arima, Aripo, Guanapo), and all interactions entered as fixed effects. We ln-transformed all traits to improve normality and homogeneity of variances and included ln-fish length as a covariate. We first evaluated the full model with all possible interactions (including interactions with the covariate).



We then removed interactions with little statistical significance ( $F$ -value  $< 1.0$ ) and reran analyses to converge on the best fitting model (lowest AIC values). We also performed complimentary analyses that included ln-brain size as a covariate instead of body length. The results from both analyses were similar. In the results section, we focus on results that included total length as a covariate but also present the analyses using brain size as the covariate (see Table S1). Based upon the number of analyses performed for these data, we expected to observe approximately four significant results by chance.

### ***Common garden specimens***

Methods from the common garden experiments were previously published (Walsh & Reznick, 2008) and are briefly summarized here. We collected wild-caught killifish from RO and HP sites in the Arima and Guanapo rivers in July 2005. We returned the fish to the laboratory and established laboratory stocks from approximately 20 fish per population (10 males and 10 females, 72 fish total). We generated the first laboratory generation by randomly pairing wild-caught males and females from the same locality in 9-l aquaria with an artificial spawning substrate. The eggs that were collected from each pairing were placed in Petri dishes in a methylene blue solution. All newly hatched larvae from each pairing were then placed in aquaria at a maximum density of eight fish per tank and were fed an ad libitum diet of liver paste and brine shrimp nauplii. We generated the second laboratory generation by pairing mature females from each lineage in the first generation with mature males from the same locality but different lineage. Overall, the experiment included fish from six pairings per population. For the common garden experiment, all offspring were reared at densities of eight fish per 9-l aquarium and fed ad libitum. Beginning at an age of 20 days, eight fish from each pairing were individually placed in separate 9-l aquaria. Each tank was supplied with a clay pot for refuge and an artificial spawning substrate. The fish from each pairing were equally divided between two food treatments: (a) a 'high food' ration that approximates growth in HP sites and (b) a low food ration that mimics the growth naturally observed in RO sites (Walsh & Reznick, 2008). All fish

were provided with quantified portions of liver paste in the morning and brine shrimp nauplii in the afternoon. We then reared all killifish to maturity in order to quantify a diversity of life-history traits (see Walsh & Reznick, 2008 for details regarding quantification of life-history traits). Males were immediately euthanized and preserved in 5% formalin following maturation. All preserved fish were stored for approximately eight years prior to being photographed for eye size, weighed, and dissected for assessments of brain size and structure.

We dissected the brain from all preserved specimens beginning in August 2015 (see Walsh et al., 2016). The brain was removed by cutting from the top of each gill slit and then removing the lower jaw and the tissue between the roof of the mouth and the braincase. Each brain was blotted dry, and we then photographed the dorsal surface of each brain (Figure S1-a). We then measured the width of the telencephalon, optic tecta, cerebellum, and dorsal medulla via ImageJ. All measurements were performed by individuals unaware of population of origin or food treatment. Note that we only have images associated with the dorsal surface of the brain and could therefore not calculate the volume of the brain structures. To address possible issues with comparing width (dorsal surface) to structure volume, we ran correlations between structure width and structure volume for each brain structure using wild caught data. All correlations were significant ( $p < .05$ , Table S2). The total sample size of common garden-reared specimens was 87 males (Arima high predation = 22, Arima *Rivulus*-only = 23, Guanapo high predation = 19, Guanapo *Rivulus*-only = 23).

We evaluated killifish from HP and RO sites for differences in the width of the telencephalon, optic tectum, cerebellum, and dorsal medulla via linear mixed models (SPSS v. 26 IBM Corporation) with fish community (high predation, *Rivulus*-only), food level (high, low), river (Arima, Guanapo), and all interactions entered as fixed effects. We ln-transformed all traits to improve normality and homogeneity of variances and included ln-fish length as a covariate. Similar to the analyses for the wild-caught fish, we evaluated the full model with all possible interactions. We then removed interactions with little statistical significance ( $F$ -value  $< 1.0$ ) and

reran analyses with reduced models to find the best fit model (lowest AIC values). Since the age of these fish was known (see Walsh & Reznick, 2008), we included their age at maturation as a covariate in the initial analyses. The influence of age at maturation was not significant for all traits. We therefore removed this covariate from the analyses. We also ran complimentary analyses that included ln-brain size as a covariate instead of body length and found results were similar for both (see Table S3). Based upon the number of analyses performed for these data, we expected to observe approximately three significant results by chance.

### ***Covariation between eye size and brain size***

To further evaluate the extent to which the brain structures evolve in concert or independently with eye size, and also determine the structures that contribute to variation in brain and eye size, we first performed multiple regressions with ln-transformed eye size as the dependent variable and each ln-transformed brain structure and ln-transformed brain size as the independent variables (SPSS v.26 IBM Corporation). Then, we separated the data by population and ran regressions to evaluate potential differences in the relationship between brain size, brain structure, and eye size across RO and HP sites. All regressions were performed with ln-length as a covariate. To determine whether the connection between eye size and brain size differ between populations, we then specifically evaluated the 'predation × brain' interaction via general linear models with ln-eye size as the dependent variable, predation as a fixed effect, and ln-brain size or brain structure as a covariate. All of these analyses were performed separately for wild caught and common garden-reared fish.

## **RESULTS**

### ***Brain structure***

#### *Wild caught.*

We observed a significant ( $p < .05$ ) 'river × predation × length' interaction for the volume of all measured brain structures (dorsal medulla, cerebellum, optic tectum, and telencephalon;

Table 1), due to the differing allometries between total length and brain size or structure across rivers and populations (Figure 1, Figure S2). We also observed a significant 'river x predation' interaction for the volume of all measured brain structures (Table 1). These significant interactions were due to contrasting patterns of divergence in brain structures between HP and RO sites among the focal rivers. For example, RO fish from the Arima River exhibited a 17% larger dorsal medulla volume compared with Arima HP fish while the differences in dorsal medulla volume were smaller (Guanapo) or in the opposite direction (Aripo) in the other rivers (Figure S2). Overall, there was a significant ( $p < .05$ ) difference in the volume of the optic tectum but not the dorsal medulla, cerebellum, or telencephalon between HP and RO sites (Table 1, Figure 1). The volume of the optic tectum was 23% greater in RO fish than HP fish (Figure 1). Although the differences were nonsignificant, RO fish qualitatively exhibited a larger dorsal medulla, cerebellum, and telencephalon compared with HP fish (Figure 1).

#### *Common Garden.*

We observed significant ( $p < .05$ ) differences in the width of all four brain structures between the focal populations (optic tectum, telencephalon, dorsal medulla, and cerebellum; Table 2, Figure 1). RO fish exhibited larger brain regions than HP fish for all measured structures (Figure 1). The width of the optic tectum, telencephalon, dorsal medulla, and cerebellum was 14%, 22%, 21%, and 11% greater in RO fish than HP fish, respectively. The 'river x predation' and 'river x predation x length' interactions were not significant and not included in the final model for any of the brain structures (Figure 1; Figure S3). The contrasting food treatments were not significant (Table 2). All 'predation x food' interactions were not significant and not included in the final models ( $F\text{-value} < 1.0$ ), but the 'predation x food x length' interaction was significant for the cerebellum (Table 2).

#### ***Covariation between brain size and eye size***

##### *Wild Caught.*

The results of a multiple regression revealed a significant ( $p < .05$ ) link between overall brain size and eye size and dorsal medulla and eye size (Figure 2, Table S4). When separating the multiple regressions by population, we observed that the connection between the dorsal medulla and relative eye size was significant for RO fish ( $p = .006$ ; Figure S4). All other connections between brain structures and eye size stemming from the multiple regressions were not significant. None of the 'predation  $\times$  brain structure' interactions from the GLMs were significant.

#### *Common Garden.*

The multiple regressions revealed a significant positive relationship between the size of the telencephalon, optic tectum, cerebellum, and eye size (Figure 2, Table S4). The connections between brain size and eye size and dorsal medulla and eye size stemming from the multiple regressions were not significant (Figure 2). When separating the multiple regressions by population, none of the links between brain size or structure and eye size were significant (Figure S5). None of the 'predation  $\times$  brain structure' interactions were significant.

## **DISCUSSION**

The evolution of larger brains is associated with shifts in brain architecture in Trinidadian killifish. Fish stemming from common garden experiments revealed that killifish from RO sites have evolved a wider (based upon diameter) telencephalon, optic tectum, cerebellum, and dorsal medulla than fish from HP sites (Figure 1, Figure S3). These patterns were similar in wild-caught specimen but were larger in magnitude and more consistent in common garden-reared fish. In the wild-caught fish, the overall differences between HP and RO sites and the allometric relationships between brain structure and eye size often varied across rivers. Yet, the consistent pattern of divergence between RO and HP fish observed in the second-generation common garden-reared fish indicates such differences are likely genetic in origin (Figure 1). We also observed a significant connection between brain and eye size; in the common garden-

reared fish we found a positive correlation between the size of the telencephalon, optic tectum, cerebellum, but not overall brain size, and eye size (Figure 2). This provides evidence for evolutionary covariation between the components of the brain and eye size. However, it is important to note that we failed to observe a consistent pattern between the wild-caught and common garden-reared specimens and that we cannot rule out the influence of grandparental or transgenerational effects on the differences in brain regions for common garden-reared fish. Collectively, our data indicate that phenotype (wild caught) does not necessarily predict genotype (common garden) in brain structure or in the connection between brain and eye size.

What ecological features might lead to the contrasting patterns of brain architecture evolution observed between wild-caught and common garden-reared fish? As described in the introduction, it is important to note that differences in predation are associated with correlated shifts in ecological factors that may influence the expression of brain size, brain structure, and eye size. Increased rates of predation in high predation sites is correlated with increased light and resource availability (El-Sabaawi et al., 2012; Grether et al., 2001; Reznick, Butler IV, & Rodd, 2001). Killifish are known to mostly forage on invertebrates (Fraser et al., 1999), and invertebrate abundance varies with predation intensity (El-Sabaawi et al., 2012). Invertebrate abundance is 40%–173% greater in HP sites than RO sites (El-Sabaawi et al., 2012). Furthermore, canopy cover is 5%–27% more open in HP sites than RO sites in the same rivers used in this study (El-Sabaawi et al., 2012). These differences in canopy cover and resource availability are also known to vary across streams. For example, the Guanapo RO sites have 7% more open canopy than Arima and Aripo RO sites, while invertebrate abundance is 80% higher in Guanapo RO sites compared with the Aripo RO (El-Sabaawi et al., 2012). These differences across streams may alter the expression of traits at the phenotypic level. We explored potential connections between the published estimates of light and food availability (El-Sabaawi et al., 2012) and the brain and eye size values in the current study via multiple

regressions. Such exploratory analyses did not yield significant connections between the environmental data and the neurosensory traits of killifish (Table S5).

The disconnection between our wild-caught specimens and common garden-reared samples is not necessarily surprising because a growing number of studies have shown that brain size (and brain components) is plastic and sensitive to changes in the environment. For example, brain mass was shown to be plastic across and within populations of an African cichlid (*Pseudocrenilabrus multicolor victoriae*) that experience differing oxygen levels (Crispo & Chapman, 2010). Differences in the size and plasticity of brain size, optic tectum, and olfactory bulbs were documented in stickleback from marine and pond habitats that were reared in differing social environments (Gonda et al., 2009a, 2009b). Another study in extremophile fish (*Poecilia Mexicana*) found plasticity in brain region volumes (i.e., cerebellum, optic tectum, telencephalon) across varying levels of light and sulfide exposure (Eifert et al., 2015). Competition and predation were shown to induce plastic responses in brain size and structures (i.e., optic tectum, medulla oblongata) in tadpoles (Gonda et al., 2010). This work collectively signals that a multitude of ecological variables likely influenced the patterns of variation in the components of the brain in the wild-caught specimens.

One surprising aspect of our results is that we did not find a connection between overall brain size and eye size in common garden-reared fish. However, we did find connections between eye size and three brain structures—telencephalon, optic tectum, and cerebellum. We expected to see positive relationships between eye size and the telencephalon and optic tectum, but not necessarily between eye size and the cerebellum. However, the connection between eye size and cerebellum is potentially intuitive given that the cerebellum is involved in coordinating motor activity (Broglia et al., 2003) and more specifically may play a role in spatial awareness, spatial orientation, and eye movement (Kotrschal et al., 1998). The cerebellum may also be indirectly correlated with eye size via connections to the telencephalon and optic tectum. Studies have indeed shown positive associations between cerebellum size and optic tectum,

telencephalon size (Huber & Rylander, 1992; Van Staaden et al., 1995), suggesting a functional unit upon which selection acts upon in similar ways. These results suggest that selection (directly or indirectly) acts upon specific regions of the brain, and not overall brain size, to enhance visual capabilities. Our results also expand current understanding of the connection between eye and brain size by identifying links between eye size and specific brain structures. Our results now call for more mechanistic studies that specifically quantify the functional implications of variation in brain architecture and eye size and their potential connections with visual acuity and behavioral aspects of visual performance.

There are multiple plausible adaptive explanations for the observed differences in brain structure between RO and HP killifish and the observed connection between brain architecture and eye size. Previous work has suggested that competition for food and mates requires high cognitive abilities (Barkley & Jacobs, 2007; Jacobs, 1996; Jacobs et al., 1990). This is important because RO sites are characterized by high killifish density, low resources, and presumably intense competition for both food and mates compared with fish in HP sites (Fraser et al., 1999; Gilliam et al., 1993). Thus, larger brain structures that help overcome these obstacles are likely favored in RO sites. That is, the evolution of larger brain structures that are connected to learning, movement, and coordination may be driven by selection for increased investment in foraging and mating capabilities that enhance fitness in high competition environments. For example, cognitive function is controlled by the telencephalon (Bshary et al., 2002; Vargas et al., 2009) and the telencephalon is also associated with learning and spatial behaviors such as foraging and mating (Broglia et al., 2003; Portavella et al., 2002). Male killifish can maximize their reproductive fitness by mating with as many females as possible, which likely requires them to move frequently throughout the stream. Research has indeed shown that males exhibit greater movement than females in natural streams (K. J. Howell & M. R. Walsh, unpublished data). Thus, an increase in the size of the telencephalon may enhance spatial memory and mating opportunities and therefore reproductive fitness. The connection between brain



structures and eye size is also potentially adaptive. Previous work in killifish has shown that increases in eye size are associated with greater survival and enhanced growth in sites that lack predators (Beston & Walsh, 2019). This suggests that the increased competition for food in RO sites selects for increased eye size and that brain structures may evolve as an indirect byproduct. Experimental tests are now needed to determine the fitness advantages of the connection between brain and eye size.

## **Conclusions**

Here, we found that decreased predation was associated with the evolution of larger components of the brain in fish that were reared for multiple generations in the laboratory (Figure 1). These same data also revealed positive correlations between brain components (but not brain size) and eye size, providing evidence for covariation between brain architecture and eye size (Figure 2). However, inconsistent patterns between wild-caught and common garden-reared fish highlight that varying ecological conditions across streams may alter phenotypic expression of traits. Overall, our results provide support for covariation in brain component and eye size evolution, and we propose that selection, directly or indirectly, acts upon specific brain regions rather than overall brain size to increase visual system function. More tests are now needed to understand the fitness and functional advantages of selection for larger eyes, brains, and brain structures.

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## **DATA AVAILABILITY STATEMENT**

All data have been made available on Dryad. <https://doi.org/10.5061/dryad.2547d7wpd>.

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## TABLES

**Table 1.** Results of linear mixed models for brain regions of wild caught fish with ln-length included as a covariate.

<b>Brain Region</b>	<b>Predictor</b>	<b>df</b>	<b>F</b>	<b>p-value</b>
<i>Telencephalon</i>	River	2	2.5050	0.086
	Predation	1	0.806	0.371
	<b>River x predation</b>	2	11.046	0.000
	River x length	2	2.418	0.093
	<b>River x predation x length</b>	3	9.065	0.000
<i>Dorsal Medulla</i>	River	2	1.557	0.215
	Predation	1	0.014	0.907
	<b>River x predation</b>	2	5.925	0.003
	<b>River x predation x length</b>	5	2.648	0.026
<i>Optic Tectum</i>	River	2	1.559	0.214
	<b>Predation</b>	1	6.349	0.013
	<b>River x predation</b>	2	14.896	0.000
	<b>Predation x length</b>	1	5.787	0.018
	<b>River x predation x length</b>	4	9.232	0.000
<i>Cerebellum</i>	<b>River</b>	2	3.904	0.023
	Predation	1	1.975	0.162
	<b>River x predation</b>	2	8.392	0.000
	<b>River x length</b>	2	3.518	0.032
	<b>River x predation x length</b>	3	7.425	0.000

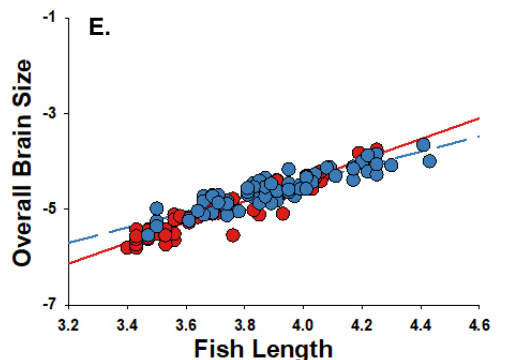
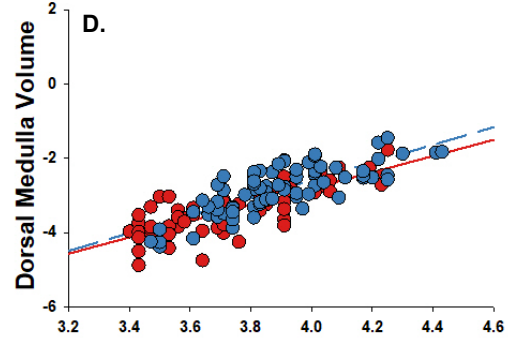
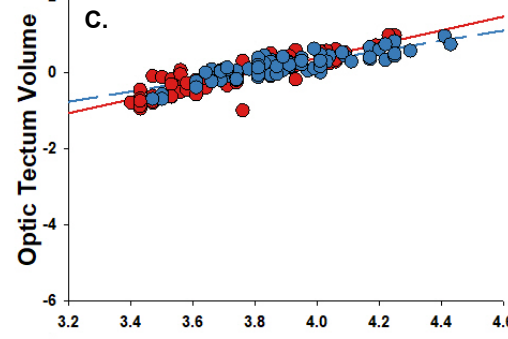
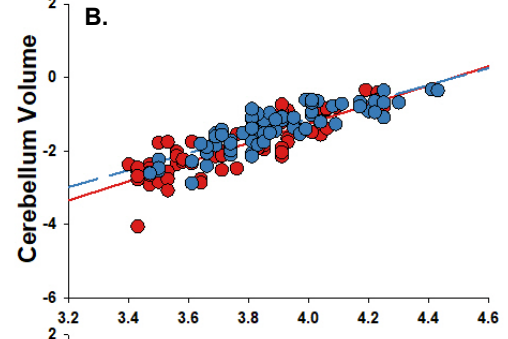
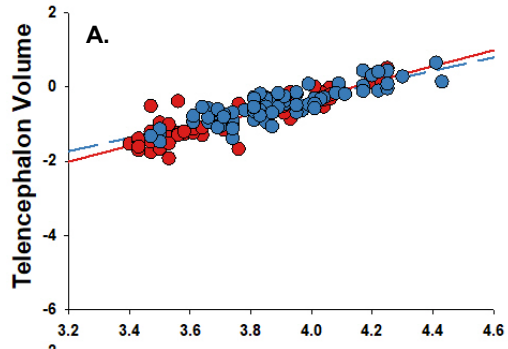
**Table 2.** Results of linear mixed models for brain regions of common garden fish with ln-length included as a covariate.

<b>Brain Region</b>	<b>Predictor</b>	<b>df</b>	<b>F</b>	<b>p-value</b>
<i>Telencephalon</i>	River	1	1.581	0.212
	<b>Predation</b>	1	10.144	0.002
	Food	1	2.314	0.132
	River x length	1	1.616	0.207
<i>Dorsal Medulla</i>	River	1	0.088	0.768
	<b>Predation</b>	1	4.667	0.034
	Food	1	3.312	0.073
<i>Optic Tectum</i>	River	1	0.233	0.631
	<b>Predation</b>	1	9.796	0.002
	Food	1	1.804	0.183
<i>Cerebellum</i>	River	1	1.123	0.293
	<b>Predation</b>	1	11.647	0.001
	Food	1	0.128	0.722
	<b>Food x predation x length</b>	3	3.920	0.012

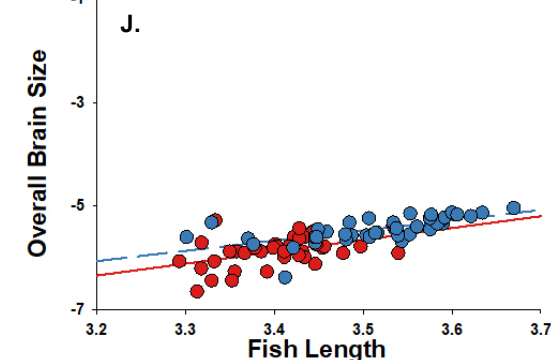
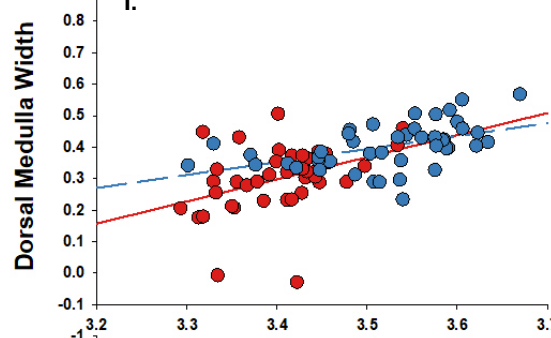
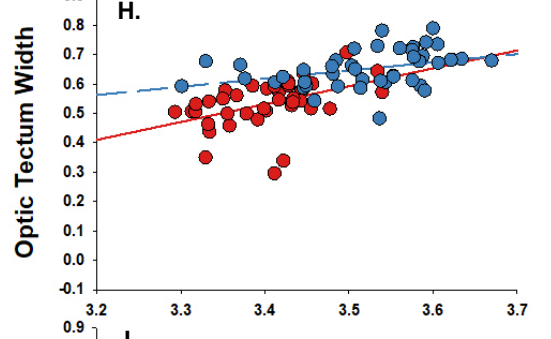
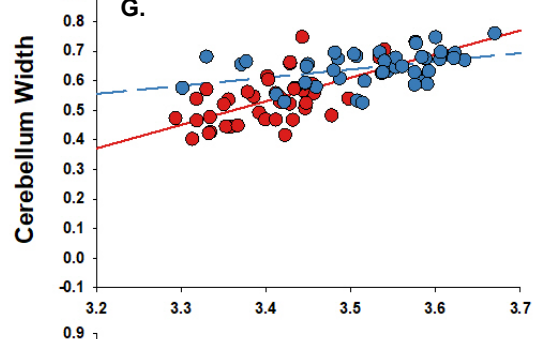
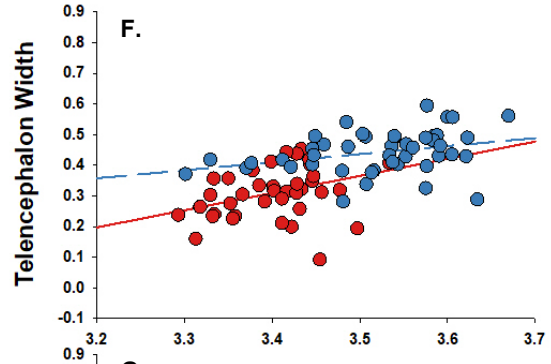
## FIGURES

**Figure 1.** Regressions between fish length (x-axis) and overall brain size or brain structure for wild caught fish and common garden fish (all data was ln-transformed). The left column shows wild caught fish (panels A-E), and the right column shows common garden fish correlations (F-J). RO data is represented by blue circles and the dashed blue line and the HP population is represented by the red circles and solid red line.

### Wild Caught

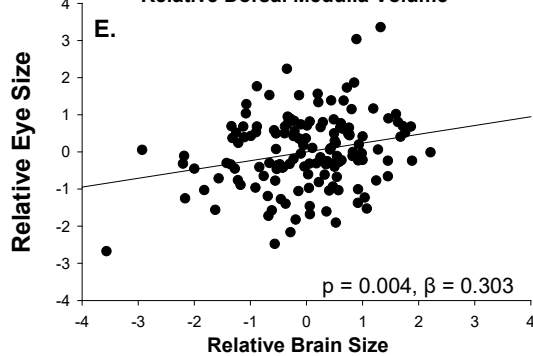
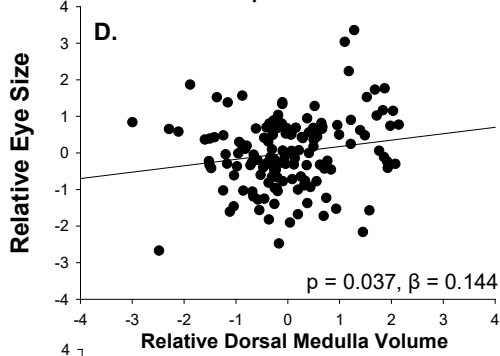
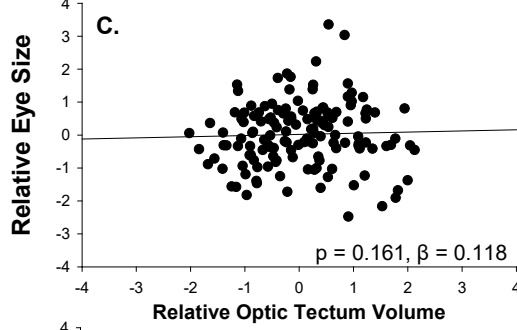
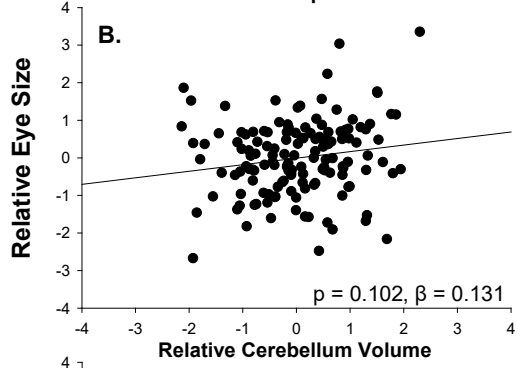
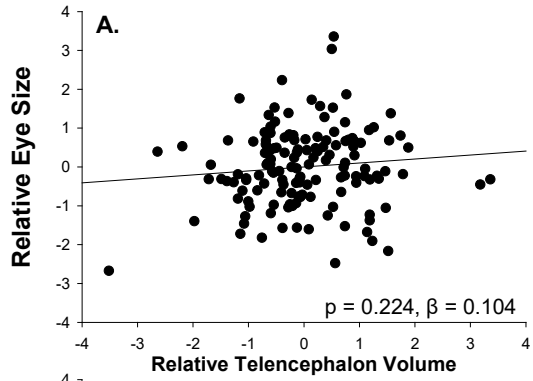


### Common Garden

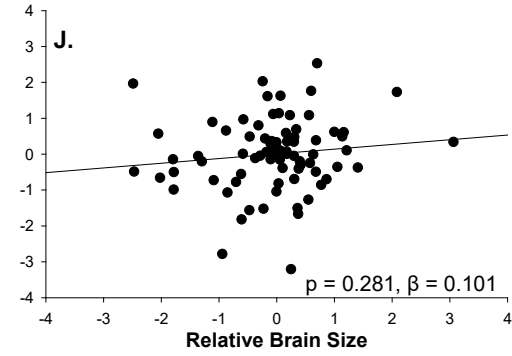
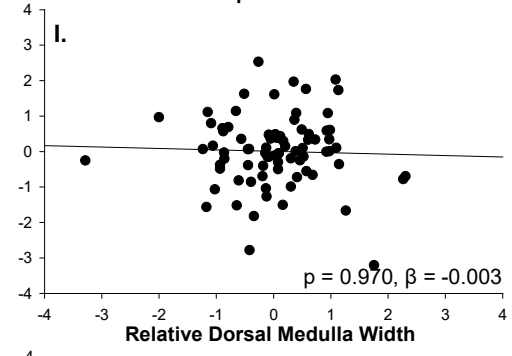
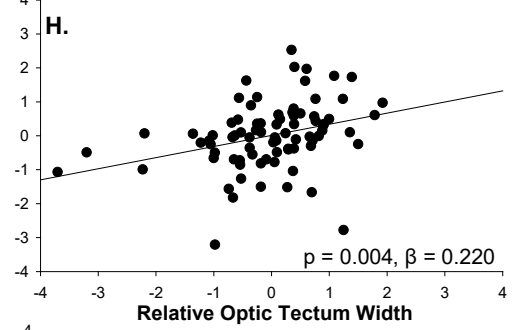
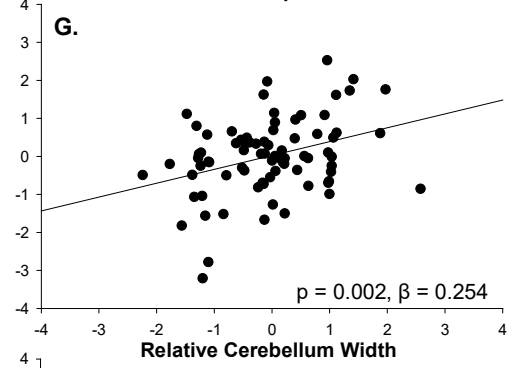
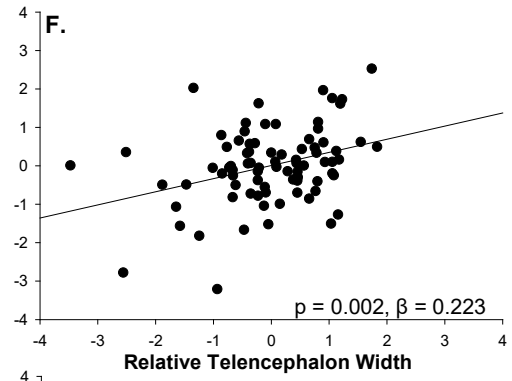


**Figure 2.** Regressions between relative brain region or relative brain size, and relative eye size. Relative brain structures were generated by outputting the residuals from regressions between an individual ln-brain structure vs. ln-fish length. Relative brain size and relative eye size were generated by outputting residuals from regressions between ln-eye or ln-brain size and ln-fish length. The left column represents the wild caught fish (panels A-E), and the right column shows the common garden fish data (F-J).

### Wild Caught



### Common Garden



## SUPPORTING INFORMATION

**Supplemental Table 1.** Results from linear mixed models for brain regions of wild caught fish with ln-brain size as the covariate.

<b>Brain Region</b>	<b>Predictor</b>	<b>df</b>	<b>F</b>	<b>p-value</b>
<i>Telencephalon</i>	River	2	0.539	0.585
	<b>Predation</b>	1	4.506	0.036
	<b>River x predation</b>	2	8.477	0.000
	Predation x brain	1	3.930	0.050
	<b>River x predation x brain</b>	4	5.953	0.000
<i>Dorsal Medulla</i>	River	2	0.267	0.766
	Predation	1	2.888	0.092
	Predation x brain	1	2.112	0.149
	<b>River x predation x brain</b>	4	2.867	0.026
<i>Optic Tectum</i>	River	2	0.498	0.609
	Predation	1	0.128	0.721
	<b>River x predation</b>	2	6.266	0.003
	<b>River x predation x brain</b>	5	4.017	0.002
<i>Cerebellum</i>	River	2	1.532	0.220
	Predation	1	0.433	0.512
	<b>River x predation</b>	2	5.216	0.007
	River x brain	2	1.620	0.202
	<b>River x predation x brain</b>	3	4.348	0.006

**Supplemental Table 2.** Correlations between the width and volume of each brain structure (wild caught data only).

	<b>Telencephalon</b>	<b>Dorsal Medulla</b>	<b>Optic Tectum</b>	<b>Cerebellum</b>
<i>Pearson correlation (r)</i>	0.407	0.416	0.456	0.309
<i>p-value</i>	0.000	0.000	0.000	0.000

**Supplemental Table 3.** Results of linear mixed models for brain regions of common garden fish with ln-brain size as the covariate.

<b>Brain Region</b>	<b>Predictor</b>	<b>df</b>	<b>F</b>	<b>p-value</b>
<i>Telencephalon</i>	River	1	0.003	0.954
	<b>Predation</b>	1	8.503	0.005
	Food	1	0.832	0.364
	River x Food	1	1.550	0.217
<i>Dorsal Medulla</i>	River	1	1.201	0.277
	Predation	1	3.664	0.059
	Food	1	0.327	0.569
	Food x predation	1	2.859	0.095
	Predation x brain	1	3.204	0.078
	<b>River x predation x brain</b>	2	4.421	0.015
	Food x predation x brain	2	1.475	0.235
<i>Optic Tectum</i>	River	1	1.992	0.162
	<b>Predation</b>	1	14.019	0.000
	Food	1	0.065	0.800
<i>Cerebellum</i>	<b>River</b>	1	4.043	0.048
	Predation	1	0.230	0.633
	Food	1	0.100	0.753
	<b>River x predation</b>	1	5.636	0.020
	River x brain	1	3.555	0.063
	River x predation x brain	2	2.792	0.068

**Supplemental Table 4.** Multiple regressions between brain size or structure and eye size with ln-length included as a covariate for both wild caught and common garden specimens. The r-square values and overall p-values represent the predictive ability of the full model. Significant p-values for brain structures are bolded.

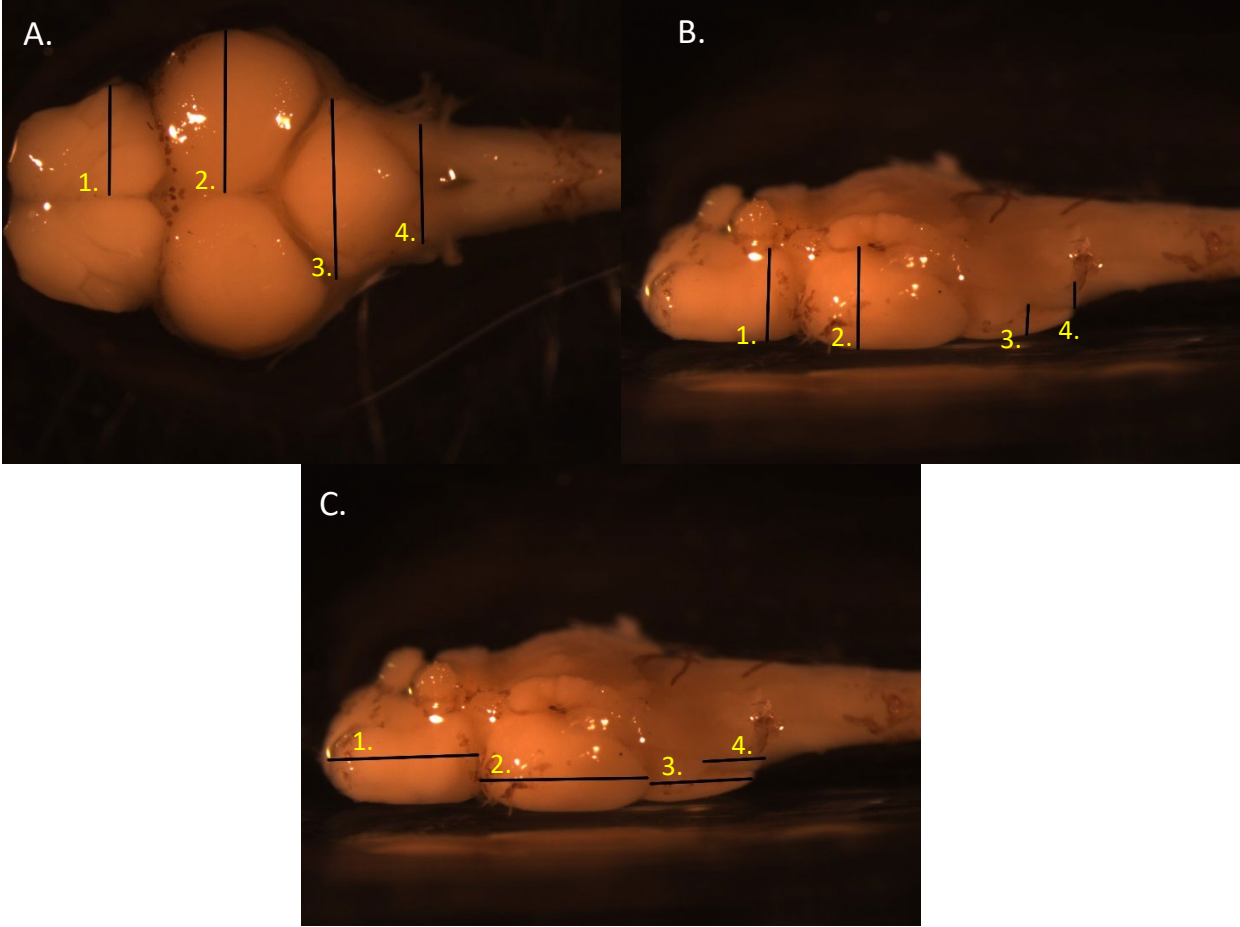


Brain Region	R square	Overall p	Wild-Caught				Common Garden			
			t	$\beta$	p-value	R square	Overall p	t	$\beta$	p-value
<i>Telencephalon</i>	0.774	0.000	1.222	0.104	0.224	0.771	0.000	3.917	0.223	<b>0.002</b>
<i>Ln Length</i>			9.212	0.786	0.000			10.354	0.721	0.000
<i>Dorsal Medulla</i>	0.779	0.000	2.103	0.144	<b>0.037</b>	0.741	0.000	-0.038	-0.003	0.970
<i>Ln Length</i>			11.125	0.761	0.000			11.866	0.862	0.000
<i>Optic Tectum</i>	0.775	0.000	1.410	0.118	0.161	0.768	0.000	3.011	0.220	<b>0.004</b>
<i>Ln Length</i>			9.245	0.775	0.000			9.770	0.714	0.000
<i>Cerebellum</i>	0.776	0.000	1.645	0.131	0.102	0.771	0.000	3.249	0.254	<b>0.002</b>
<i>Ln Length</i>			9.631	0.766	0.000			8.687	0.678	0.000
<i>Overall Brain</i>	0.785	0.000	2.889	0.303	<b>0.004</b>	0.731	0.000	1.085	0.101	0.281
<i>Ln Length</i>			5.696	0.597	0.000			8.296	0.775	0.000

**Supplemental Table 5.** Regressions between environmental factors, brain size and structure, and eye size. Environmental data is an average from multiple samples at each site (see El-Sabaawi et al. 2012), therefore all eye and brain data were averaged from ln-transformed data in order to run multiple regression.

	<b>Environmental Factor</b>	<b><math>\beta</math></b>	<b>t</b>	<b>p-value</b>
<i>Telencephalon</i>	Algae Abundance	-0.369	-1.748	0.331
	Invertebrate Abundance	-1.184	-3.744	0.166
	Light (% Canopy)	-0.032	-0.128	0.919
<i>Dorsal Medulla</i>	Algae Abundance	0.247	1.014	0.496
	Invertebrate Abundance	-1.479	-4.052	0.154
	Light (% Canopy)	0.348	1.211	0.439
<i>Optic Tectum</i>	Algae Abundance	-0.531	-0.481	0.714
	Invertebrate Abundance	0.169	0.102	0.935
	Light (% Canopy)	0.220	0.170	0.893
<i>Cerebellum</i>	Algae Abundance	-0.051	-0.135	0.915
	Invertebrate Abundance	-1.336	-2.365	0.255
	Light (% Canopy)	0.111	0.248	0.307
<i>Overall Brain</i>	Algae Abundance	-0.014	-0.202	0.873
	Invertebrate Abundance	-0.294	-3.800	0.164
	Light (% Canopy)	-0.169	-2.311	0.260
<i>Eye</i>	Algae Abundance	0.057	0.207	0.870
	Invertebrate Abundance	0.000	0.000	1.000
	Light (% Canopy)	-0.465	-1.632	0.350

**Supplemental Figure 1.** A) Dorsal view of the brain with width measurements B) Lateral view of height measurements C) Lateral view of length measurements. Brain components are represented by numbers: (1) telencephalon, (2) optic tectum, (3) cerebellum, (4) dorsal medulla.

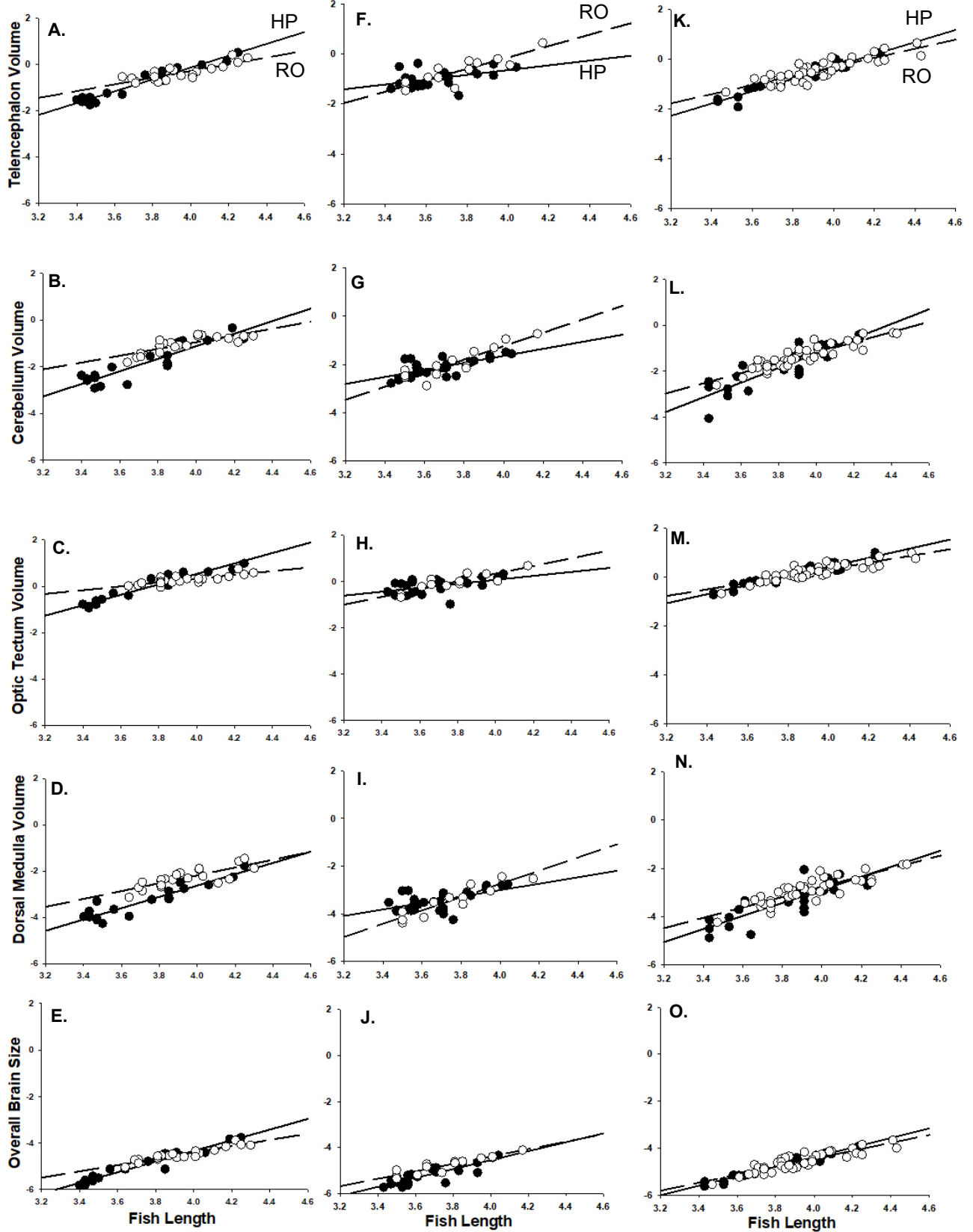


**Supplemental Figure 2.** Regressions of fish length (x-axis) and overall brain size or brain structure volume separated by river for wild caught fish. HP populations are represented by the black circles and solid black lines; RO populations are represented by the open circles and the dashed lines.

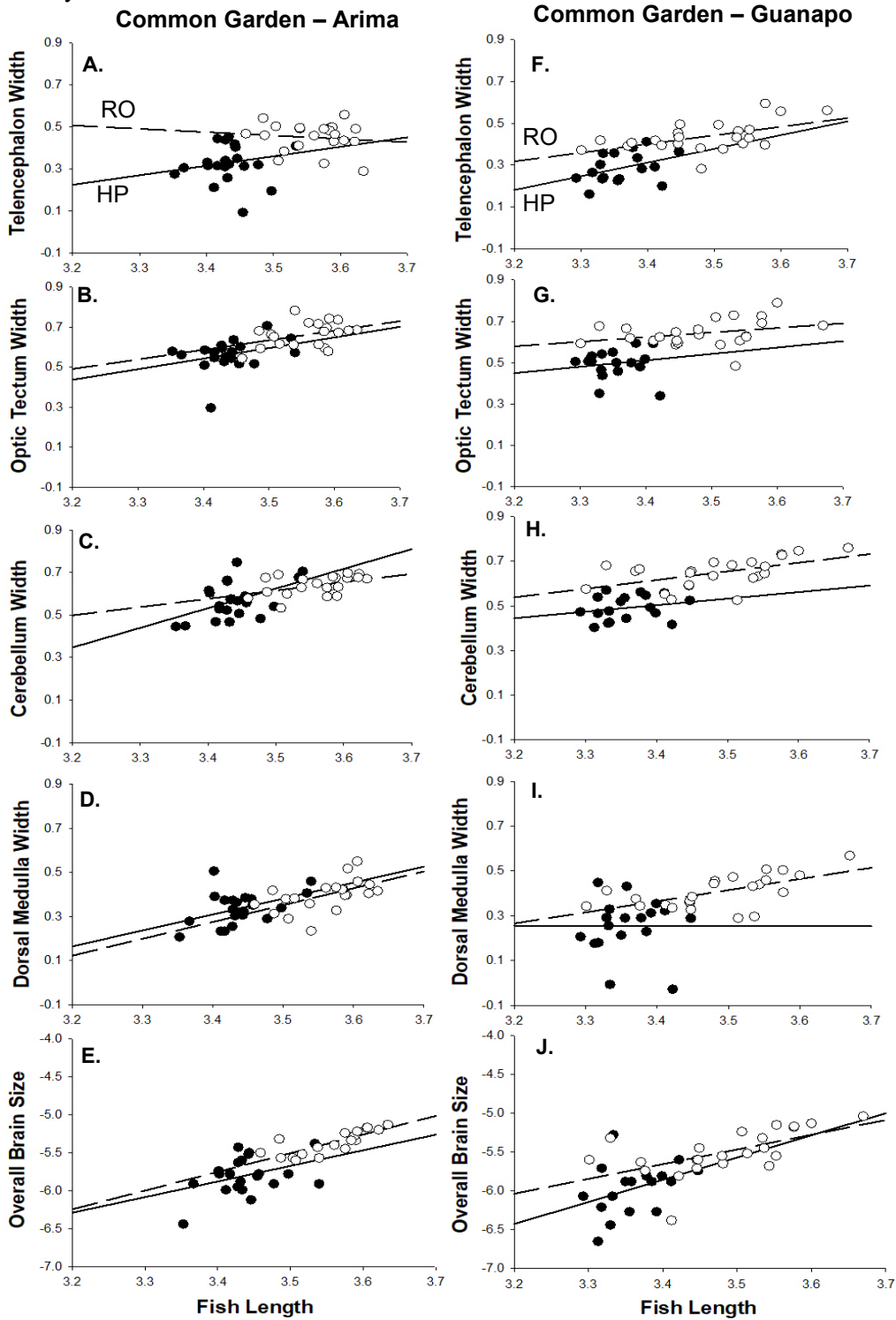
Wild Caught - Arima

Wild Caught - Aripo

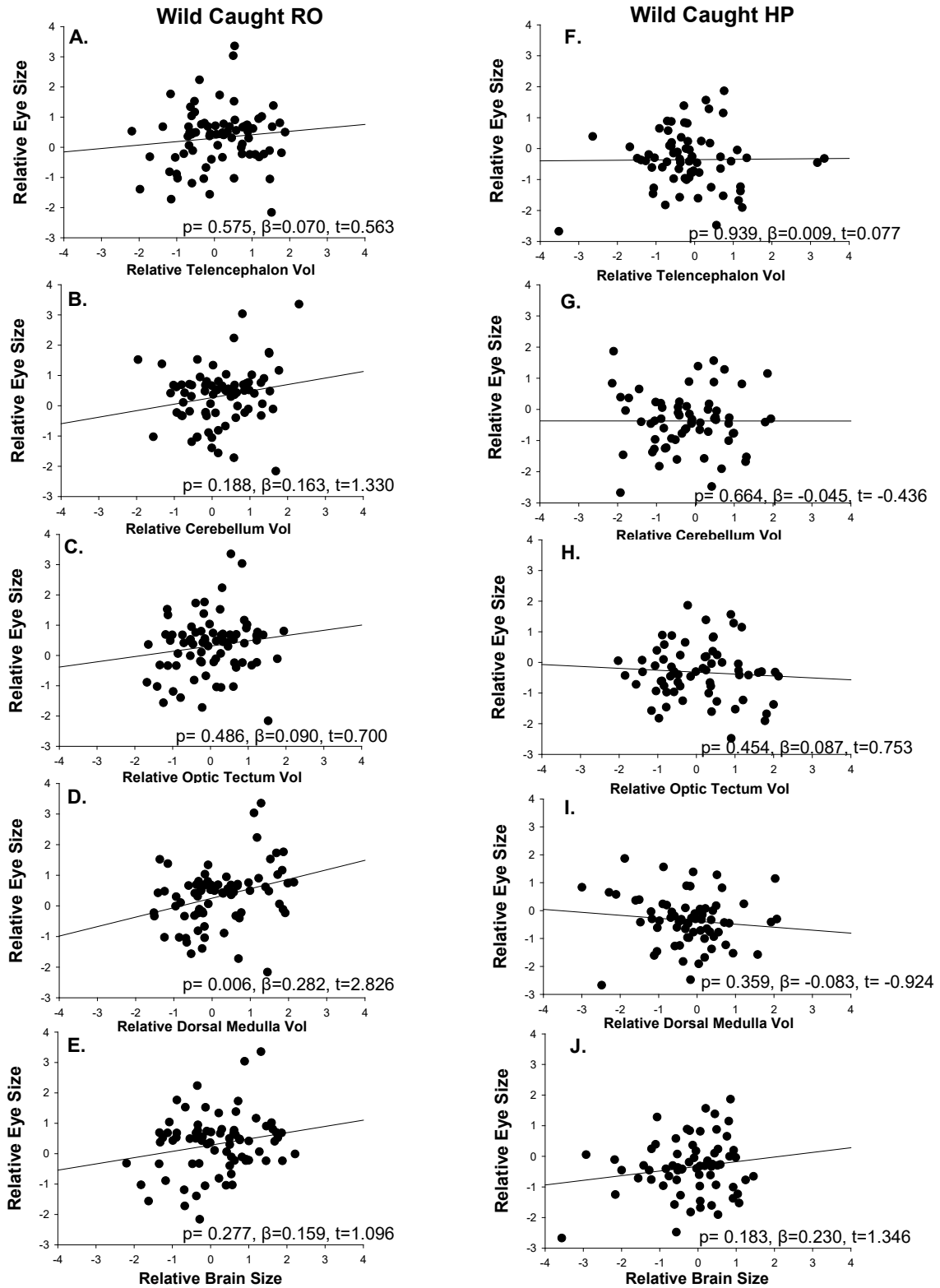
Wild Caught - Guanapo



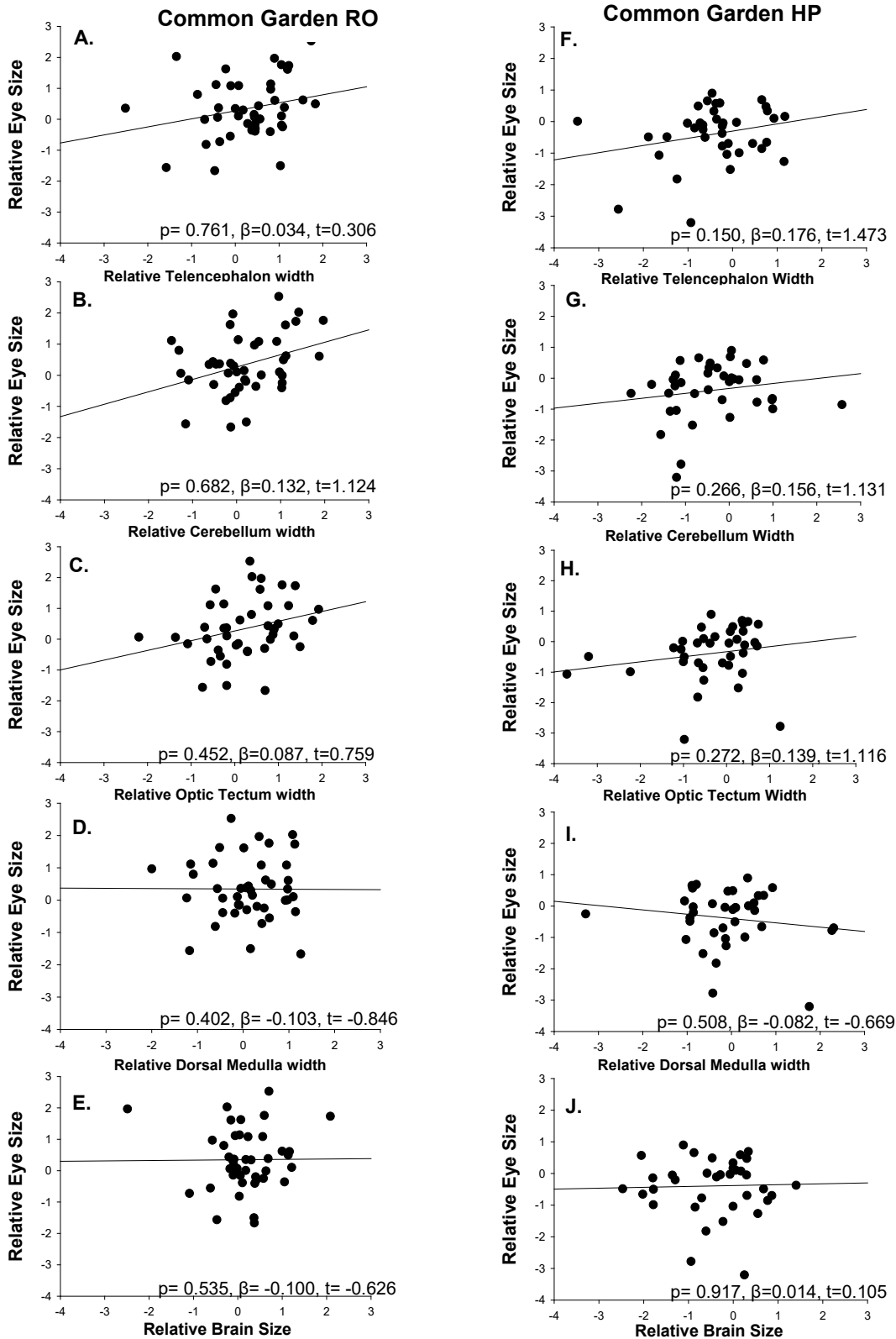
**Supplemental Figure 3.** Regressions between fish length (x-axis) and overall brain size or brain structure width separated by river and predation for common garden fish. Arima River is the left column (panels A-E), and Guanapo River is the right column (panels F-J). HP populations are represented by the solid circles and solid black lines; RO populations are represented by the open circles and the dashed lines.



**Supplemental Figure 4.** Regressions between relative brain region, relative brain size, and relative eye size by population for wild caught fish. The left column represents RO fish (panels A-E), and the right column shows HP fish (F-J).



**Supplemental Figure 5.** Regressions between relative brain region or relative brain size, and relative eye size for common garden fish. The left column represents RO fish (panels A-E), and the right column shows HP fish (F-J).





## CHAPTER 2:

### **Sex-specific evolution of brain size and structure and covariation with eye size in Trinidadian killifish\***

**Kaitlyn J. Howell<sup>1</sup>, Shannon M. Beston<sup>1</sup>, Matthew R. Walsh<sup>1</sup>**

Howell, KJ, Beston, SM, Walsh, MR. 2022. Sex-specific evolution of brain size and structure and covariation with eye size in Trinidadian killifish, *Biol. J. Linn. Soc.*, <https://doi.org/10.1093/biolinnean/blac033>

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## **ABSTRACT**

Links between contrasting ecological conditions and evolutionary shifts in neurosensory components, such as brain and eye size, are accumulating. Whether selection operates in a different manner on these traits between sexes is unclear. Trinidadian killifish (*Anablepsoides hartii*) are found in sites with and without predators. Male killifish from sites without predators have evolved larger brains and eyes than males from sites with predators. These differences in brain size are present early in life but disappear in adult size classes. Here, we evaluated female brain growth allometries to determine whether females exhibit similar size-specific differences in brain size between sites that differ in predation intensity. We also quantified brain size and structure and eye size to determine whether these structures co-evolved in a sex-specific manner. We found that female brain growth allometries did not differ across populations. Yet, female killifish from sites without predators exhibited a larger cerebellum, optic tectum, and dorsal medulla early in life (before maturation), although such differences disappeared in larger size classes. Females from sites with predators exhibited similar patterns in brain growth to males in those sites; therefore, shifts in brain size and structure are driven by differences between sexes in sites without predators. We also found evidence for covariation between brain and eye size in both sexes despite different levels of variation in both structures, suggesting that these structures might covary to fluctuating degrees in sex-specific ways. We conclude that differential investment in brain tissue in sites without predators might be linked to varying reproductive and cognitive demands between the sexes.

## **INTRODUCTION**

It is well established that features of neurosensory systems (i.e., brain size, eye size) vary across taxa (Aiello & Wheeler, 1995; Land & Fernald, 1992; Striedter, 2005; Walls, 1942). The drivers of the observed variation in brain size has been the focus of much research (Aiello

& Wheeler, 1995; Allman, McLaughlin, & Hakeem, 1993; Amiel, Tingley, & Shine, 2011; Axelrod, Laberge, & Robinson, 2021; Bauchot, Bauchot, Platel, & Ridet, 1977; Dunbar, 1998; González-Lagos, Sol, & Reader, 2010; Hoops et al., 2017; Sol, Bacher, Reader, & Lefebvre, 2008; Sol, Duncan, Blackburn, Cassey, & Lefebvre, 2005; Striedter, 2005). Explanations for variation in brain size include life history strategy (Allman et al., 1993; Deaner, Barton, & van Schaik, 2003; Yu et al., 2018), shifts in cognition (Jacobs, Gaulin, Sherry, & Hoffman, 1990; MacLean et al., 2014; Sol et al., 2005), and ecological conditions (Huber, van Staaden, Kaufman, & Liem, 1997; Safi & Dechmann, 2005). More recently, research has shown that brain size can diverge within species (Axelrod, Laberge, & Robinson, 2018; A. Gonda, Trokovic, Herczeg, Laurila, & Merilä, 2010; Abigél Gonda, Herczeg, & Merilä, 2009, 2011; Kolm, Gonzalez-Voyer, Brelin, & Winberg, 2009; Roth & Pravosudov, 2009). For instance, work by Gonda et al. (2011) revealed phenotypic differences in brain size between populations of marine and pond nine-spined sticklebacks. In addition to habitat, studies have shown that ecological variables such as competition and predation can alter the plasticity and evolution of brain size and brain structure within species (A. Gonda et al., 2010; Kotrschal, Deacon, Magurran, & Kolm, 2017).

Studies that explore the ecological drivers of variation in eye size are also accumulating (Hall, 2008; Land & Fernald, 1992; Walls, 1942). Specifically, studies have linked variation in light (Hall, 2008; Hall & Ross, 2007; Kröger & Fernald, 1994; Veilleux & Lewis, 2011), competition (Beston & Walsh, 2019; Beston, Wostl, & Walsh, 2017), and predation (Beston, Dudycha, Post, & Walsh, 2019; Glazier & Deptola, 2011; Nilsson, Warrant, Johnsen, Hanlon, & Shashar, 2012) to changes in eye size. This is important because increases in eye size have been linked to improved vision (Caves, Sutton, & Johnsen, 2017; Motani, Rothschild, & Wahl, 1999; Ross & Kirk, 2007) and shifts in foraging, mating, and predatory behavior that enhance fitness (Garamszegi, Møller, & Erritzøe, 2002; Hall & Ross, 2007; Møller & Erritzøe, 2014;

Thomas, Székely, Powell, & Cuthill, 2006). Similar to brain size and structure, such variation in eye size has also been observed within species (Beston et al., 2019, 2017; Beston & Walsh, 2019; Glazier & Deptola, 2011; Kröger & Fernald, 1994). The growing amount of research highlighting similar ecologically mediated shifts in brain and eye size suggests that selection could potentially favor coordinated shifts in these structures. Studies have begun to illuminate potential covariation between eye and brain size (Burton, 2008; Corral-López, Garate-Olaizola, Buechel, Kolm, & Kotrschal, 2017; Garamszegi et al., 2002; Howell, Beston, Stearns, & Walsh, 2021), but more are needed to further investigate this connection.

A growing number of studies have indicated that cognitive demands may differ between the sexes. That is, brain size may diverge in a sex-specific manner (Hoops et al., 2017; Iwaniuk, 2001; Kotrschal, Räsänen, Kristjánsson, Senn, & Kolm, 2012). For example, Iwaniuk (2001) found that male ground squirrels that experience indirect and direct male-male competition had larger brains than females. In brown trout, mating strategy has been connected to sex-specific differences in brain size and structure; non-migratory female trout had larger brains than non-migratory males, while males had larger telencephalons regardless of mating strategy (Kolm et al., 2009). Sex-specific brain variation has also been correlated with care type and diet in fish and sperm competition in birds (Garamszegi, Eens, Erritzøe, & Møller, 2005; Gonzalez-Voyer, Winberg, & Kolm, 2009). Furthermore, work has revealed sex-specific shifts in eye size that may be due to differences in behavior (Rutowski, 2000) or differences in predator-induced plasticity (Meuthen, Baldauf, Bakker, & Thünken, 2018; Svanbäck & Johansson, 2019). Many such studies make comparisons across species but our understanding of sex-specific patterns of brain size evolution and, in particular, sex-specific connections between brain and eye size evolution within a species is more limited (Buechel et al., 2019; Burger, Saucier, Iwaniuk, & Saucier, 2013; Herczeg, Välimäki, Gonda, & Merilä, 2014; Kotrschal et al., 2013; Park & Bell, 2010).

Trinidadian killifish (historically '*Rivulus hartii*' Boulenger, 1890 but now *Anablepsoides hartii*) are found in fish communities that vary in predation intensity. This includes downstream, lowland 'high predation' (HP) sites where killifish co-occur with multiple species of piscivorous fish (e.g., *Hoplias malabaricus*, *Crenicichla frenata*) and upstream, '*Rivulus*-only' (RO) sites where killifish are the only fish species present (*Rivulus*-only is used given historical precedent; Gilliam et al. 1993; Fraser et al. 1999; Walsh and Reznick 2008). Killifish in RO sites are found at higher densities and thus experience strong intraspecific competition compared to fish found in sites with predators (Gilliam et al., 1993; Walsh & Reznick, 2008). In HP sites, killifish experience increased mortality rates likely due to the presence of piscivorous fish (Furness & Reznick, 2014). Research has shown that variation in predation intensity is associated with evolved differences in life history traits (Walsh, Fraser, Bassar, & Reznick, 2011; Walsh & Reznick, 2008, 2009, 2010, 2011). More recent work has also revealed an association between increased predation and evolutionary shifts in brain size, brain architecture, and eye size (Beston & Walsh, 2019; Beston et al., 2017; Howell et al., 2021; Walsh, Broyles, Beston, & Munch, 2016). Male killifish from sites without predators have evolved a larger brain, larger brain structures (specifically telencephalon, cerebellum, optic tectum, and dorsal medulla), and larger eye size (Beston et al., 2017; Howell et al., 2021; Walsh et al., 2016). However, these differences in brain size between populations depend upon body size; they are apparent early in life for small size classes of killifish (~30mm in total length) but then disappear as the fish grow into adult size-classes (by the time killifish are ~52 mm in total length). This is likely because male killifish from HP sites exhibit higher rates of brain cell proliferation and steeper allometric growth trajectories than males from sites without predators (Dunlap, Corbo, Vergara, Beston, & Walsh, 2019). There is also a positive covariation between brain components (telencephalon, optic tectum, cerebellum) and eye size in males (Howell et al., 2021).

Here, we utilized wild caught and second-generation common garden reared specimens to quantify brain growth allometries and connections between brain and eye size in female killifish from HP and RO sites. We assume that any differences that exist in the common garden specimens are genetic in origin. Females invest much more heavily into reproduction when compared with males (Walsh & Reznick, 2008) which may influence allocations towards brain and eye tissue (Aiello & Wheeler, 1995). In fact, we previously failed to detect differences in female brain size in newly mature individuals from RO and HP sites (Walsh et al., 2016). Here we examined female brain growth allometries across their entire range of body sizes and quantified the relationship between female brain size, brain structure, and eye size. This will allow us to compare brain growth allometries across sexes and determine if covariation between brain components and eye size evolves in a sex-specific manner.

## **METHODS**

### *Wild caught specimens:*

We collected killifish from high predation and Rivulus-only sites from four rivers (Arima, Aripo, Guanapo, and El Cedro) during May-June 2016. Fish were euthanized immediately upon collection using MS-222, preserved in 10% formalin, and then stored in 70% ethanol until they were measured for total length, photographed for assessment of eye size, and their brains were dissected during Summer-Fall 2016. Eye size was measured using the diameter of the eye cavity at the widest part for each photograph (Fig. S1). Brains were dissected by cutting from the top of each gill slit and then removing the lower jaw and the tissue between the roof of the mouth and the braincase. Excess tissue was removed from the brain as much as possible without damaging any structures. Dissections were performed by the same individual to ensure all brains were trimmed consistently. Brains were blotted dry, weighed, and then preserved in 70% ethanol until photographs were taken (males in 2019 and females in 2021; we assume any shrinkage over time is similar across populations). We photographed the dorsal and lateral

surface of each brain to measure brain structures. We measured the width, height, and length of the telencephalon, optic tectum, cerebellum, and dorsal medulla via ImageJ to quantify the volume of each via the ellipsoid model:  $V = (L \times W \times H) \pi/6$  (Fig S1). This approach has been shown to provide an accurate estimate of the volume of fish brain structures (Huber et al., 1997; Pollen et al., 2007). To ensure these calculated volumes accurately reflect brain size, we ran correlations between each brain structure and brain mass; all correlations were significant ( $p < 0.01$ ; Table S1).

We first evaluated differences in female brain size between populations and then made quantitative comparisons with males by analyzing both sexes together. We analyzed variation in female brain size via general linear models (SPSS v. 26 IBM Corporation) with river (Arima, Aripo, Guanapo, El Cedro) and fish community (high predation, Rivulus-only) entered as fixed effects and total length as a covariate. Because we were interested in female brain growth across their entire body size range, we also entered the 'predation x length' interaction as a fixed effect. Fish length and brain size were ln-transformed to improve normality and homogeneity of variance. We ran a linear regression between ln-fish length and ln-brain size and computed residuals to identify any outliers; residuals  $\pm 3$  were considered outliers and removed (3 brain outliers removed). Our sample size included 200 female killifish.

We also evaluated differences in female brain structure allometries between populations via general linear models, following the same methods described above but with overall brain size entered as a covariate (instead of length). Brain structure volume was ln-transformed to improve normality and variance. Our sample size for brain structure analysis varied slightly by structure as we did not include structures that were damaged or otherwise unable to be measured (telencephalon  $n=167$ , optic tectum and cerebellum  $n=168$ , dorsal medulla  $n=164$ ).

We then performed analyses that included the previously published data on males to make direct comparisons between the sexes (see Howell et al., 2021). These general linear

models included river, predation, and sex as fixed effects. For overall brain size, we used  $\ln$ -length as the covariate and for each brain structure we used  $\ln$ -brain as the covariate. We were interested in differences between the sexes in brain growth across the range of body sizes. As a result, we included the 'predation x sex' and the 'predation x sex x length' interactions (or 'predation x sex x brain' for structures) as fixed effects. Our sample size for brain size included 172 males, for a total of 372 killifish; sample size for each brain structure included 143 males.

Additionally, we performed multiple linear regressions to evaluate the extent to which female brain size and structures covary with eye size. Eye size ( $\ln$ -transformed) was the dependent variable with  $\ln$ -transformed brain size and each  $\ln$ -transformed brain structure as the independent variable (SPSS v26 IBM Corporation). We then separated the data by population and ran regressions to evaluate differences in brain size, structure, and eye size across predation regimes. The regression between brain size and eye size included  $\ln$ -total length as a covariate while the regressions between brain structures and eye size included  $\ln$ -brain size as a covariate.

#### *Common Garden specimens:*

The methods for common garden experiments were previously published (Walsh & Reznick, 2008) and are briefly summarized here. We collected wild caught killifish from HP and RO sites in the Arima and Guanapo rivers in July 2005 and established laboratory stocks from approximately 20 fish per population (10 males, 10 females, 72 fish total). We then produced the first laboratory generation by randomly pairing wild-caught males and females from the same locality. Eggs were collected from each pairing and upon hatching offspring were placed in aquaria at a maximum density of eight fish per tank and were fed an ad libitum diet of brine shrimp nauplii and liver paste. We produced the second laboratory generation by pairing mature females from the first generation with mature males from the same locality but different lineage. All offspring were reared at densities of eight fish per 9-l aquaria and fed ad libitum. Once fish



were 20 days old, eight fish from each pairing were individually placed in separate 9-l aquaria. Then, the fish from each pairing were divided equally into two food treatments: (a) a 'high food' ration that approximated growth in HP sites and (b) a 'low food' ration that approximated the growth naturally observed in RO sites (Walsh & Reznick, 2008). Fish were fed measured portions of liver paste and brine shrimp nauplii and reared until maturity in order to quantify life history traits (see Walsh and Reznick 2008 for details on trait quantification). After maturation, males were immediately euthanized and preserved in 5% formalin. Eggs were collected from females for two weeks after maturation, and then females were euthanized and preserved in 5% formalin. All preserved fish were stored for about eight years prior to being photographed for eye size, weighed, and dissected for brain size and structure measurements. We assumed equal shrinkage of samples between populations and sexes; any fish that were dried out or damaged were not measured. Data on eye size was limited to male killifish as we lacked lateral images to measure the eye size of females.

Brains were dissected from preserved specimens beginning in August 2015 (see Walsh et al. 2016). We removed the brain, gently blotted the brain dry, and then photographed the dorsal surface of each. Again, brains were dissected by one individual to ensure consistency. This allowed us to measure the width of the telencephalon, cerebellum, optic tectum, and dorsal medulla via ImageJ. Note that we only have images associated with the dorsal surface of the brain and therefore could not calculate the volume of the brain structures for common garden fish. Our sample size included 77 females for each of the four brain structures.

We evaluated differences in common garden reared female brain structures between populations via linear mixed models (SPSS v. 26 IBM Corporation) with river (Arima, Guanapo), fish community (high predation, Rivulus-only), and the 'predation x length' interaction all entered as fixed effects. We included parental lineage (family) nested within the 'river x population' interaction as a random effect and overall brain size (ln-transformed) as a covariate. Analysis of

overall brain size was not included because it has been previously evaluated in common garden females (see Walsh et al. 2016).

Lastly, we directly compared the growth of the brain structures between males and females using linear mixed models. We included river, predation, and sex as fixed factors, family nested in 'predation x river' as a random effect, and ln-brain size as the covariate. Since we were interested in differences in brain growth across the range of body sizes between the sexes, we also included the 'predation x sex' and the 'predation x sex x brain' interactions as fixed effects. Our sample size included 85 males, for a total of 163 killifish for each brain structure.

## RESULTS

### Wild caught specimen:

#### *Female-only brain size and structure:*

Female brain size did not vary significantly by predator community or river of origin ( $p > 0.05$ , Table 1). The 'predator x length' interaction was also non-significant ( $p > 0.05$ , Fig 1A). We instead observed significant ( $p < 0.05$ ) differences between the fish communities for three female brain structures (optic tectum, cerebellum, and dorsal medulla). Differences in the telencephalon between fish communities was marginally non-significant ( $p = 0.056$ ; Table 1). We also observed significant 'predation x length' interactions for the optic tectum, cerebellum, and dorsal medulla ( $p < 0.05$ , Table 1). The differences in allometry were consistent across all brain structures – steep brain structure growth in HP populations and shallower relationships between brain size and brain structure in RO populations (Fig. 1B-E). RO females exhibited a larger telencephalon (10%), optic tectum (7%), cerebellum (12%), and dorsal medulla (5%) in smaller size classes (35mm), but such differences disappeared or were reversed when fish were approximately 41-42mm in total length (except for differences in optic tectum, which disappeared around 37mm; Fig. 1B-E).

### *Female brain and eye size correlations:*

The results of the multiple regressions revealed a significant positive ( $p < 0.05$ ) link between overall brain size and eye diameter (Table 2; Fig. 2A). The regressions between female brain structures (telencephalon, optic tectum, cerebellum, dorsal medulla) and eye size were not significant (Table 2; Fig. 2B-E). After separating the multiple regressions by population, we found that the connections between the optic tectum and eye size and dorsal medulla and eye size were significant for HP fish ( $p=0.030$ ,  $p=0.037$  respectively), and the connection between overall brain size and eye size was significant for RO fish ( $p=0.021$ ).

### *Comparisons between sexes:*

In the analyses that included the data for males and females, overall brain size varied significantly by sex, predation, and river ( $p < 0.05$ , Table 3). Furthermore, the 'predation x sex' interaction was also significant ( $p < 0.05$ , Fig S2). Males exhibited larger differences in brain size between populations than females, but the overall patterns were similar for both sexes. Overall, small males have ~1% larger brain size compared to small females at 35mm total length. Additionally, the 'predation x sex x length' interaction was significant. RO males and females have larger brains than HP males and females, respectively, at smaller sizes but then differences disappear or reverse as they grow. The differences in males and females between populations early in life are larger in males than females. At 35mm, males from RO sites have brains that are 4.2% larger than HP males, while females only exhibit a 1.4% shift in brain size (Fig. S2).

All brain structures exhibited significant 'predation x sex' interactions ( $p < 0.05$ ) and significant 'predation x sex x brain' interactions ( $p < 0.05$ ; Table 3) except for the telencephalon, which was slightly non-significant ( $p=0.06$ ). RO females exhibit larger brain structures compared to HP females at smaller brain sizes, but differences disappeared as overall brain size increased. The 'predation x sex x brain' interaction was likely significant because males exhibit much smaller differences in brain structure allometries across sites compared to females (Fig.

S2). Sex was also a significant predictor for each structure except for the telencephalon, while predation was significant only for cerebellum and optic tectum volume (Table 3).

*Common garden specimen:*

*Female-only brain structure:*

We did not detect significant effects associated with predator community, river, or the 'predation x brain' interactions for the telencephalon (mean± std. deviation: RO=0.542±0.085, HP=0.52±0.090), optic tectum (RO=0.708±0.084, HP=0.730±0.068), cerebellum (RO=0.738±0.075, HP=0.740±0.059), or dorsal medulla (RO=0.508±0.076, HP=0.513±0.077) ( $p>0.05$ ; Table 4).

*Comparisons between sexes:*

Predator community, river, family, and sex were not significant predictors for each measured brain structure ( $p>0.05$ ). Neither the 'predation x sex' interaction nor the 'predation x sex x brain' interaction were significant for any of the brain structures ( $p>0.05$ ; Table 5).

## **DISCUSSION**

Our results show that female brain size (for both wild-caught and common garden reared specimens) does not differ significantly between sites with and without predators (Fig. 1A). However, female brain structures (cerebellum, optic tectum, and dorsal medulla) do vary across populations in a size-specific fashion (Fig. 1B-E). Wild-caught killifish from RO sites exhibited a larger cerebellum, optic tectum, and dorsal medulla early in life (in small size classes of fish). Such differences then disappear as the fish grow and develop (~41mm). This is because females from sites with predators had much steeper allometric slopes than RO females for each measured brain region (Fig. 1B-E). Overall, HP females exhibit a rate of brain structure growth that is approximately 1.5-3 times greater than RO females (based on allometric slopes). This contrasts with the patterns observed in males – males exhibit differences in brain growth allometry across predation regimes but brain structure growth allometries do not differ between

HP and RO sites (Dunlap et al., 2019; Howell et al., 2021). Additionally, we also observed a significant connection between brain and eye size; in wild-caught females, overall brain size was positively correlated with eye size (Fig. 2A). This aligns with patterns observed in wild-caught males, as males also exhibited positive covariation between brain size and eye size.

Wild-caught females exhibit differences in brain structures between sites with and without predators in small, juvenile size-classes. In particular, females in RO sites exhibited larger cerebellum, optic tectum, and dorsal medulla when compared with small females from HP sites. Females likely invest much more energy into reproduction early in life than males; therefore, one possibility is that RO females are unable to spend as much energy on overall brain size early in life as RO males do. Given that RO sites are characterized by higher killifish density, lower resources, and increased competition compared to HP sites (Gilliam et al., 1993; Fraser et al., 1999), selection may still favor increases in female brain structures that are connected to enhanced cognitive abilities such as learning, coordination, and movement to help ameliorate the negative consequences of residing in a high competition environment. It is also unclear why we observed differences in female brain structures but not overall brain size between the populations. One possibility is that other unmeasured brain structures could be smaller in RO females (e.g., energy tradeoffs), which ultimately led to the lack of overall differences in brain size between the fish communities despite differences in the telencephalon, optic tectum, and dorsal medulla. Further investigation into possible differences in brain anatomy and neural morphology (i.e., neurogenesis, neuron density, cell proliferation, etc.) are needed.

Differences in brain size and structure between sexes is driven by RO fish, as males and females from HP sites exhibit very similar brain growth trajectories (Fig. S3). That is, early in life, male killifish in RO sites exhibit larger brains than males from HP sites and females from both sites. Why do male and female killifish from sites without predators invest differentially in brain tissue? One plausible explanation is that females invest more energy into reproduction (i.e. egg

production and reproductive tissue) than males (Parker, 1970; Hayward & Gillooly, 2011) resulting in a trade-off between brain tissue and reproductive investment early on in life. Differences in brain size have indeed been linked to shifts in reproductive effort. For example, guppies that were artificially selected for a larger relative brain size produced fewer offspring (Kotrschal et al., 2013). But another study showed that killifish with higher rates of reproductive effort exhibit relatively larger brains than killifish with lower reproductive rates (Sowersby et al., 2021). Our results revealed the opposite pattern in brain size – fish from HP sites (which exhibit a 'faster' life-history and higher reproductive effort, Walsh & Reznick, 2009) exhibit smaller relative brains than fish from RO sites (which exhibit a 'slower' life-history). However, we find a similar trend as Sowersby et al., (2021) when looking at brain growth allometries; killifish with higher rates of reproductive effort (HP fish) exhibit higher rates of brain growth than those with lower reproductive effort (RO fish). This indicates that the connection between life history and brain size is complex. Furthermore, contrasting reproductive behaviors between males and females may also alter investment in brain tissue (Jacobs, 1996). One example of this is in nine-spined stickleback, where males perform courtship, defend territories, and build nests while females choose mates and produce eggs (Herczeg et al., 2014). Male stickleback exhibited larger brain size and larger brain structures (telencephalon, cerebellum, hypothalamus) compared to females. A larger brain size early in life for RO males may be positively related to their ability to forage and find mates. This high competition environment may drive strong selection on aspects of behavior and increased cognition, particularly in males. The end result is that, as in sticklebacks, the sexual dimorphism in brain size and structure observed in RO sites may be due to increased competition for mates in conjunction with increased reproductive investment in females.

In wild-caught fish, we found that females exhibited similar positive covariation between overall brain size and eye size as males despite much smaller differences in female brain size between HP and RO sites. This pattern in females is interesting because we failed to detect

significant differences in overall brain size across populations. However, previous work showed females exhibit significant differences in eye size across populations (Beston et al., 2017). Specifically, RO females have eyes that are 12% larger than HP females, while males only exhibit a 7% shift in eye size. Together, this indicates that even minor variation in brain size may lead to covariation with eye size and suggests that these structures can covary to differing degrees within species, particularly in a sex-specific manner. Regardless, the connection between brain and eye size is potentially adaptive in both sexes. Increases in killifish eye size have been linked to enhanced growth and improved survival in RO sites (Beston & Walsh, 2019), suggesting that increased competition in these sites selects for larger eye size and that brain size may evolve as an indirect byproduct of selection. If this is the case, then correlation between brain and eye size may be driven by functional or genetic links between structures. Alternatively, the covariation could be explained by similar selection for brain and eye size across predation regimes. We further explored the cause of this correlation using additional linear regressions between brain and eye size with predation regime added as a predictor variable. This analysis did not change the significant correlation between brain and eye size (Table S2) and therefore suggests that these structures may covary genetically or functionally in females. However, more research is needed to better understand this connection.

It is important to note that we found contrasting patterns of female brain structure variation between wild caught and common garden fish. That is, the differences in brain structures were not observed in the common garden reared fish. One potential explanation is that the trends for wild caught fish are influenced by a multitude of ecological variables (see Howell et al. 2021 for further discussion). This may indicate shifts in female brain architecture are environmentally driven and not maintained across generations in lab reared fish. However, the common garden fish were 40mm in total length, on average. The data from the wild caught females show that differences in brain structures disappear as fish attain that size class. As a result, it is not necessarily surprising that we failed to detect differences in brain structure in the

lab reared fish. In general, the genetic basis of population divergence in female brain structure requires more study.

## **Conclusions**

We found that female brain size did not differ across predation regimes when all size classes were considered, but that wild-caught female brain structure allometries did vary across predation regimes. Additionally, male and female wild-caught killifish both exhibit covariation between brain size and eye size despite exhibiting shifts of varying magnitudes in these neurosensory structures. This indicates that brain and eye size may covary to differing degrees in a sex-specific manner. Overall, these results are some of the first to indicate differential brain tissue investment between sexes across their lifespan. We hypothesize that the variation between sexes in sites without predators is linked to differences in reproductive allocation, and/or differential impacts of competition. However, further research is now needed to directly measure such energetic costs in male and female killifish.

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## **DATA ACCESSIBILITY**

All data are available at the Dryad Digital Repository,  
<https://doi.org/10.5061/dryad.9w0vt4bhs>.

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## TABLES

**Table 1.** Results from general linear models evaluating differences in brain size and brain structure growth allometries in wild caught females. Significant p-values are bolded.

Brain Region	Predictor	df	Mean Squares	F	p-value
<b>Overall Brain</b>	River	3	0.046	1.539	0.206
	Predation	1	0.081	2.717	0.101
	Predation x length	1	0.067	2.241	0.136
<b>Telencephalon</b>	River	3	1.064	7.618	<b>0.000</b>
	Predation	1	0.516	3.693	0.056
	Predation x brain	1	0.432	3.095	0.080
<b>Optic Tectum</b>	River	3	0.240	2.173	0.093
	Predation	1	2.368	21.444	<b>0.000</b>
	Predation x brain	1	1.924	17.420	<b>0.000</b>
<b>Cerebellum</b>	River	3	0.204	1.319	0.270
	Predation	1	2.723	17.636	<b>0.000</b>
	Predation x brain	1	2.558	16.564	<b>0.000</b>
<b>Dorsal Medulla</b>	River	3	1.268	5.635	<b>0.001</b>
	Predation	1	1.527	6.789	<b>0.010</b>
	Predation x brain	1	1.401	6.227	<b>0.014</b>

**Table 2.** Results from multiple linear regressions between brain size and eye size with Ln-length included as covariate, and between brain structures and eye size with Ln-brain included as covariate, in wild caught females. The r-square values and overall p-values represent the predictive ability of the full model.

Brain Region	R square	Overall p	t	$\beta$	p-value
<i>Telencephalon Ln Brain</i>	0.690	0.000	0.423	0.029	0.672
			11.870	0.808	0.000
<i>Optic Tectum Ln Brain</i>	0.690	0.000	-0.794	-0.044	0.428
			15.458	0.857	0.000
<i>Cerebellum Ln Brain</i>	0.689	0.000	-0.312	-0.019	0.756
			13.904	0.843	0.000
<i>Dorsal Medulla Ln Brain</i>	0.693	0.000	0.387	0.020	0.699
			15.545	0.821	0.000
<i>Overall Brain Ln Length</i>	0.836	0.000	3.471	0.276	<b>0.001</b>
			7.365	0.585	0.000

**Table 3.** Results from general linear models including both male and female wild-caught killifish. Significant p-values are bolded.

Brain Region	Predictor	df	Mean Squares	F	p-value
<i>Brain Size</i>	River	3	0.071	2.426	0.065
	Predation	1	0.678	23.036	<b>0.000</b>
	Sex	1	0.105	3.559	0.060
	Predation x sex	1	0.196	6.663	<b>0.010</b>
	Predation x sex x length	3	0.269	9.145	<b>0.000</b>
<i>Telencephalon</i>	River	3	0.369	3.351	<b>0.019</b>
	Predation	1	0.045	0.409	0.523
	Sex	1	0.108	0.985	0.322
	Predation x sex	1	0.801	7.281	<b>0.007</b>
	Predation x sex x brain	3	0.273	2.479	0.061
<i>Optic Tectum</i>	River	3	0.110	1.445	0.230
	Predation	1	1.277	16.716	<b>0.000</b>
	Sex	1	0.863	11.306	<b>0.001</b>
	Predation x sex	1	1.127	14.753	<b>0.000</b>
	Predation x sex x brain	3	0.739	9.674	<b>0.000</b>
<i>Cerebellum</i>	River	3	0.419	3.012	<b>0.030</b>
	Predation	1	0.655	4.711	<b>0.031</b>
	Sex	1	1.510	10.850	<b>0.001</b>
	Predation x sex	1	2.491	17.905	<b>0.000</b>
	Predation x sex x brain	3	1.351	9.709	<b>0.000</b>
<i>Dorsal Medulla</i>	River	3	0.620	2.935	<b>0.034</b>
	Predation	1	0.040	0.191	0.662
	Sex	1	2.883	13.652	<b>0.000</b>
	Predation x sex	1	2.401	11.372	<b>0.001</b>
	Predation x sex x brain	3	1.383	6.550	<b>0.000</b>

**Table 4.** Results from linear mixed models evaluating differences in brain structures and allometry in common garden reared females. Wald Z values are included only for random effects. Note that the full model with random effect for the optic tectum could not be computed so family was removed for this model (the results are the same as a general linear model).

Brain Region	Predictor	Numerator	Denominator	F	Wald Z	p-value
		df	df			
<i>Telencephalon</i>	River	1	17.223	1.617	-	0.220
	Predation	1	65.189	0.003	-	0.958
	Predation x brain	1	65.597	0.001	-	0.978
	Family	-	-	-	0.433	0.665
<i>Optic Tectum</i>	River	1	72	0.017	-	0.897
	Predation	1	72	0.002	-	0.963
	Predation x brain	1	72	0.000	-	0.985
	Family	-	-	-	-	-
<i>Cerebellum</i>	River	1	15.871	2.166	-	0.161
	Predation	1	65.832	3.341	-	0.072
	Predation x brain	1	66.238	3.355	-	0.072
	Family	-	-	-	0.496	0.620
<i>Dorsal Medulla</i>	River	1	17.104	0.212	-	0.651
	Predation	1	70.902	1.845	-	0.179
	Predation x brain	1	70.950	1.861	-	0.177
	Family	-	-	-	1.143	0.253



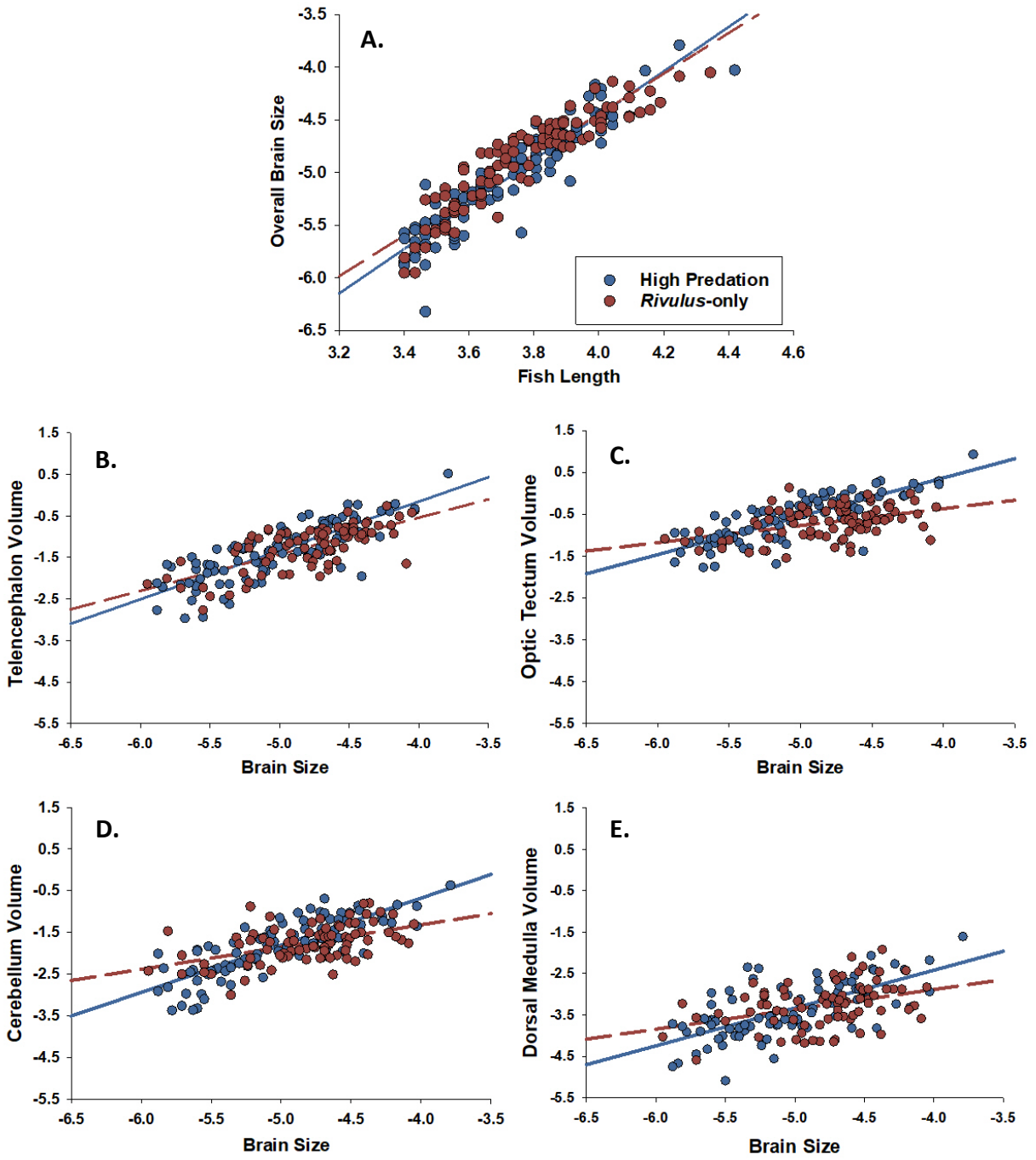
**Table 5.** Results from linear mixed models including both male and female common garden killifish. Wald Z values are included for random effects.

<b>Brain Region</b>	<b>Predictor</b>	<b>Numerator df</b>	<b>Denominator df</b>	<b>F</b>	<b>Wald Z</b>	<b>p-value</b>
<b><i>Telencephalon</i></b>	River	1	21.921	0.345	-	0.563
	Predation	1	146.031	0.048	-	0.826
	Sex	1	152.335	0.066	-	0.797
	Predation x sex	1	145.680	0.002	-	0.966
	Predation x sex x brain	3	147.911	0.135	-	0.939
	Family	-	-	-	0.625	0.532
<b><i>Optic Tectum</i></b>	River	1	19.816	0.756	-	0.395
	Predation	1	147.714	0.261	-	0.610
	Sex	1	152.796	0.000	-	0.995
	Predation x sex	1	147.483	0.063	-	0.803
	Predation x sex x brain	3	149.256	0.190	-	0.903
	Family	-	-	-	0.801	0.423
<b><i>Cerebellum</i></b>	River	1	20.549	0.184	-	0.672
	Predation	1	152.999	2.765	-	0.098
	Sex	1	151.187	0.325	-	0.569
	Predation x sex	1	152.999	1.787	-	0.183
	Predation x sex x brain	3	152.152	1.118	-	0.344
	Family	-	-	-	1.698	0.089
<b><i>Dorsal Medulla</i></b>	River	1	22.601	0.493	-	0.490
	Predation	1	149.720	2.377	-	0.125
	Sex	1	152.976	0.246	-	0.621
	Predation x sex	1	149.590	0.016	-	0.899
	Predation x sex x brain	3	150.708	1.308	-	0.274
	Family	-	-	-	1.017	0.309

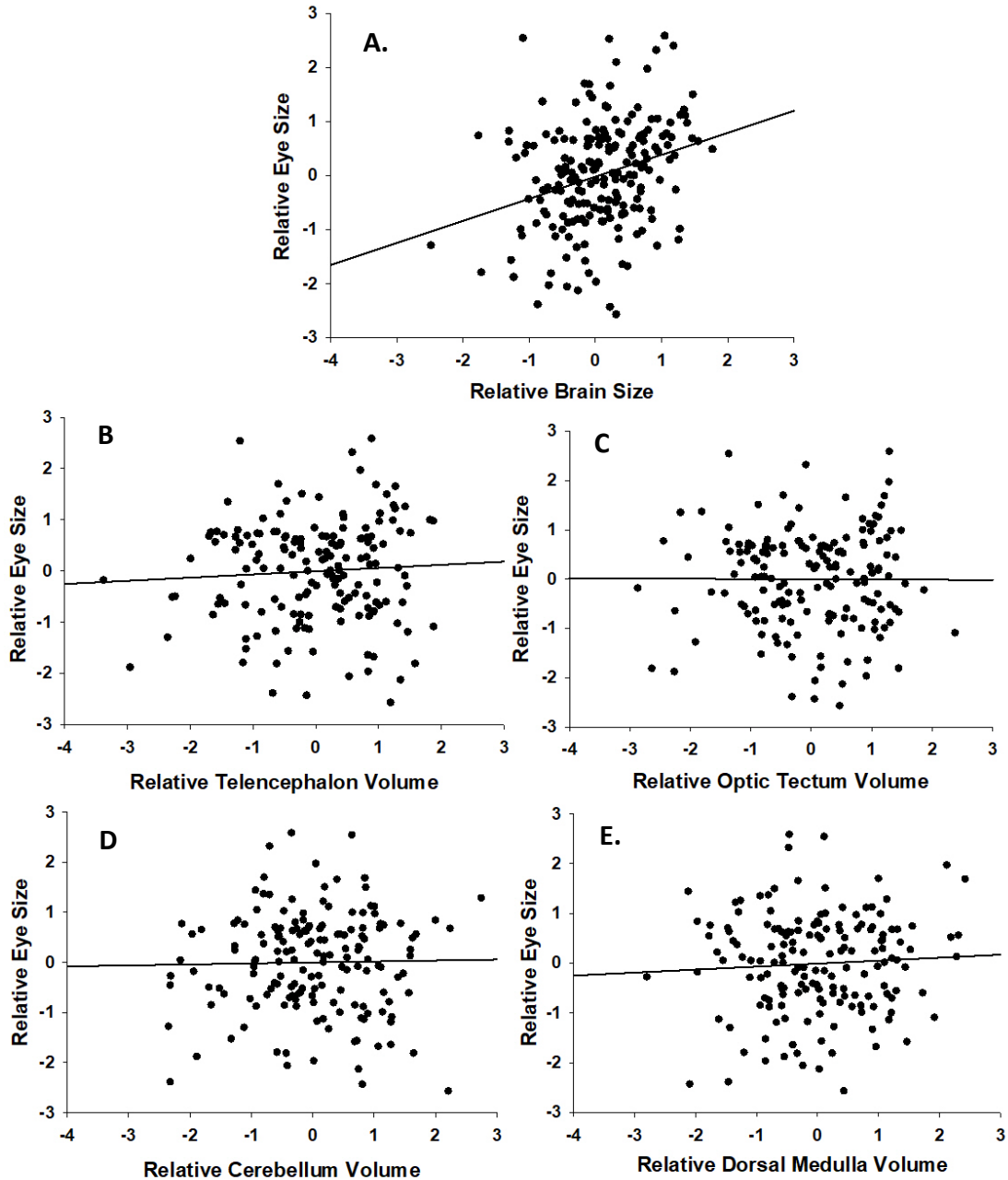
## FIGURES

**Figure 1.** Brain size and structure allometries of wild-caught females separated by population. A, brain size (natural logarithmic scale, in grams) is on the y-axis and fish length (natural logarithmic scale, in millimeters) on the x-axis. B–E, brain structure volume (natural logarithmic scale, in millimeters cubed) is on the y-axis and brain size (natural logarithmic scale, in grams) on the x-axis. **A.** relationship between fish length and brain size in high-predation (HP) females ( $y = -12.74 + 2.07x$ ) and *Rivulus*-only (RO) females ( $y = -12.13 + 1.92x$ ). Differences in female brain size across populations disappear at ~60 mm total length. **B.** differences in female telencephalon volume disappear at ~42 mm total length (RO,  $y = -7.42 + 1.63x$ ; HP,  $y = -11.13 + 2.62x$ ). **C.** differences in female optic tectum across populations disappear at ~37 mm (RO,  $y = -3.2 + 0.66x$ ; HP,  $y = -8.03 + 2x$ ). **D.** size differences in cerebellum volume disappear at ~42 mm (RO,  $y = -5.57 + 1.01x$ ; HP,  $y = -11.07 + 2.48x$ ). **E.** differences in female dorsal medulla disappear at ~ 42 mm (RO,  $y = -6.77 + 0.92x$ ; HP,  $y = -10.95 + 2.04x$ ).

### Females

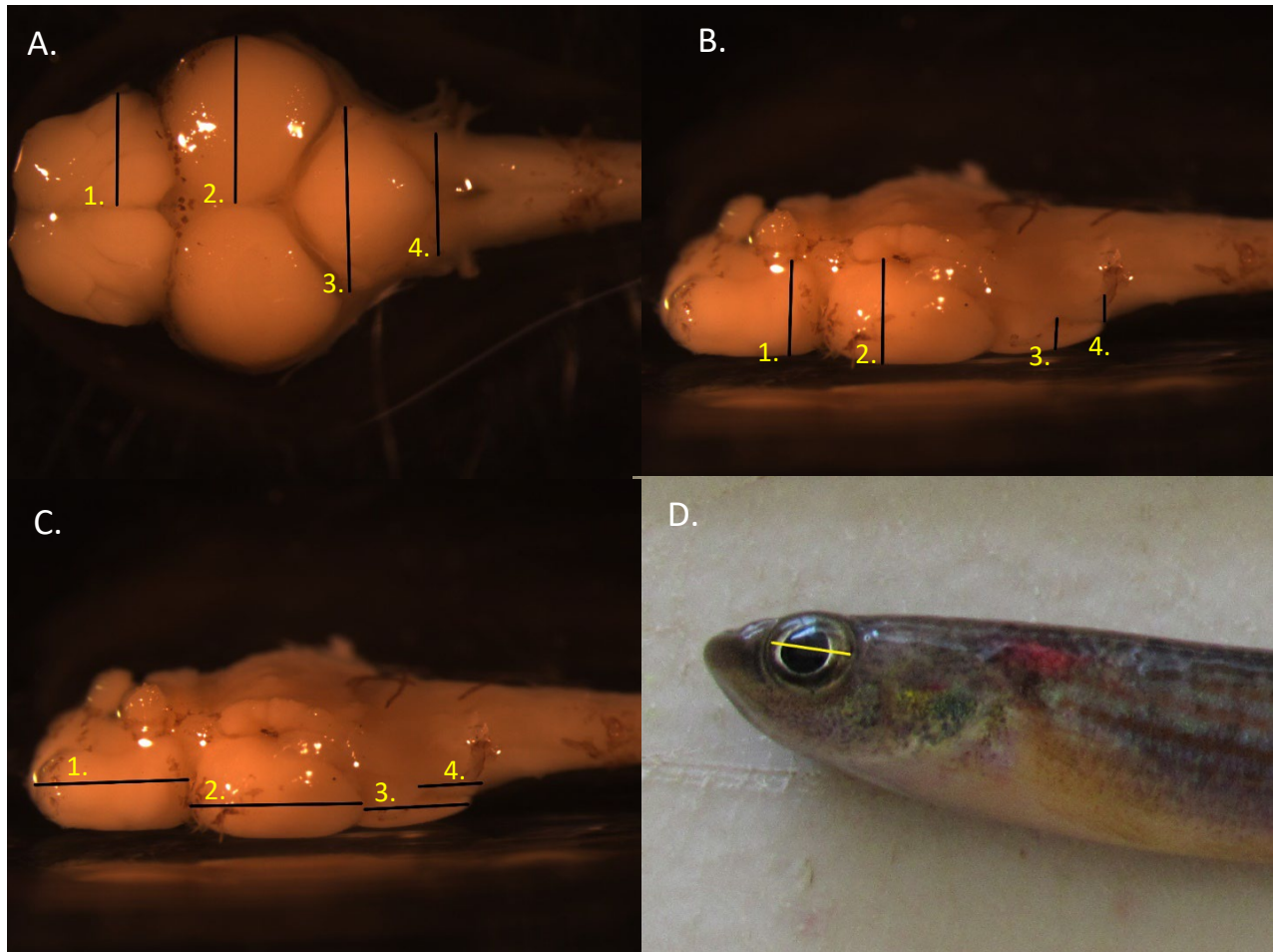


**Figure 2.** Regressions between relative brain size (A) or relative brain region (B–E) and relative eye size of wild-caught female killifish. Relative brain structures were generated by outputting the standardized residuals from regressions between an individual ln-brain structure vs. ln-fish length. Relative brain size and relative eye size were generated by outputting residuals from regressions between ln-eye or ln-brain size and ln-fish length.

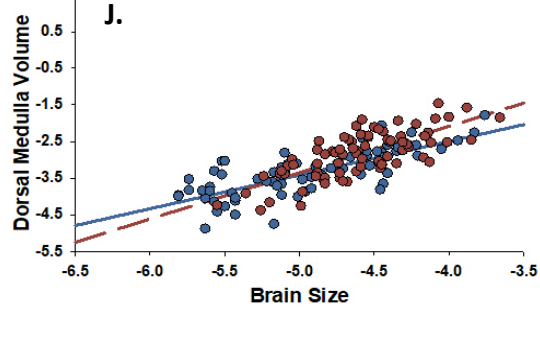
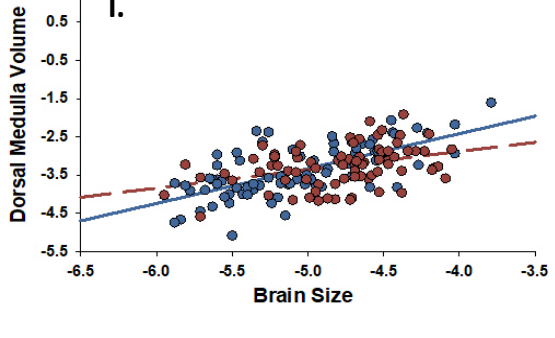
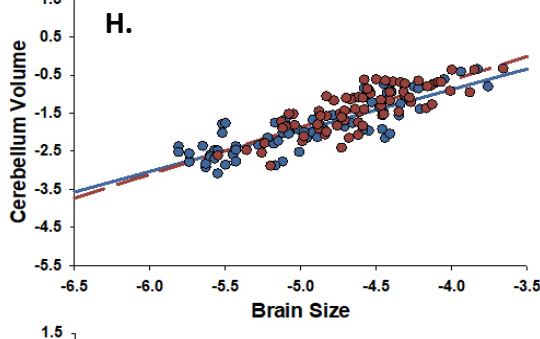
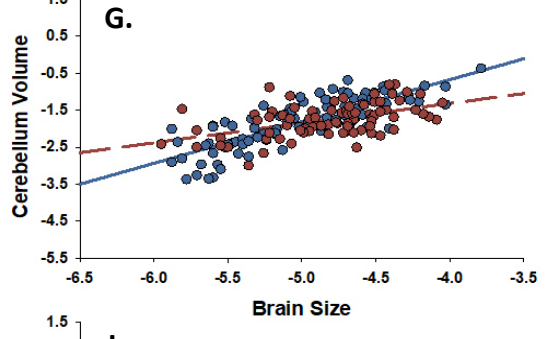
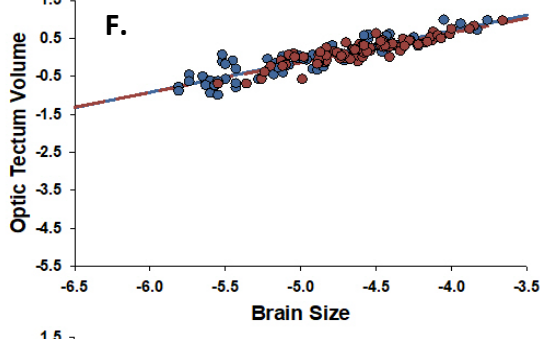
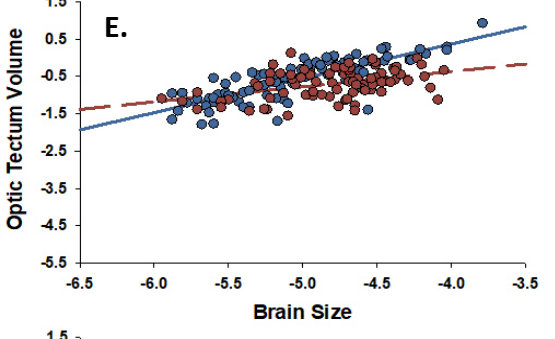
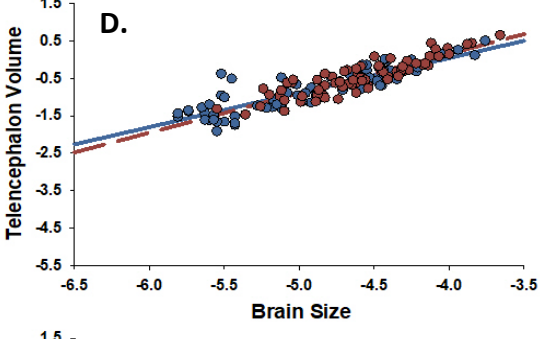
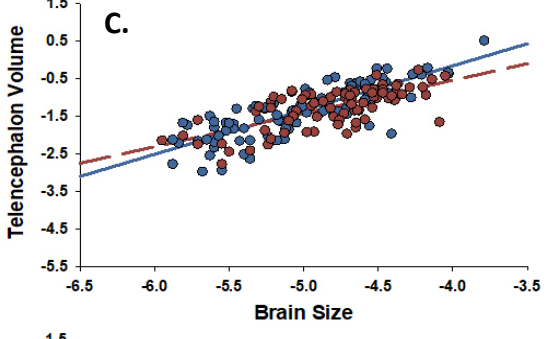
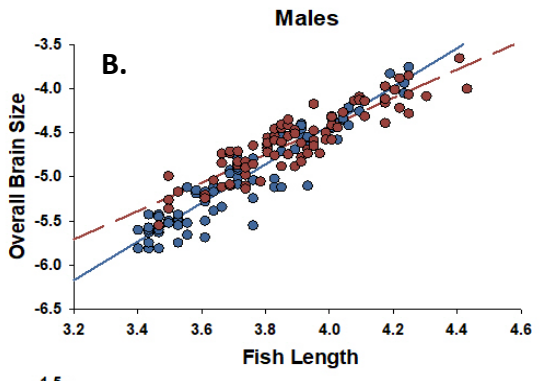
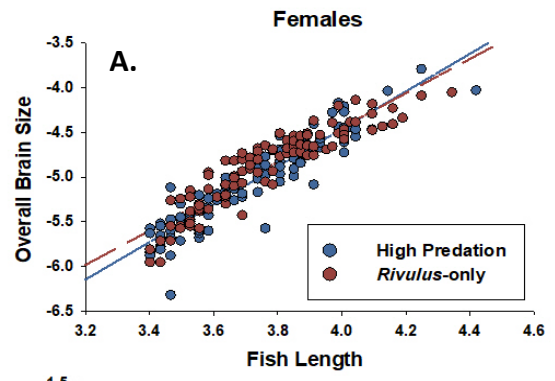


## SUPPORTING INFORMATION

**Supplemental Figure 1.** A) Dorsal view of the brain with width measurements B) Lateral view of height measurements C) Lateral view of length measurements. D) Eye size was measured using diameter at the widest part for each photograph (indicated by the yellow line). Brain components are represented by numbers: (1) telencephalon, (2) optic tectum, (3) cerebellum, (4) dorsal medulla.

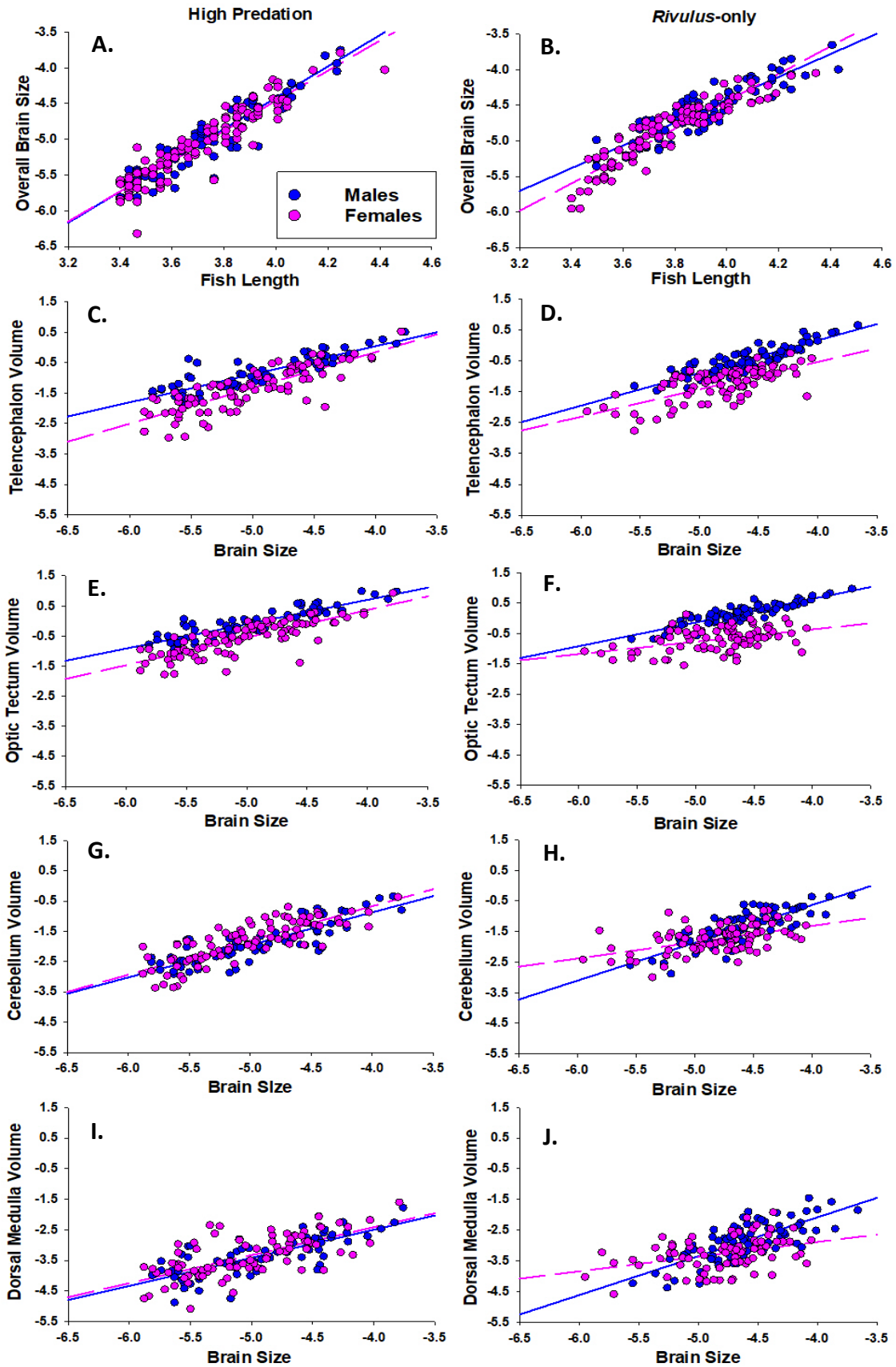


**Supplemental Figure 2.** Overall brain size and structure allometries compared in wild caught fish across sexes, females (left) and males (right). Brain size (ln g) is on the y-axis with fish length (ln mm) on the x-axis (A, B). For brain structure volumes (ln mm<sup>3</sup>), brain size (ln g) is on the x-axis (C-J). Fish from high predation sites are represented by the solid blue line and blue dots; *Rivulus*-only are represented by the dashed red line and red dots.



**Supplemental Figure 3.** Overall brain size and structure allometries compared in wild caught fish across predation regimes, high predation (left) and *Rivulus*-only (right). Brain size (ln g) is on the y-axis with fish length (ln mm) on the x-axis (A, B). For brain structure volumes (ln mm<sup>3</sup>), brain size (ln g) is on the x-axis (C-J). Males are represented by the solid blue line and blue dots; females are represented by the dashed pink line and pink dots.





**Supplemental Table 1.** Correlations between the brain mass and structure volume of each brain structure (female wild caught data only).

	<b>Telencephalon</b>	<b>Optic Tectum</b>	<b>Cerebellum</b>	<b>Dorsal Medulla</b>
<i>Pearson correlation (r)</i>	0.736	0.619	0.643	0.573
<i>p-value</i>	0.000	0.000	0.000	0.000

**Supplemental Table 2.** Results from multiple linear regressions with predation regime included, for wild-caught females only. Regression between brain size and eye size included as Ln-length covariate, and regressions between brain structures and eye size included Ln-brain as covariate. The r-square values and overall p-values represent the predictive ability of the full model.

<b>Brain Region</b>	<b>R square</b>	<b>Overall p</b>	<b>t</b>	<b>β</b>	<b>p-value</b>
<i>Telencephalon</i>	0.736	0.000	1.228	0.030	0.221
<i>Predation</i>			-5.323	-0.109	0.000
<i>Ln Brain</i>			11.870	0.808	0.000
<i>Optic Tectum</i>	0.733	0.000	1.040	0.029	0.300
<i>Predation</i>			-5.148	-0.111	0.000
<i>Ln Brain</i>			13.062	0.384	0.000
<i>Cerebellum</i>	0.732	0.000	0.268	0.006	0.789
<i>Predation</i>			-5.094	-0.104	0.000
<i>Ln Brain</i>			13.904	0.843	0.000
<i>Dorsal Medulla</i>	0.745	0.000	0.904	0.020	0.367
<i>Predation</i>			-5.696	-0.115	0.000
<i>Ln Brain</i>			14.932	0.386	0.000
<i>Overall Brain</i>	0.768	0.000	2.334	0.078	<b>0.021</b>
<i>Predation</i>			-7.663	-0.129	0.000
<i>Ln Length</i>			9.248	0.723	0.000

## **CHAPTER 3:**

# **Increased brain size is associated with enhanced jumping behavior in Trinidadian killifish**

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## **ABSTRACT**

Increased brain size is associated with an improved ability to colonize and survive in novel environments across species. Yet, experimental tests of the connection between vertebrate brain size and dispersal are notably lacking. Trinidadian killifish (*Anablepsoides hartii*) exhibit a jumping behavior that allows them to aerially forage on terrestrial prey and disperse into novel environments. Additionally, killifish are found in sites that vary in predation intensity; decreased predation is associated with evolved brain size differences. Males in sites without predators have evolved larger relative brains than males in sites with predators. Here we evaluate the connection between brain size and jumping behavior in wild-caught and lab acclimated killifish. We observed a significant, positive relationship between relative brain size and propensity to jump and disperse into a novel environment in wild-caught killifish. The propensity to jump did differ between populations from divergent predatory regimes, more fish from sites without predators successfully jumped than fish from sites with predators. However, the link between increased brain size (and brain structure) and propensity to jump was stronger in fish from sites with predators. Such results suggest that selection for jumping behavior may partially explain the evolution of brain size in this system.

## INTRODUCTION

It has long been known that organisms display extensive variation in brain size (Bauchot et al., 1977; Crile & Quiring, 1940; Jarvis et al., 2005; Mink et al., 1981; Striedter, 2006; Taylor & van Schaik, 2007). Research has shown that increased brain size is associated with characteristics that improve fitness, such as enhanced cognitive abilities, intelligence, learning capability and population persistence (Barrickman, Bastian, Isler, & van Schaik, 2008; Benson-Amram, Dantzer, Stricker, Swanson, & Holekamp, 2016; Kotrschal et al., 2013; Kotrschal, Buechel, et al., 2015a; Kotrschal, Corral-Lopez, Amcoff, & Kolm, 2015b; MacLean et al., 2014; Overington, Morand-Ferron, Boogert, & Lefebvre, 2009; Reader, Hager, & Laland, 2011; Shultz & Dunbar, 2006; Tebbich & Bshary, 2004, but also see Drake, 2007). In particular, several studies have shown that increased brain size is associated with an improved ability to colonize and survive in novel environments (Amiel et al., 2011; Sol et al., 2007, 2008, 2012; Sol & Lefebvre, 2000). This leads to the prediction that a large brain is central to the ability of organisms to disperse and colonize new environments. However, the majority of work to date has evaluated this hypothesis at the macroevolutionary scale (Amiel et al., 2011; Sol et al., 2007, 2008, 2012; Sol & Lefebvre, 2000). Experimental tests of the connection between brain size, behavior, and dispersal are now needed.

In addition to a link between overall brain size and dispersal, specific structures of the brain may be important for improving fitness and colonization. For example, the telencephalon has been linked to memory, learning, cognition, and behaviors such as predator avoidance, mating, and foraging in teleost fish (Broglia et al., 2003; Portavella et al., 2002). The cerebellum is also involved in memory and learning through the execution of motor activity (Broglia et al., 2003), while the medulla and optic tecta are involved with auditory function and processing visual stimuli, respectively (Broglia et al., 2003; K. Kotrschal et al., 1998; Tomchik & Lu, 2005). Thus, increases in these brain structures may improve, directly or indirectly, dispersal and

survival. However, few studies have specifically investigated this connection (A. Kotrschal, Buechel, et al., 2015; Roth & Pravosudov, 2009).

The Trinidadian killifish (*Anablepsoides hartii*; historically '*Rivulus hartii*') exhibits an impressive dispersal ability and is able to colonize novel upstream environments above barrier waterfalls. As a result, they are located across a diversity of fish communities that differ in predation intensity (Fraser et al., 1999; Gilliam et al., 1993; Walsh & Reznick, 2008). This includes lowland 'high predation' (HP) sites whereby killifish co-occur with several large species of piscivorous fish (*Crenicichla frenata*, *Hoplia malabaricus*). Killifish are also located in upstream sites where they are the only fish species present (hereafter: '*Rivulus* only' or RO sites; this is used given historical precedent). Research has shown that increased rates of predation are associated with evolutionary divergence in life history traits (Walsh & Reznick, 2008, 2009, 2010, 2011; Walsh, Fraser, Bassar, & Reznick, 2011). Recent work has also revealed differences in brain size and brain structure between high predation and *Rivulus*-only sites; male killifish from *Rivulus*-only sites have evolved larger brains than fish from high predation sites (Walsh et al. 2016). The patterns are similar in females, but the differences are smaller and not significant. Furthermore, there is also evidence that the brain structures have diverged between HP and RO sites. Several brain structures are larger in male (telencephalon, optic tectum, cerebellum, and dorsal medulla) and female (optic tectum, cerebellum, dorsal medulla) killifish from *Rivulus*-only sites versus fish from high predation sites (Howell et al., 2021, 2022). This system thus presents the opportunity to test the connection between brain size and behavior and whether evolved differences in brain size influence this relationship.

Here, we test the connection between brain size and propensity for killifish to jump and disperse into a novel environment using laboratory assays on lab acclimated and wild-caught fish from HP and RO sites (Fig. S1). Jumping behavior serves as a proxy for multiple behaviors; this includes dispersal capability since killifish are known to jump into the air and over land (Seghers, 1978). Jumping ability also represents a proxy for foraging as killifish aerially prey

upon terrestrial food items above the stream surface (Seghars, 1978). This study addresses two main questions: 1) Are increased brain size and increased brain structures positively associated with increased jumping behavior in killifish? and 2) Are evolved differences in brain size and structure associated with differences in the jumping ability of killifish between high predation and *Rivulus*-only sites? Given the results of prior comparisons across species (Amiel et al., 2011; Sol et al., 2007, 2008, 2012; Sol & Lefebvre, 2000), we predict that increases in brain size should increase the likelihood that killifish would disperse into a novel environment. Additionally, because killifish colonized *Rivulus*-only sites by dispersing upstream and killifish from *Rivulus*-only populations exhibit larger brains, we predict that killifish from RO sites will exhibit an increased propensity to jump and take less time to successfully jump.

## **MATERIALS AND METHODS**

### *Lab Acclimated:*

We assayed the jumping behavior of killifish from high predation and *Rivulus*-only sites using wild caught and laboratory acclimated individuals. Lab acclimated killifish were collected from high predation and *Rivulus*-only populations in the Arima and Aripo rivers in January 2017. Approximately 133 fish were transported to our lab at the University of Texas at Arlington and were maintained in 37-L aquaria with a maximum density of 10 fish per tank. Fish were fed a diet of liver paste ad libitum daily. These fish were tested approximately 12-13 months after being transported to the lab.

To assay the jumping behavior of killifish, a 9-L aquarium was placed inside a 37-L tank, centered to the back of the tank (Fig. S1). The 9-L tank was filled with approximately 7-L of water and the larger tank was filled with approximately 9-L of water. An air stone was placed in the front corner of the 9-L tank to encourage killifish to jump by disturbing the surface of the water (see Fig S1). Individual fish were dip netted from their stock tank and placed in the center of the small tank (hereafter native tank). Fish were then monitored for one hour to observe if

they would jump from their native tank into the foreign tank. Fish that remained in the native tank after an hour were recorded as not jumping and fish that jumped to the foreign tank were recorded as jumping. The time it took for killifish to successfully jump was also recorded. For lab acclimated individuals, we assessed jumping behavior for 5 males and 5 females per locality (river and population, 4 total) for a total of 40 lab acclimated fish (5 fish x 2 sexes x 2 rivers x 2 populations = 40 fish).

After the trials, all individuals were euthanized using MS-222 and preserved in 70% ethanol. These fish were subsequently measured for total length and wet weight; brain size was quantified by dissecting the brain from each individual and measuring the wet weight of each brain. Brains were removed by cutting at the top of each gill slit, removing the lower jaw, and then removing tissue between the roof of the mouth and the braincase. Excess tissue was then gently removed from the brain so as not to damage the brain. Dissections were done by one individual to ensure consistency. Note that the brain for one fish was damaged during dissection and the data for this individual was therefore excluded the analyses. We also photographed the dorsal and lateral surface of each brain to measure brain structures (Fig. S2). We measured the width, height, and length of the telencephalon, optic tectum, cerebellum, and dorsal medulla via ImageJ to quantify the volume of each via the ellipsoid model:  $V = (L \times W \times H) \pi / 6$ .

#### *Wild Caught:*

Wild caught fish were collected from high predation and *Rivulus*-only sites in the Arima and Aripo streams in November-December 2018. For each site 21 males were collected and transported back to the field lab where fish were maintained individually in 2-L tanks. Only males were used because only male killifish exhibit differences in overall brain size across populations. Fish were fed fish flakes ad libitum daily. Killifish were tested approximately 2-10 days after being caught.

To assay jumping behavior, we used a similar set up as lab acclimated fish, as described above, except that the aquaria varied in size. For wild caught assays, a 15-L



aquarium was placed inside a 52-L aquarium, centered to the back of the tank. Killifish were again monitored for an hour to determine if they would jump from the native tank to the foreign tank and how long it took them to do so. For wild caught individuals, we assessed jumping behavior for 21 males per locality (river and population, 4 total) for a total of 84 wild caught fish (21 fish x 2 rivers x 2 populations = 84 fish).

After the trials were completed, all individuals were euthanized using MS-222 and preserved in 10% formalin. Preserved fish were then transported back to our lab at the University of Texas at Arlington and kept in 70% ethanol. Fish were dissected in February-May 2019 for quantification of brain size and measured for total length and wet weight. Dissections were completed by one individual to ensure consistency when trimming and removing brains. We again photographed the dorsal and lateral surfaces of the brain to measure the telencephalon, optic tectum, cerebellum, and dorsal medulla (Fig. S2).

#### *Data analysis:*

We evaluated the factors that predicted the extent to which killifish jumped out of its native tank and dispersed into a new environment using binary logistic regression (SPSS v.26). For each trial, we classified whether the fish had jumped out of its tank after a one-hour period and entered this information as the dependent variable (0 vs. 1). For the lab acclimated fish, we included relative brain size or relative brain structure, stream (Arima, Aripo), predator community (high predation, *Rivulus*-only), and sex as predictor variables. We also included the 'predation x brain' and 'brain x sex' interactions. For wild caught fish, we included relative brain size or structure, predator community, and the 'brain size (or structure) x predation' interaction as predictor variables. Stream was removed as a predictor for the wild-caught data because only five of 42 killifish from the Aripo stream successfully jumped, making the data highly imbalanced. We ran separate binary logistic regressions for brain size and each brain structure. We calculated relative brain size and relative brain structure using the standardized residuals from the regression between brain size or brain structure (ln-transformed) and total length (ln-

transformed) and then incorporated these values into the model. We also ran models with standardized residuals that were output from regressions between ln-brain structure and ln-brain size, but this had little effect on model selection and did not impact the overall significance of the results, therefore we focus on residuals using ln-length. We used likelihood ratio tests to determine the final model using backward model selection to determine the final model. We confirmed the goodness of fit of the data with a non-significant Hosmer-Lemeshow test.

For both lab acclimated and wild caught killifish, we also evaluated factors that predicted the time it took fish to successfully jump using general linear models. We included time as the dependent variable and predation, brain size or structure residual, and the 'brain x predation' interaction as predictors. We ran separate linear models for brain size and each brain structure. For fish that did not jump we used the maximum value of the duration of a trial for those data points.

## **RESULTS**

### *Lab acclimated.*

Overall, 40% of lab acclimated killifish successfully jumped from their native tank. The best fitting model from the logistic regression analyses for brain size retained the 'brain size x predation' interaction as the only predictor variable. This indicated the connection is stronger in high predation populations because jumping behavior was more dependent on increased relative brain size in HP fish compared to RO fish (Table 1; Fig. 1A-B). Model selection for the telencephalon retained relative telencephalon volume and stream in the best fitting model (Table 1). The best fitting model for cerebellum retained only the 'cerebellum x predation' interaction as a predictor variable while model selection for both the optic tectum and dorsal medulla did not retain any predictors in the final model (Table 1). The 'cerebellum x predation' interaction also indicated that the connection between cerebellum and the propensity to jump was stronger in high predation sites (Fig. S3).

Results from general linear models indicated that brain size and telencephalon volume were significant predictors of the time it took fish to jump ( $p < 0.05$ ; Table 2, Fig 1C). As brain size and telencephalon volume increased, fish took less time to jump. None of the other brain structures (optic tectum, cerebellum, dorsal medulla) or predictors (predation, predation interactions) were significant predictors of time to jump in any of the linear models.

#### *Wild-caught.*

In total, 36% of wild-caught killifish jumped. The best fitting model from the logistic regression analyses for brain size retained brain size and the 'brain size x predation' interaction as significant predictor variables (Table 3). The significant 'brain size x predation' interaction was driven by high predation populations as the link between increased brain size and jumping behavior was stronger in HP fish (Fig. 2). In model selection for each brain structure, none of the brain structures were retained in the best fitting models nor was predation or the 'brain structure x predation' interaction retained in any final model (Table 3).

We found that the 'brain x predation' interaction was the only significant predictor for the time it took wild-caught fish to successfully jump (Table 4). In HP fish, a larger brain decreased the time to jump, while the trend was opposite in RO fish (Fig 2C-D). Brain size and predation were slightly non-significant in the general linear model (Table 4;  $p = 0.066$ ). None of the brain structures were significant although the 'dorsal medulla x predation' interaction was slightly non-significant ( $p = 0.07$ ).

## **DISCUSSION**

Our results showed that increased brain size is associated with a significant increase in the propensity for wild-caught killifish to jump from their native environment into a novel environment. We also found a stronger connection between brain size and jumping in high predation fish for both lab acclimated and wild caught trials (Fig. 1, 2). Furthermore, the relationship between relative brain size and the duration of time before successfully jumping

from the native tank differed between the fish communities. There was a positive relationship between brain size and time to successfully jump in *Rivulus*-only fish, but this relationship was negative for fish from high predation sites (Fig. 2C-D). Given the evolved differences in brain size observed between HP and RO sites (Walsh et al., 2016), these results contradict our a priori expectations. Below, we consider: (1) the potential fitness advantages of jumping behavior in killifish; (2) why fish from high predations exhibit a stronger connection between brain size and the propensity and duration of time to jump from their native habitat.

Much research has shown that increased brain size is associated with enhanced cognition and learning (Sol et al., 2012, 2016). Our results revealed a positive association between relative brain size and the likelihood that killifish will jump. There are two potential fitness advantages associated with jumping in killifish. First, it may allow for killifish to explore a novel environment. This includes the potential opportunity to colonize environments that are upstream above barrier waterfalls. Jumping behavior in killifish may also enhance rates of energy intake by consuming terrestrial insects above the surface of the water (Seghars, 1978). We induced killifish to jump in this study by disturbing the surface of the water at the corner of the experimental unit (by bubbling air with an air stone, see Fig. S1). This experimental set-up thus mimics the disturbance caused by waterfalls and the behavior measured in this study could reflect killifish attempting to disperse into a new environment. Though, we cannot rule out that killifish are attempting to forage when they jump from the water. Ants and winged insects (dipterans) are a large part of killifish diets (Fraser et al., 1999), and therefore surface disturbance could mimic such insects falling onto the surface of the water.

Killifish from high predation and *Rivulus*-only sites did differ in jumping behavior. However, trends were not what we expected given known differences in brain size (Walsh et al., 2016). While killifish from *Rivulus*-only sites did jump more than those from high-predation (in lab acclimated trials 45% of RO fish jumped compared to 35% HP and in wild-caught trials 43% of RO fish and 29% of HP fish jumped), the relationship between brain size and propensity to

jump was driven by the positive trend observed in killifish from high predation sites. The stronger connection between brain size and jumping behavior in high predation killifish could be an environmentally-induced response to escape from the threat of predation (Baylis, 1983; Seghers, 1978). It may also increase the likelihood that they colonize new, upstream environments. If HP killifish with increased brain size are more successful at jumping and dispersing over upstream barriers then this may, in part, explain the evolution of a larger brain in killifish from *Rivulus*-only sites (Walsh et al., 2016). But why is the connection between brain size and jumping behavior weaker in RO fish, despite having evolved larger brains? One explanation is that there is no or weak selection for dispersal due to the lack of predators in RO sites. Research has shown that RO fish move less than fish from HP sites (Gilliam & Fraser, 2001). Thus, selection for dispersal is likely stronger in HP sites due to the presence of predators and increased mortality. Trends in the time it took fish to successfully jump were also not what we expected across populations (Fig. 2C-D). In high predation sites, as brain size increased the time to successfully jump decreased; in *Rivulus*-only sites, this trend was opposite. Given that high predation killifish exhibit higher rates of cell proliferation despite smaller brains (Dunlap et al., 2019), it's possible that differences in spatial ability are related to variation at different levels of neural morphology (e.g., neuron density, neurogenesis, synaptogenesis, etc., van Praag et al., 2000) in addition to or instead of brain size. Additional tests to determine why declines in predation are associated with the evolution of a larger brain are needed.

We also found positive associations between brain structures and jumping behavior. In lab acclimated killifish, increased telencephalon volume was associated with a significant increase in both jumping behavior and a decrease in time to jump while increased cerebellum volume in high predation fish was associated with an increased propensity to jump (Fig. S3). This is not necessarily surprising given that these structures are particularly linked to spatial learning, cognition, and motor activity (Broglia et al., 2003; Rodríguez et al., 2005). The stronger

connection between brain size and structure (specifically the cerebellum) and jumping in high predation killifish indicates that differences in cognition are complex. Specifically, increases in brain size may not cause shifts in all cognitive abilities. Rather, certain behaviors may be driven by environmental factors that don't necessarily select for increases in brain size (or structure).

It has long been known that fish exhibit the capacity to jump (Aronson, 1971; Dempster et al., 2011; Gibb et al., 2011; Lauritzen et al., 2010; Uchida et al., 1990). For example, jumping behavior has been quantified in salmonids, killifish, mosquitofish, sunfish, and zebrafish among others (Gibb et al., 2011; Lauritzen et al., 2010; Prenosil et al., 2016; Uchida et al., 1990). Most of this work has focused on the importance of jumping to the completion of life history stages. For instance, many salmonids need to scale barrier waterfalls during their freshwater migration to spawn. Thus, jumping is critical in such species. Yet, the heritability of jumping behavior for other species of fish is not known. Historically, the long-standing assumption was that the vast majority of behaviors displayed by animals were learned and environmentally-induced (Bell et al., 2009). However, it is now becoming increasingly clear that many behaviors are heritable and shaped by natural selection (Bell et al., 2009; Brown et al., 2007; Dingemanse et al., 2002). For example, studies have shown that behaviors such as risk-taking (Van Oers et al., 2004), foraging (Martins et al., 2005; Missoweit et al., 2007) and migration (Møller, 2001) are heritable. This is interesting because jumping behavior in killifish is not driven by the need to complete a particular life history stage but instead is more likely explained by the need to forage or disperse. Future work needs to determine if the extent to which jumping behavior is heritable depends upon why organisms exhibit this behavior and the role of environment and heritability in determining specific behaviors.

## **Conclusions.**

We tested the connection between brain size and the jumping behavior of Trinidadian killifish. We found that increased brain size did predict jumping ability in wild-caught fish, and

that this interaction varied by population. We also found that a larger telencephalon and cerebellum were associated with increased propensity to jump. The connection between larger brains and jumping behavior was stronger in high predation fish indicating that jumping behavior may be a driver of the evolved differences in brain sizes between killifish from high predation and *Rivulus* only sites (Walsh et al 2016). Continuing tests of the connection between brain size, behavior, predation, and dispersal are needed.

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## TABLES

**Table 1.** Results of the logistic regression analyses for the lab-acclimated fish. Bolded predictors were retained in the final model selection. The values for the other predictor variables reflect the results of the full model.  $\beta$  - coefficient, S.E. – standard error, df – degrees of freedom,  $\text{Exp}(\beta)$  – odds ratio.

<i>Brain Region</i>	<i>Predictors</i>	$\beta$	<i>S.E.</i>	<i>df</i>	<i>p-value</i>	<i>Exp (<math>\beta</math>)</i>
<i>Overall Brain Size</i>	Stream	0.398	0.850	1	0.640	1.489
	Predation	-0.966	0.877	1	0.271	0.381
	Sex	-0.441	0.916	1	0.631	0.644
	Brain Size	0.195	0.834	1	0.815	1.215
	<b>Brain x Predation</b>	<b>1.437</b>	<b>0.806</b>	<b>1</b>	<b>0.075</b>	<b>4.206</b>
	Brain x Sex	0.500	1.447	1	0.730	1.648
<i>Telencephalon</i>	<b>Stream</b>	<b>2.045</b>	<b>1.246</b>	<b>1</b>	<b>0.101</b>	<b>7.728</b>
	Predation	-1.570	1.235	1	0.204	0.208
	Sex	-.421	1.243	1	0.735	0.656
	<b>Telencephalon</b>	<b>1.692</b>	<b>0.819</b>	<b>1</b>	<b>0.039</b>	<b>5.432</b>
	Tel x Sex	-1.952	2.460	1	0.427	0.142
	Tel x Predation	-.750	1.752	1	0.668	0.472
<i>Cerebellum</i>	Stream	0.393	1.072	1	0.714	1.481
	Predation	-1.038	1.136	1	0.361	0.354
	Sex	-0.058	1.003	1	0.954	0.944
	Cerebellum	-0.766	0.970	1	0.430	0.465
	Cer x Sex	1.495	1.566	1	0.340	4.458
	<b>Cer x Predation</b>	<b>1.640</b>	<b>0.971</b>	<b>1</b>	<b>0.091</b>	<b>5.157</b>
<i>Optic Tectum</i>	Stream	0.824	1.037	1	0.427	2.280
	Predation	-0.449	1.031	1	0.663	0.638
	Sex	-0.493	0.982	1	0.616	0.611
	Optic Tectum	-0.283	1.178	1	0.810	0.754
	OT x Sex	0.927	1.431	1	0.517	2.526
	OT x Predation	0.824	1.419	1	0.561	2.280
<i>Dorsal Medulla</i>	Stream	1.179	1.098	1	0.283	3.251
	Predation	-0.262	0.883	1	0.767	0.770
	Sex	-0.834	1.112	1	0.453	0.434
	Dorsal Medulla	0.508	0.855	1	0.553	1.662
	DM x Sex	-0.673	1.187	1	0.571	0.510
	DM x Predation	-0.406	1.110	1	0.714	0.666

**Table 2.** General linear models with the time to jump for lab acclimated as the dependent variable. Brain size and each brain structure were run in separate models. Only brain size and telencephalon volume were significant.

<i>Predictors</i>	<i>df</i>	<i>F</i>	<i>p-value</i>
<b><i>Brain size</i></b>	<b>1</b>	<b>4.700</b>	<b>0.037</b>
<i>Predation</i>	1	1.562	0.220
<i>Brain x Predation</i>	1	0.180	0.674
<b><i>Telencephalon</i></b>	<b>1</b>	<b>4.472</b>	<b>0.045</b>
<i>Predation</i>	1	1.195	0.286
<i>Tel x Predation</i>	1	0.793	0.382
<i>Cerebellum</i>	1	0.036	0.851
<i>Predation</i>	1	0.681	0.418
<i>Cer x Predation</i>	1	1.811	0.192
<i>Optic Tectum</i>	1	0.351	0.559
<i>Predation</i>	1	0.603	0.445
<i>OT x Population</i>	1	0.176	0.679
<i>Dorsal Medulla</i>	1	3.102	0.091
<i>Predation</i>	1	1.169	0.291
<i>DM x Predation</i>	1	0.440	0.514

**Table 3.** Results of the logistic regression analyses for the wild caught fish. Bolded predictors were retained in the final model selection. The values for the other predictor variables reflect the results of the full model.  $\beta$  - coefficient, S.E. – standard error, df – degrees of freedom,  $\text{Exp}(\beta)$  – odds ratio.

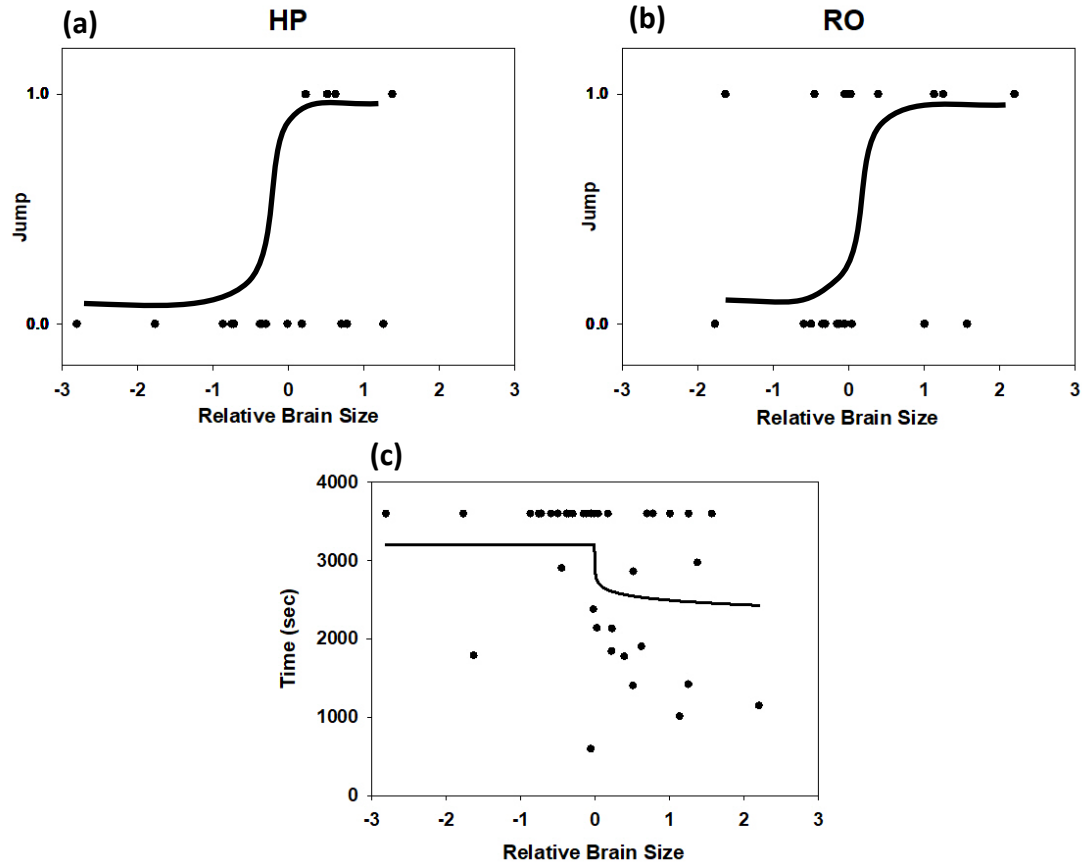
<i>Brain Region</i>	<i>Predictors</i>	$\beta$	<i>S.E.</i>	<i>df</i>	<i>p-value</i>	<i>Exp (<math>\beta</math>)</i>
<i>Overall Brain Size</i>	Predation	-0.606	0.481	1	0.208	0.546
	<b>Brain Size</b>	-0.824	0.389	1	<b>0.041</b>	0.461
	<b>Brain x Predation</b>	1.065	0.517	1	<b>0.045</b>	2.860
<i>Telencephalon</i>	Predation	-0.543	0.469	1	0.247	0.581
	Telencephalon	-0.099	0.314	1	0.754	0.906
	Tel x Predation	0.257	0.480	1	0.591	1.294
<i>Cerebellum</i>	Predation	-0.625	0.475	1	0.188	0.535
	Cerebellum	-0.397	0.376	1	0.290	0.672
	Cer x Predation	0.742	0.503	1	0.140	2.100
<i>Optic Tectum</i>	Predation	-0.543	0.476	1	0.254	0.581
	Optic Tectum	0.435	0.387	1	0.261	1.544
	OT x Predation	-0.243	0.513	1	0.635	0.784
<i>Dorsal Medulla</i>	Predation	-0.691	0.490	1	0.158	0.501
	Dorsal Medulla	-.0742	0.429	1	0.084	0.476
	DM x Predation	1.206	0.559	1	<b>0.031</b>	3.340

**Table 4.** General linear models with the time it took wild caught fish to jump as the dependent variable. Brain size and each brain structure were run in separate models. Only the ‘brain x predation’ interaction was significant (bolded).

<i>Predictors</i>	<i>df</i>	<i>F</i>	<i>p-value</i>
<i>Brain size</i>	1	3.463	0.066
<i>Predation</i>	1	3.476	0.066
<b><i>Brain x Predation</i></b>	<b>1</b>	<b>8.687</b>	<b>0.004</b>
<i>Telencephalon</i>	1	0.099	0.754
<i>Predation</i>	1	2.643	0.108
<i>Tel x Predation</i>	1	1.249	0.267
<i>Cerebellum</i>	1	2.245	0.138
<i>Predation</i>	1	1.214	0.274
<i>Cer x Predation</i>	1	0.488	0.487
<i>Optic Tectum</i>	1	0.674	0.414
<i>Predation</i>	1	2.670	0.106
<i>OT x Predation</i>	1	0.019	0.889
<i>Dorsal Medulla</i>	1	0.358	0.551
<i>Predation</i>	1	2.108	0.150
<i>DM x Predation</i>	1	3.364	0.070

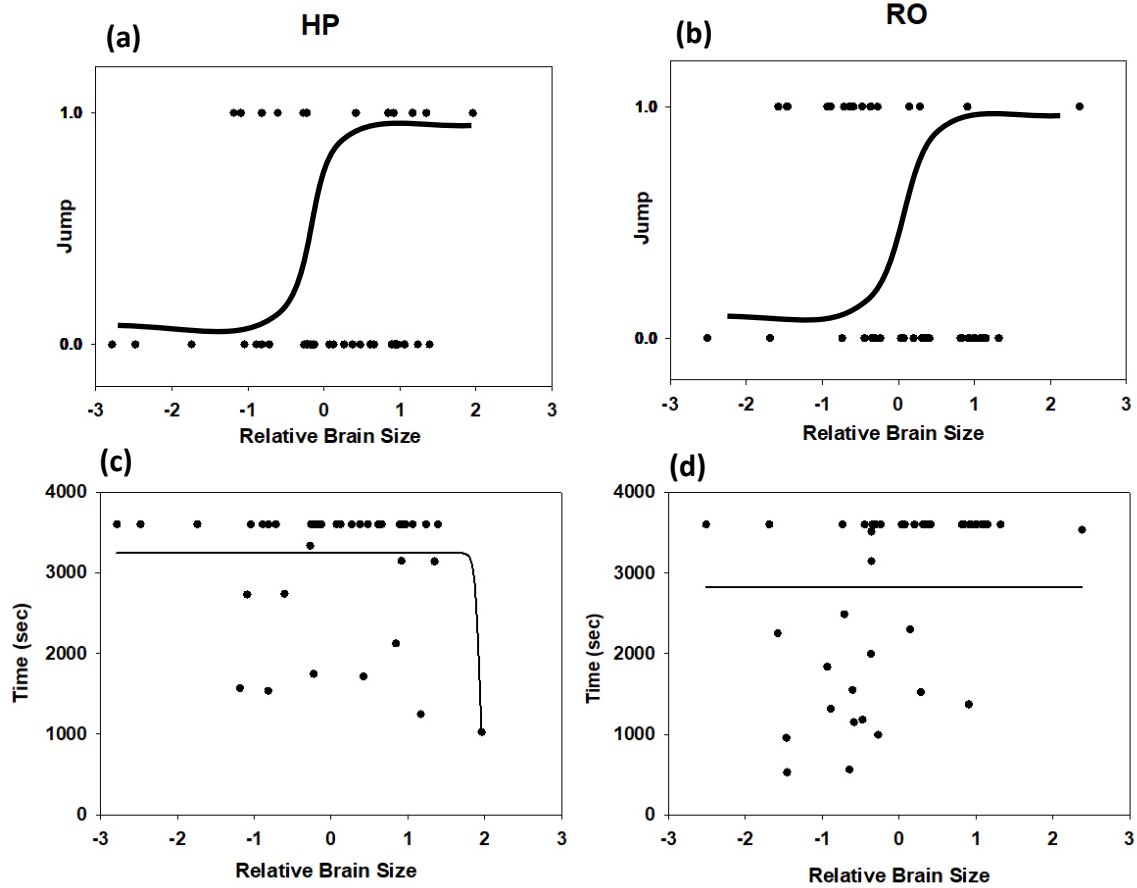
## FIGURES

**Figure 1.** Increased brain size is positively associated with jumping behavior in lab acclimated killifish (a, b). (a) The association between brain size and jumping is driven primarily by HP killifish. (b) Regression between brain size and if RO killifish jumped. (c) Increased brain size is negatively associated with the time lab acclimated killifish took to successfully jump. That is, as brain size increased killifish generally took less time to successfully jump. Line represents a logistic curve fit to the data. We included the maximum time value (3600 seconds) for fish that did not jump.



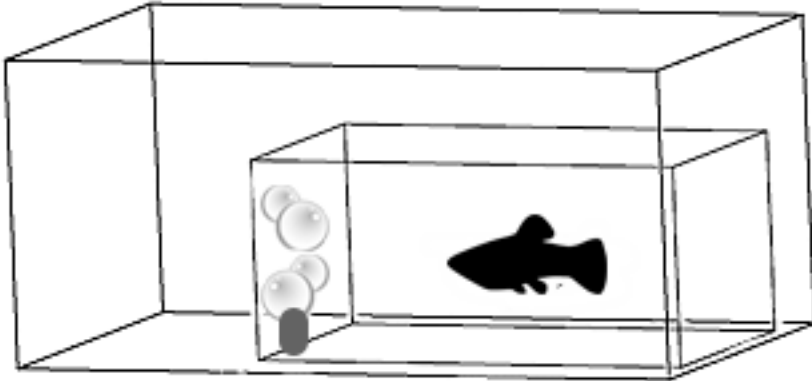


**Figure 2.** Increased brain size is positively associated with jumping behavior in wild caught killifish. (a-b) Regressions between relative brain size and whether a fish successfully jumped from its tank, separated by population. The relationship between brain size and time to successfully jump depended upon population. (c) Brain size and time to jump are negatively associated in HP killifish. As brain increased, the time to successfully jump decreased. Line represents a logistic curve fit to the data. (d) Brain size and time to jump are positively associated in RO killifish. As brain increased, the time to jump successfully also increased. The maximum time value (3600 seconds) was included for fish that did not jump.

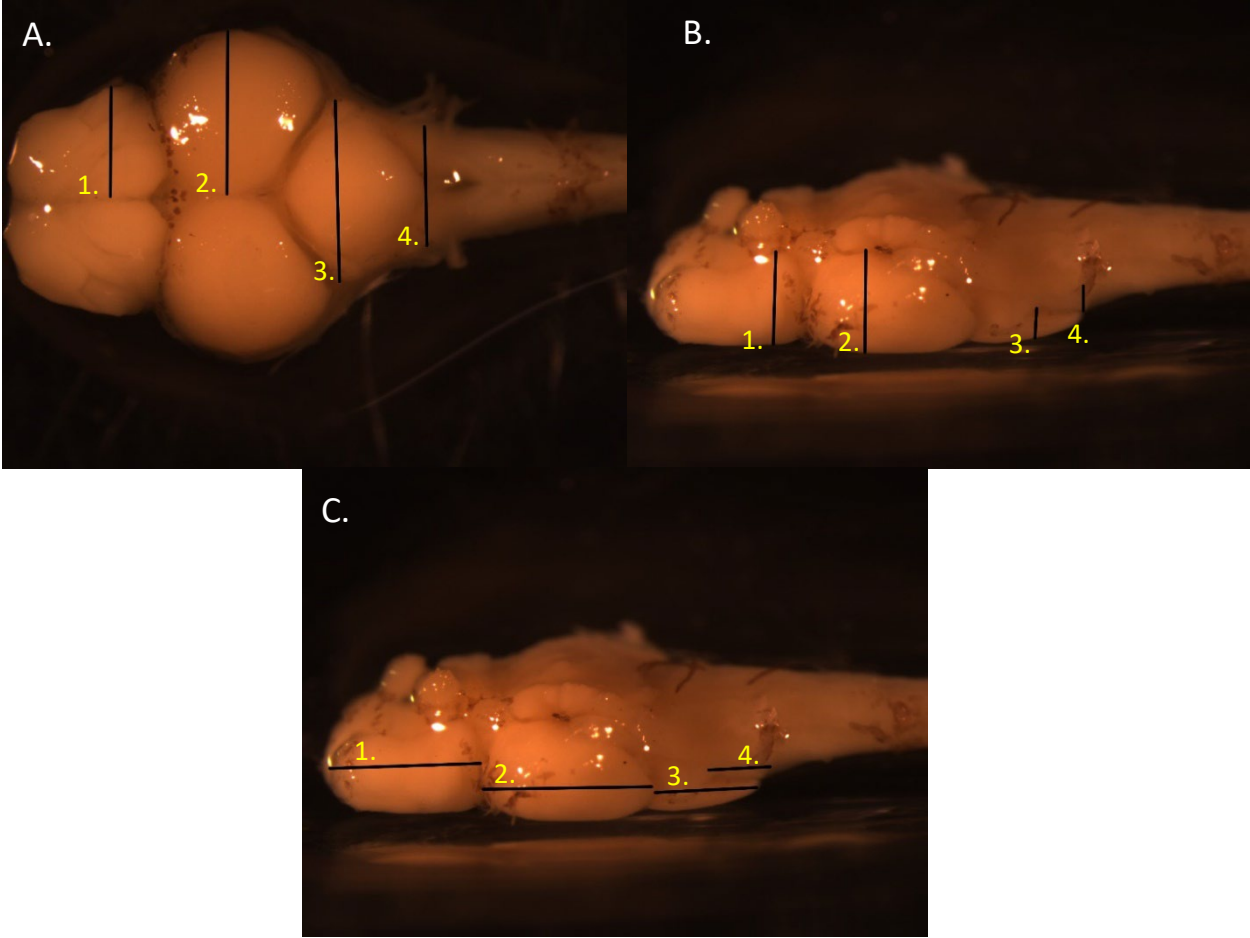


## SUPPORTING INFORMATION

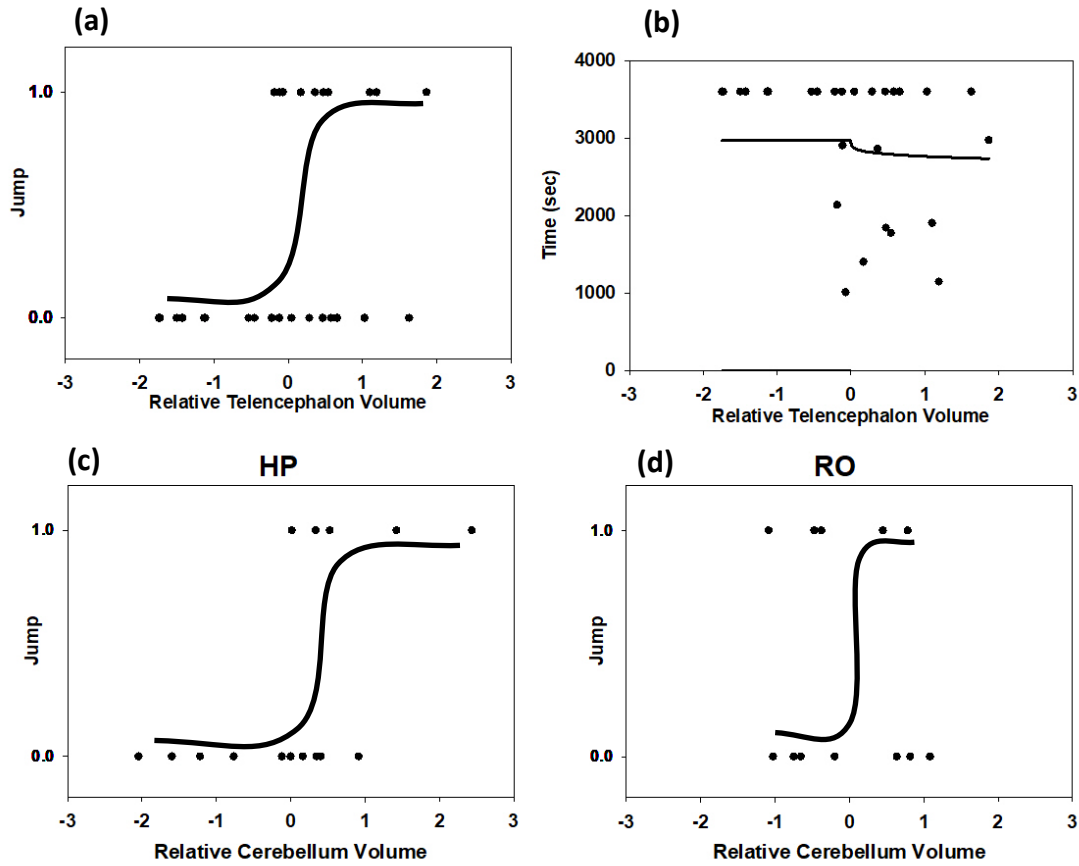
**Supplemental Figure 1.** A schematic of the experimental aquaria set up. Bubbles represent the location of the air stone.



**Supplemental Figure 2.** A) Dorsal view of the brain with width measurements B) Lateral view of height measurements C) Lateral view of length measurements. Brain components are represented by numbers: (1) telencephalon, (2) optic tectum, (3) cerebellum, (4) dorsal medulla.



**Supplemental Figure 3.** Significant results from brain structure analysis from lab acclimated fish. Relative telencephalon volume was a significant predictor for both propensity to jump (panel A) and the time it took a fish to successfully jump (panel B). The cerebellum was also a significant predictor of propensity to jump depending on population (panels C, D).



## CHAPTER 4:

### Experimental evidence demonstrating larger brains increase fitness in novel environments in Trinidadian killifish (*Anablepsoides hartii*)

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## **ABSTRACT**

Scientists have long investigated the benefits of increased brain size. One proposed explanation is that large brains buffer an organism against complex, novel, or changing environments through behavioral flexibility and that this should then reduce mortality and enhance fitness. Experimental tests of this hypothesis are lacking. Trinidadian killifish (*Anablepsoides hartii*) present a natural system within which to test the connection between brain size and fitness in a novel environment. Killifish are found in sites that vary in predation intensity; decreased predation is associated with evolved brain size differences. Males and, to a lesser extent, females, in sites without predators have evolved larger relative brains than fish in sites with predators. Here, we experimentally tested if larger brains increase fitness in novel environments by transplanting marked fish from sites with predators to sites without predators and tracking rates of individual growth for one month. We found that transplanted killifish that were recaptured exhibited a larger mean brain size than transplanted fish not recaptured. We also found that killifish transplanted to new environments exhibited a positive relationship between brain size and growth while resident fish did not. Such results provide experimental support that larger brains increase fitness in novel environments, and indirect support that bigger brains improve establishment success.

## INTRODUCTION

The evolutionary drivers of vertebrate brain size have long intrigued scientists. It has been hypothesized that a larger brain may influence key components of organismal fitness. Organisms with larger brains exhibit greater cognitive flexibility (MacLean *et al.*, 2014; van der Bijl *et al.*, 2015; Buechel *et al.*, 2018), which can help improve an organism's response to challenges such as predation, competition, and foraging (Sol, 2009a). Such improvements can then lead to shifts in fitness. Research has indeed shown that a larger brain is positively correlated with increased survival, a longer lifespan, and an improved ability to colonize and survive in novel environments and thus population persistence (Sol & Lefebvre, 2000; Sol *et al.*, 2007, 2008; Amiel *et al.*, 2011; but see Drake, 2007). The vast majority of this work is correlational in nature (but see Kotrschal *et al.*, 2015). Experimental tests of the connection between brain size and fitness in the wild, within species, are now needed.

Trinidadian killifish (*Anablepsoides hartii*, previously *Rivulus hartii*) are found in sites that differ in predation intensity. This includes upstream, *Rivulus*-only (RO) sites where killifish are the only fish species present and downstream high-predation (HP) sites where killifish co-occur with multiple species of piscivorous fish, e.g., *Hoplias malabaricus*, *Crenicichla frenata* (Gilliam *et al.*, 1993; Fraser *et al.*, 1999; Walsh & Reznick, 2008). Killifish in RO sites exist in higher densities and experience strong selection due to intraspecific competition. Conversely, killifish in HP sites experience higher mortality due to the presence of predators (Gilliam *et al.*, 1993; Walsh & Reznick, 2008; Furness & Reznick, 2014). Variation in predation intensity has been associated with evolved differences in life-history (Walsh & Reznick, 2008, 2009, 2010, 2011; Walsh *et al.*, 2011) and brain size, brain structure, and eye size (Walsh *et al.*, 2016; Beston *et al.*, 2017; Beston & Walsh, 2019; Dunlap *et al.*, 2019; Howell *et al.*, 2021, 2022). Particularly, males from sites without predators have evolved larger brains and brain structures than males from sites with predators (Walsh *et al.*, 2016; Howell *et al.*, 2021). Females exhibit similar shifts

in brain size but the differences between the populations are much smaller (Howell *et al.*, 2022). Thus, this system offers a unique opportunity to investigate the role that intraspecific brain size variation plays in persistence and fitness.

We tested the connection between brain size, fitness (rates of growth), and colonization success in killifish via mark-recapture transplant experiments performed in replicate natural streams. We can estimate the brain size of live individuals by taking images of the semitransparent head of killifish. We transplanted fish from downstream HP sites to upstream RO sites as this mimics the natural colonization process – movement from ancestral sites to upstream sites. We also monitored the relationship between brain and growth in the resident (control) fish in RO sites. Given that increased brain size is positively associated with various aspects of fitness (Huber *et al.*, 1997; Garamszegi *et al.*, 2002; Lefebvre *et al.*, 2004; González-Lagos *et al.*, 2010), we predicted HP fish with larger relative brains would be more likely to persist and exhibit enhanced growth in novel environments.

## **METHODS**

Fish were collected from high predation and *Rivulus*-only sites in two streams (Arima and Aripo) in March 2022. All individuals were sexed, measured for total length, weighed, and received a unique mark using an elastic polymer injection for identification (Northwest Marine Technology Incorporated, Shaw Island, WA, USA); unique markings were achieved using a two-mark code, five colors, and six body positions. Photographs were taken to measure brain size using ImageJ freeware. Brain size can be quantified on live individuals because the top of the heads of killifish are transparent and we can estimate the size of the optic tectum, a structure highly correlated with overall brain mass (Näslund, 2014; Fig 1). We transplanted 149 high predation fish to *Rivulus*-only sites in the Arima stream and 134 high predation fish to RO sites in the Aripo. We marked approximately 100 RO control fish in both experimental sites (RO sites with transplanted HP fish) and marked an additional 50 RO fish that were upstream of



experimental reaches. This allows us to compare the growth of transplanted high predation fish to *Rivulus*-only fish that are interacting with transplanted fish, and additionally compare those fish to *Rivulus*-only fish presumably not in contact with transplanted fish. In Arima, the experimental reach was 82m; the upstream control pools were about 10m past the end of the experimental reach. In Aripo, the experimental reach was 100m and the control was approximately 10m upstream. We removed at least 60 fish from the focal stretch of Arima and 41 killifish from the focal stretch of Aripo in an effort to maintain density in these sites. In the Arima experimental reach, we increased density by approximately 74-89 fish; in the Aripo, experimental reach we increased density by 93 killifish. Each focal site was resampled over two nights after 28-30 days, in April 2022. We also sampled ~10m downstream of the initial focal stretch and between the end of the focal stretch and the upstream controls to recapture any fish who might have moved. Note that no HP killifish were recaptured within the upstream control. Recaptured fish were again measured for length and weight.

We measured instantaneous growth using the following formula:  $[(\ln(\text{wet weight}_{\text{recap}}) - \ln(\text{wet weight}_{\text{initial}})) / (\text{day}_{\text{recap}} - \text{day}_{\text{initial}})]$ . We used general linear models to test for differences in growth as a function of brain size (SPSS v. 26, IBM). We included predation regime (HP or RO), sex, and stream as fixed effects. Relative brain size was included as a covariate. Relative brain size (brain size controlled for body size) was output as standardized residuals from a linear regression between ln-transformed total length and ln-brain size. Residuals greater than 3 or less than -3 were considered outliers and removed before analysis (1 residual removed from Arima RO; 5 Aripo HP residuals removed). Given that we were interested in differences across sexes and predation regimes as a function of brain size, we included the 'predation x brain', 'sex x brain', and 'predation x sex x brain' interactions. To determine if responses were consistent across rivers, we also included the 'stream x predation x brain' interaction. Our total sample size was n=250.

We were also interested in the relationship between brain size and establishment success, so we compared the brain size of killifish we recaptured to those of killifish we did not recapture. We ran a general linear model with ln-brain size as the dependent variable, recapture status (0,1; 1 indicated a fish was recaptured) and predation regimes as fixed effects, and ln total length as a covariate. We included the 'predation x recapture' interaction because we wanted to know if patterns varied between RO and HP fish.

## RESULTS

We recaptured 53 Arima RO focal killifish (51% recapture rate), 22 Arima RO upstream control killifish (39% recapture rate), and 49 Arima HP transplants (33% recapture rate). For the Aripo river, we recaptured 41 focal killifish (37% recapture rate), 17 RO control (36% recapture rate), and 81 HP transplants (60% recapture rate). The relationship between relative brain size and rates of growth differed between the fish communities. This is because we observed a significant ( $p < 0.05$ ) 'predation x brain' and 'stream x predation x river' interaction (Table 1). The connection between brain size and growth was positive in fish transplanted from HP to RO sites in both streams (Fig. 2), but the same trends varied across streams for RO fish. Arima RO fish exhibited a slightly positive relationship between brain size and growth while Aripo RO fish exhibited a strongly negative trend (Fig. 2).

We evaluated the average relative brain size of the fish that were marked at the beginning of the experiment but not recaptured and compared that with the average relative brain size of the fish that were recaptured. The average brain sizes between these two groups differed in the transplanted HP fish but not in the resident RO fish. This is because the 'predation x recapture' interaction was significant (Table 2). HP fish that were recaptured exhibited a larger mean brain size than HP fish not recaptured (Fig. 3), while RO fish did not exhibit differences in mean brain size across recapture status.

## DISCUSSION

Our results clearly demonstrate that fish transplanted from sites with predators to sites without predators exhibit increased growth when they have larger relative brains (Fig. 2). The relationship between brain size and growth in the resident fish was much weaker or the opposite (Fig. 2). We performed these transplant experiments during the dry season of Trinidad, which is characterized by low food availability for killifish and thus intense competition for resources in RO sites (DN Reznick, *unpublished data*). As a result, the strong positive relationship between brain size and growth in the transplanted killifish shows that fish with a larger brain are better able to forage in high competition environments. Additionally, we found that the average brain size of HP fish that were recaptured was larger than the average for those not recaptured (Fig. 3). These results collectively support the idea that larger brains can lead to higher establishment success and ultimately fitness (Sol, 2009b).

Why might a larger brain allow killifish to better persist and forage in a new environment? One hypothesis is that larger brains may buffer an organism from environmental challenges through flexibility in the utilization of information and the production of behavioral responses. This behavioral flexibility then presents the opportunity to create adapted responses to unusual or novel challenges (Sol, 2009a). One potential flexible behavior that could be linked to fitness in killifish is jumping behavior. Jumping behavior serves a proxy for both dispersal and foraging in killifish because they are known to jump into the air and over land and aerially prey upon terrestrial food items above the stream surface (Seghers, 1978). In a previous study, we found a positive link between brain size and propensity to jump and explore a novel environment (KJ Howell, *unpublished data*). Additionally, this connection was stronger in killifish from HP sites. While speculative, this could provide a potential mechanistic link between brain size, behavior, and fitness if such jumping behavior increases dispersal and foraging success.

One surprising aspect of our results was that the relationship between brain size and growth in the resident killifish differed between replicate streams. Resident killifish in the Arima river displayed a slightly positive relationship (Fig. 2A) between brain size and growth while resident fish in the Aripo river exhibited a negative connection between increased brain size and growth rate (Fig. 2B). Why do the Aripo RO fish exhibit a strongly negative relationship? To further investigate this question, we conducted an additional analysis to compare the RO focal group (i.e., fish in the main stretch where HP fish were transplanted) and RO upstream control groups (i.e., fish that were above the stretch of river that received the transplanted fish). Interestingly, we found significant differences in the relationship between brain size and growth between the two groups (Table S1). RO fish from the focal stretch of Aripo river appear to drive the overall negative trend while RO fish from the upstream control display a positive relationship between brain size and growth (Fig. S1). This suggests that focal Aripo RO fish in contact with transplanted HP fish experienced negative effects of interacting with the transplanted fish. This could be due, in part, to differences in habitat across the streams. The focal section of Aripo included in this study is generally much narrower with less ideal habitat for killifish than the stretch of river utilized in the Arima river (e.g., Aripo consists of more riffles, fewer and shallower pools). Differences between streams are heightened during the months we sampled because water levels tend to be much lower during the dry season. Additionally, it's possible that density played a stronger role in the Aripo site. While we transplanted 134 HP fish to Aripo RO, less than 50 Aripo RO fish were removed from the focal stretch. We were unable to catch enough RO focal fish to mark and remove due to lower population numbers compared to Arima. However, in Arima we transplanted 149 HP fish and were able to remove at least 60 killifish from the focal stretch. If Aripo river is unable to support as many killifish as Arima, then density fluctuations may have a bigger impact. The role density and other environmental factors play in the connection between brain size and fitness requires further investigation.

## Conclusions

We experimentally tested the connection between brain size and fitness in natural populations of killifish and found that fish transplanted to novel environments exhibited a positive relationship between increased growth and increased relative brain size. We also found recaptured HP fish exhibited a larger mean brain size than HP fish not recaptured. However, we did not find consistent patterns between brain size and fitness in fish native to focal sites nor did we find differences in mean brain size between RO fish recaptured and not recaptured. These results provide experimental support that larger brains can increase fitness and indirect support that larger brains increase establishment success. Investigation into the mechanistic basis of this connection between brain size and fitness is now needed.

## ACKNOWLEDGEMENTS

We thank Anne Christian and Meghan Korte for help in the field and Janice Pham for help in the lab. This work was funded by a Society for the Study of Evolution R.C. Lewontin Early Award and by a Sigma Xi Grant in Aid of Research awarded to KJH.

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## TABLES

**Table 1.** Results of instantaneous growth analysis. Significant p-values are bolded.

<b>Predictor</b>	<b>df</b>	<b>F</b>	<b>p-value</b>
<i>Stream</i>	1	17.428	<b>0.000</b>
<i>Sex</i>	1	0.007	0.933
<i>Predation</i>	1	4.419	<b>0.037</b>
<i>Brain</i>	1	0.538	0.464
<i>Sex x Predation</i>	1	1.206	0.273
<i>Predation x Brain</i>	1	10.750	<b>0.001</b>
<i>Predation x Sex x Brain</i>	1	0.286	0.593
<i>Stream x Predation x Brain</i>	2	4.433	<b>0.013</b>

**Table 2.** Results of recapture analysis with brain size included as the dependent variable. Significant p-values are bolded.

<b>Predictor</b>	<b>df</b>	<b>F</b>	<b>p-value</b>
<i>Predation</i>	1	1.336	0.248
<i>Recapture</i>	1	7.398	<b>0.007</b>
<i>Predation x Recapture</i>	1	4.803	<b>0.029</b>
<i>lnTL</i>	1	632.835	<b>0.000</b>

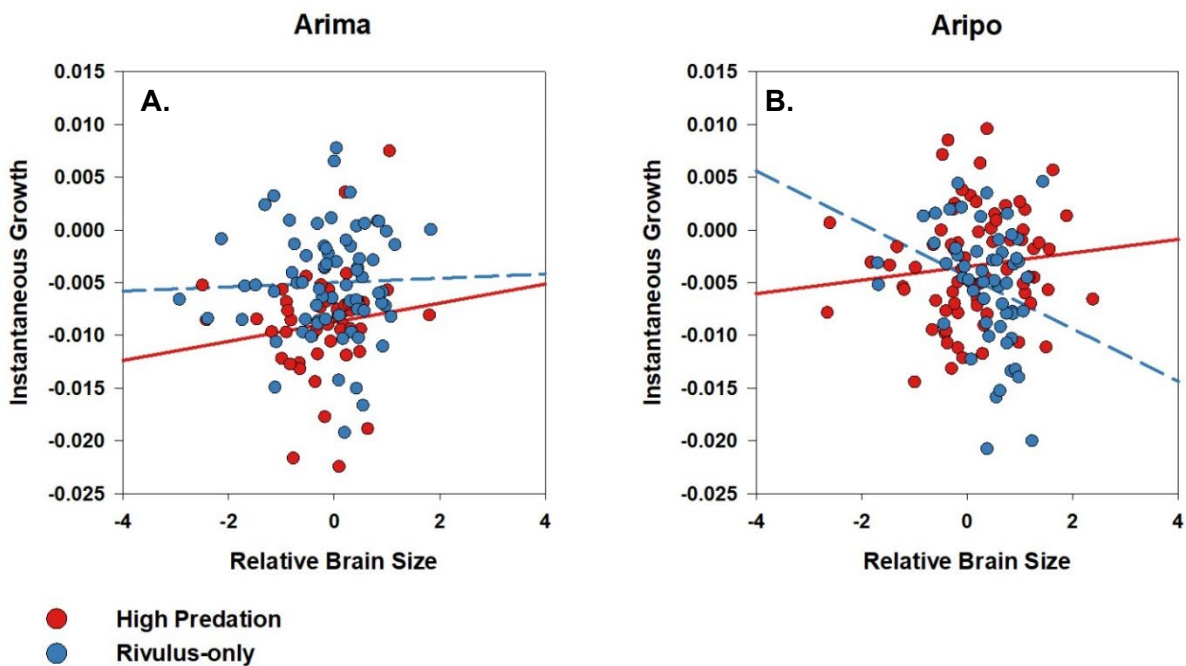


## FIGURES

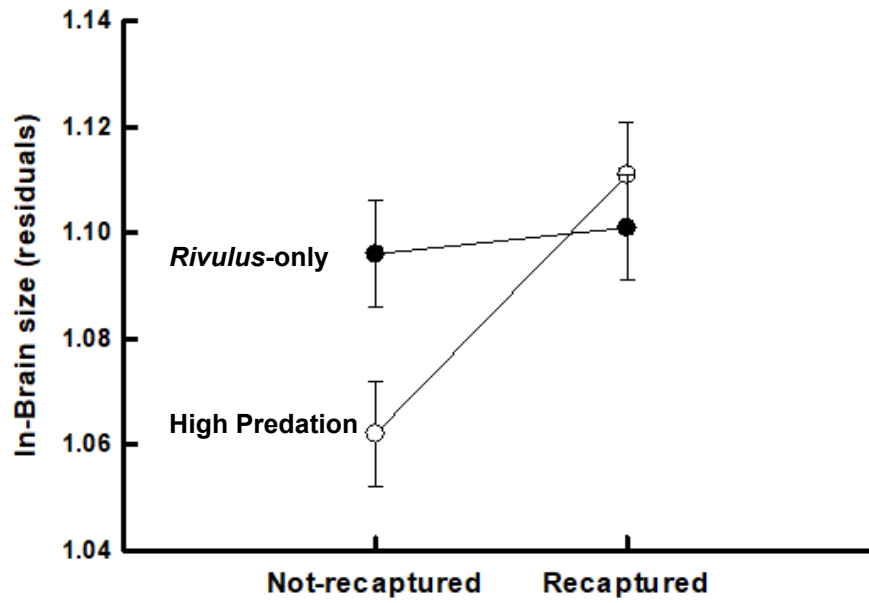
**Figure 1.** Example of measuring brain size on a live killifish. The red line indicates the width of the optic tectum.



**Figure 2.** The relationship between relative brain size (x-axis) and instantaneous growth rate (y-axis) across streams and separated by populations. High predation fish are represented by the solid red line and red circles with *Rivulus*-only fish represented by the dashed blue line and blue circles.



**Figure 3.** Mean relative brain size (y-axis) compared to recapture status (x-axis), separated by fish community. ‘Recaptured’ indicates fish were caught during recapture sampling; ‘Not-recaptured’ indicates killifish that were not caught during recapture sampling. *Rivulus*-only is represented by the solid black circles and High predation is represented by the open circles. Errors bars represent 1.0S.E.

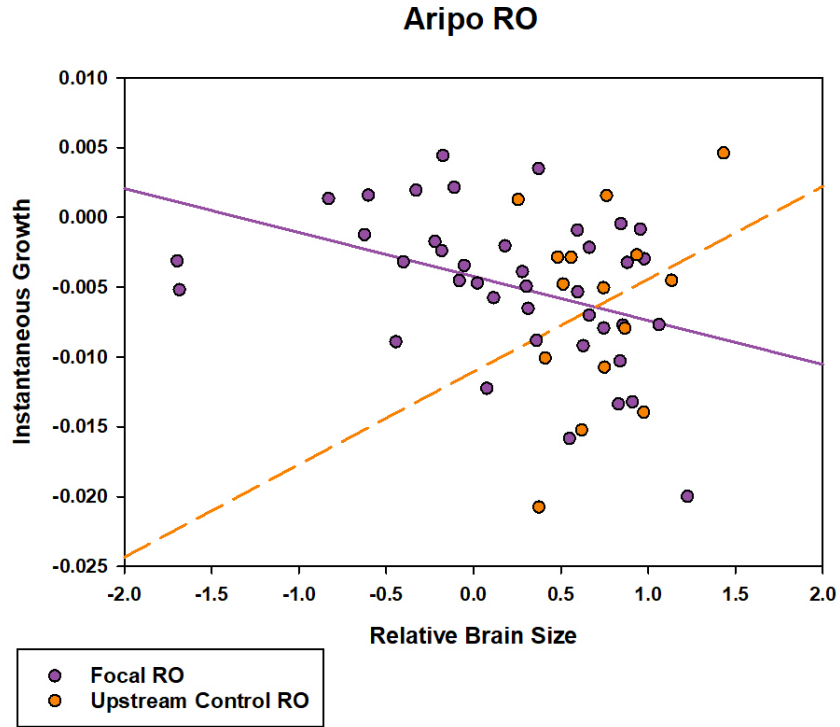


## SUPPORTING INFORMATION

**Supplemental Table 1.** Results from a general linear model including only Aripo RO fish. Groups are defined as Aripo RO focal and Aripo RO upstream control.

Predictor	df	F	p-value
<i>Group</i>	1	2.909	0.094
<i>Sex</i>	1	0.394	0.533
<i>Brain</i>	1	0.143	0.707
<i>Group x Brain</i>	1	4.181	<b>0.046</b>
<i>Sex x Brain</i>	1	1.731	0.194

**Supplemental Figure 1.** The relationship between relative brain size (x-axis) and instantaneous growth rate (y-axis) for focal Aripo RO and upstream control RO fish. Focal killifish are represented by the solid purple line and purple circles with upstream control fish represented by the dashed orange line and orange circles.



## CHAPTER 5:

### **Experimental test of the influence of light availability on the evolution of eye size and behavior in *Daphnia***

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## ABSTRACT

1. There exists extensive variation in eye size. Much work has illustrated a connection between light availability and differences in eye size across taxa. Experimental tests of the role of the light environment on the evolution of eye size are lacking.

2. Here we performed a selection experiment that examined the influence of light availability on the evolution of eye size and the connection between eye size and phototactic (anti-predator) behavior in *Daphnia*. We set-up replicate experimental populations of *Daphnia*, repeatedly evaluated phenotypic shifts in eye size during the ~50-day experiment and performed common garden experiments at the middle and end of the experiment to test for evolutionary shifts in eye size and behavior.

3. Our phenotypic analyses showed that eye size rapidly diverged between the light treatments; relative eye size was consistently larger in the low versus high light treatments. Selection on eye size was also modified by variation in density as increases in density favored a larger eye. But we did not observe differences in eye size between the light treatments following two generations of common garden rearing at the end of the experiment. Thus, differences in light did not lead to genetically based shifts in eye size. We instead observed strong shifts in anti-predator behavior. *Daphnia* from the low light treatment exhibited decreased phototactic responses to light.

4. Our results show that decreased light relaxes selection on anti-predator behavior and that selection on eye size and behavior is not necessarily coupled. Such trends provide new insights into selection on eye size and behavior.

## INTRODUCTION

It is well known that organisms exhibit extensive variation in eye size (Fernald 2004a,b; Land & Nilson 2012). Increased eye size is linked to improved components of vision such as visual acuity (Ritland 1983; Motani et al. 1999; Møller & Erritzøe 2010; Land & Nilson 2012; Caves et al. 2017, 2018). Increased eye size may also increase fitness as it is correlated with shifts in foraging, mating, and anti-predator behavior (Garamszegi et al. 2002; Jander & Jander 2002; Hall & Ross 2007; Somanathan et al. 2009; Brischoux et al. 2010; Liu et al. 2012; McCoy et al. 2015). A growing body of work has shown that eye size is phenotypically plastic in response to environmental stressors such as predator cues and resource limitation (Merry et al. 2011; Lönnstedt et al. 2013; Brandon & Dudycha 2014; Brandon et al. 2015; Svanbäck & Johansson 2019; Vinterstare et al. 2020). Eye size can also evolve among ecologically-divergent populations (Glazier & Deptola 2011; Beston et al. 2017, 2019; Beston & Walsh 2019). Variation in light availability has long been implicated as a key driver of eye size evolution (Von Salvini-Plawen and Mayr 1977; Hiller-Adams & Case 1985; Fernald 2004a, b; Moser et al. 2004; Ross & Kirk 2007; Hall 2008; Tobler et al. 2008; Somanathan et al. 2009; Schmitz & Wainwright 2011; Veilleux & Lewis 2011; Hall et al. 2012). In particular, decreased light availability is typically associated with a larger relative eye size across species (Hiller-Adams & Case 1985; Garamszegi et al. 2002; Lisney & Collin 2007; Ross & Kirk 2007; Hall 2008; Veilleux & Lewis 2011; Pearce & Dunbar 2012). While these correlative studies provide connections between the light environment and eye size variation, experimental tests of the drivers of eye size evolution, including connections between the evolution of eye size and behaviors related to fitness, are lacking.

*Daphnia* (sp.) are a ubiquitous feature of lakes with well known ecological roles as the dominant grazer on phytoplankton (Carpenter et al. 1992). The visual system of *Daphnia* is characterized by a large, conspicuous compound eye that is responsive to variation in light and

motion (Frost 1975; Ringelberg 1987). Because *Daphnia* also respond behaviorally to visual features of the environment, it has been proposed that eye size may be linked to the ability of *Daphnia* to assess important features of the environment such as high-quality food in patchy environments (Hathaway & Dudycha 2018). A growing body of work has observed a link between features of the environment and selection on eye size (Brandon et al. 2015; Beston et al. 2019). This includes research demonstrating that resource availability influences investment in eye tissue (Brandon & Dudycha 2014; Walsh & Gillis 2021) and evolutionary divergence in eye size among lakes that differ in the intensity of fish predation (Beston et al. 2019). The light environment may be particularly relevant to the evolution of eye size in *Daphnia* because they routinely modify their behavior in such a manner that alters light availability. *Daphnia* naturally migrate to deeper depths of lakes during the day to avoid visual fish predators (i.e., exhibit negatively phototactic behavior) (de Meester 1991, 1993, 1996; Hembre & Peterson 2013; Stoks et al. 2016). This 'diel vertical migration' results in *Daphnia* residing in cold, dark depths during the day before returning to the surface to forage at night. Therefore, the evolution of eye size and behavior may be coupled in *Daphnia*.

Here we tested the influence of experimental manipulations of light availability on the evolution of eye size and behavior in *Daphnia*. We exposed replicate, genetically diverse laboratory populations of *Daphnia* to a 10-fold difference in light availability and evaluated relative eye size in individuals found at the top and bottom of tall cylindrical mesocosms at regular intervals throughout the course of the ~50-day experiment. We also performed common garden experiment at the middle and end of the mesocosm experiment to test for genetically based shifts in eye size and behavior. We addressed three main questions: (1) Do differences in light availability drive evolutionary shifts in eye size? (2) Is selection on eye size modified by variation in population density? (3) Does the evolution of eye size covary with shifts in behavior?

## MATERIALS AND METHODS

*Clonal populations.* This experiment utilized clones of *Daphnia pulicaria* that were either hatched from sediments cores collected from Lake Mendota and Lake Kegonsa in Wisconsin in February 2018 or clones that were collected from the water column in Spring 2019 (see Landy et al. 2020). This study included 36 clones of *Daphnia* that were divided into two genetically distinct 'populations' consisting of 19 clones from sediment cores (11 from Lake Kegonsa and 8 from Lake Mendota) and 17 clones of *Daphnia* collected live from the water column (8 from Lake Kegonsa and 9 from Lake Mendota). Each clonal population was replicated 3x per light treatment (see below). All clones were maintained in the lab in 125-ml jars in COMBO medium (Kilham et al. 1998) and fed non-limiting supply of green algae (species: *Scenedesmus obliquus*; concentration:  $\sim 1.0 \text{ mg C L}^{-1} \text{ day}^{-1}$ ) (Photoperiod 14L:10D; 16°C) for approximately two years prior to the initiation of the common garden rearing process.

*Selection experiment.* All clones were first reared in a common environment for two generations prior to the start of the selection experiment. To initiate this process, we isolated 10 mature females from the stock jars of each clonal lineage and placed two individuals per 90-ml jar filled with COMBO media. These jars were monitored daily (M-F) for the production of new offspring. Upon the production of the next clutch of offspring, we collected two neonates per jar and placed them into 90-ml jars filled with COMBO media. We considered this generation to be the 'first generation lab reared individuals'. All jars were fed a non-limiting supply of green algae (species: *Scenedesmus obliquus*; concentration:  $\sim 1.0 \text{ mg C L}^{-1} \text{ day}^{-1}$ ) (Photoperiod 14L:10D; 18°C). These jars were transferred to fresh media and algae three times per week (every M W F) and were monitored daily (M-F) for the production of offspring.

The selection experiment used 12, 150-l mesocosms (height=101.6cm, inside diameter=45.72cm) that were filled with COMBO media. These mesocosms were equally divided between two light treatments: (1) high light and (2) low light (2 clonal populations x 2



light treatments x 3 replicates = 12 mesocosms). To manipulate light availability, we added black window screen to each experimental unit to maintain similar light levels within each treatment but also generate a ~10x difference in light reaching the surface of the water between the treatments (Average light level in lux  $\pm 1.0$  S.D: High light=138.83 $\pm$ 12.17, Low light=11.17 $\pm$ 0.90). We used a secchi disc to confirm that light disappeared by the mid-point depth in all mesocosms in the low light treatment. Light still penetrated to the bottom of all tanks in the high light treatment. All mesocosms were supplied with gentle aeration.

The mesocosms were stocked with second generation lab born individuals. To generate an increased number of individuals for this experiment, we reared this generation in larger jars (500-ml). Also, to include as many individuals as possible for the mesocosm experiment, we began to collect offspring from the first-generation lab reared parents as soon as they began to produce offspring (i.e., clutch 1). We kept track of the maternal clutch number as well as the age and rearing density of the individuals that populated the selection experiment. We collected newly born individuals over a five-day period (between 2/24/2021-3/1/2021) and the experiment commenced with individuals that were 2-7 d old. The average age of individuals was very similar between the light treatments (Average age in d: high light = 4.51, Low light 4.48). The mesocosm experiment was ultimately initiated with a very similar number of individuals between the light treatments (Average no. of individuals added per mesocosm $\pm 1.0$ s.e: high light=243 $\pm$ 2; low light=241 $\pm$ 3.9;  $F_{1,10}=0.63$ ,  $p=0.45$ ). All of the common garden reared clones from a given population were represented in each replicate and we aimed to equally represent each clone as much as possible (average no. of individuals per clone added to a given replicate=13.3 individuals). The clutch number that produced the offspring that initiated the mesocosm experiment was also similar between the light treatments (Average clutch no.: high light=2.21, low light=2.23).

On 2/24/2021 we added specified quantities of green algae to each experimental unit (*Scenedesmus obliquus*; concentration:  $\sim 0.4 \text{ mg C L}^{-1}$ ) (Photoperiod 14L:10D). We allowed the algae to then grow for one week prior to adding *Daphnia*. *Daphnia* from the two clonal replicate populations were allocated equally between the light treatments. We then allowed the *Daphnia* populations to grow without any further additions of algae to the mesocosms until day 22. At this time, we added algae 1x per week to ensure sufficient algal levels ( $>0.15 \text{ mg C L}^{-1}$ ) to maintain the *Daphnia* populations throughout the duration of the experiment. We also periodically added equal amounts of fresh media to all mesocosms throughout the experiment.

Starting on day 10 of the experiment (i.e., 10 days after adding *Daphnia* to the mesocosms), we used a Van Dorn water sampler to collect *Daphnia* that were located at the top of each mesocosm (Van Dorn was submerged just below the surface) as well as *Daphnia* positioned at the bottom of each mesocosm (Van Dorn was positioned approximately two inches above the bottom before triggering the closing mechanism). We collected 2 liters of water per sample. We counted all *Daphnia* per sample and then photographed a maximum of 20-30 *Daphnia* per sample for estimates of eye and body size (via ImageJ). Initially this sampling occurred 2x per week. We continued to sample all tanks 2x per week until day 29, after which we shifted to sampling each mesocosm 1x per week until the end of the experiment (day 50). This resulted in nine estimates of *Daphnia* abundances, eye size, and body size per mesocosm by then end of the experiment.

*Statistical analyses.* Variation in abundances, *Daphnia* body size, absolute eye size, and relative eye size were analyzed using repeated measures linear mixed models via SPSS v. 27 (IBM corporation). These analyses included day of sampling as the repeated measures effect and individual mesocosm as the within-subject variable. We included light treatment (high, low), location of sampling (top, bottom), and clonal population as fixed effects. We also included interactions that were specifically related to our core hypotheses. This includes the light x day,

light x location, and light x location x day interactions. To analyze shifts in relative eye size, we ln-transformed eye and body size and included ln-body size as a covariate in the analyses. For this analysis, we also used the average value of eye and body size for each sampling date per mesocosm.

We also evaluated the connection between variation in population density and *Daphnia* body size, absolute eye size, and relative eye size. To do so, we calculated the average density, body size, and (absolute and relative) eye size for each combination of sampling date and mesocosm. We then performed Pearson correlations between density vs. body size, density vs. absolute eye size, and density vs. relative eye size. We performed these correlations separately between the high and low light treatments.

*Common garden experiment #1 (midpoint of selection experiment).* We performed a common garden experiment at the approximate mid-point of the experiment to determine if the light treatments led to shifts in relative eye size after 21 days of selection. On March 21st, 2021, we stirred each mesocosm and randomly pulled ~20 mature individuals per mesocosm. We considered each mature individual to represent a distinct clonal lineage. The number of clones that we were able to rear successfully varied among the mesocosms but was similar between the light treatments (Range of clones reared per mesocosm=6-26; average no. of clones: high light=13.8, low light=15.8). We initiated the first common garden reared generation by immediately collecting two offspring from the next clutch produced by each female and placing two individuals in a 90-ml jar (per clone and treatment). We then initiated the second generation of common garden rearing by collecting 3-5 individuals from clutch 1-2 produced by the parental females. These individuals were reared individually in 90-ml jars until day 4-6 when they were photographed for estimates of eye and body size (average age $\pm$ 1.0s.d.=4.54d $\pm$ 0.7). The total sample size was 572 individuals (high light=251 individuals, low light=321 individuals). All jars were transferred to fresh COMBO media and algae 3x per week throughout all phases of

rearing (*Scenedesmus obliquus*; concentration: 0.8mg C L<sup>-1</sup>; photoperiod 14L:10D; temperature=18°C). Variation in body size, absolute eye size and relative eye size was analyzed using linear mixed models with REML via SPSS v.27 (IBM corporation) with light treatment, clonal population, and the light x clonal population interaction entered as fixed effects. We ln-transformed eye size and ln-body length was included as a covariate in the analysis for relative eye size. Clone ID nested within mesocosm was included as a random effect.

*Common garden experiment #2 (end of selection experiment)*. We also performed a common garden experiment at the very end of the mesocosm trials to test for evolved differences in eye size and phototactic behavior in response to controlled manipulations of light availability. On day 50, we randomly pulled ~20 individuals after stirring each mesocosm and considered each individual to represent a distinct clonal lineage. We initiated the next generation of lab reared individuals by collecting all newly produced offspring from the subsequent clutch and allocating two individuals per 90-ml jar across a total of 3-5 jars (total offspring=6-10 offspring). These first generation, lab reared jars were changed to fresh media and algae 3x per week and were monitored for the production of new clutches of offspring daily. All other rearing conditions were the same as described above for the first common garden experiment.

We then assessed *Daphnia* behavior (phototactic behavior) and eye size in second generation lab reared individuals. Phototactic behavior approximates the naturally occurring vertical migration of *Daphnia* to deeper depths where predation intensity is lessened during the day (de Meester 1991, 1993, 1996). This behavior is assessed by measuring phototactic responses to light in experimental columns. The quantification of this behavior also requires that we rear groups of individuals. As a result, all newly born individuals from a given clone were collected and ~10 individuals were placed into 125-ml jars filled with COMBO media and green algae (*Scenedesmus obliquus*; concentration: 0.8mg C L<sup>-1</sup>; photoperiod 14L:10D;

temperature=18°C). Each combination of clone and mesocosm were replicated 2-3x (except for seven clones out of 212 that were only replicated 1x). To generate these second-generation jars, we began collecting offspring as soon as the first-generation individuals began to reproduce (i.e., first clutch) and continued to monitor all clones for the production of additional clutches to generate additional replicates. The vast majority of replicates (525/556 jars) were created by collecting offspring from clutch 1-5. Though a small number of jars (~5%) used offspring from clutch 6-7. The assessments of behavior ultimately included 212 clones and 556 jars that were evenly distributed between the light treatments (high light=278 jars, low light=278 jars; average no. of clones per mesocosm: high light=17.67, low light=17.67).

Phototactic behavior was measured on day 5-11 before the release of any offspring (average age in  $d \pm 1.0s.d. = 7.58d \pm 0.99$ ) by placing individuals in a 25-cm vertical column enclosed in a darkened chamber (for a 15-min acclimation period) before switching on the light (average Lux  $\pm 1.0s.d. = 70.14 \pm 8.72$ ). The vertical column was divided into a 12-cm upper compartment, a 10-cm middle compartment and a 3-cm lower compartment. Phototactic behavior was then measured by tracking the number of individuals in the top vs. bottom compartments of the chamber every minute over the course of the 10-minute trial (see (de Meester 1991, 1993, 1996). The phototactic index is specifically defined as:  $(U - L)/(U + M + L)$  where U, M, L are the number of animal observations in the upper, middle and lower compartment. The index ranges from -1 (negatively phototactic) to 1 (positively phototactic). For each trial, we calculated the average phototactic index over the entire 10-minute trial. At the conclusion of the trial, we randomly isolated one individual and photographed that individual for estimates of eye size (via ImageJ). Note that the sample sizes for the assessment of eye size were slightly larger (n=609 jars). This is because we set-up more replicates that we were ultimately able to assess for behavior due to logistical constraints, but we were able to quantify

eye size for all jars. These missing assessments of behavior were not biased towards any treatment.

Variation in body size, absolute eye size, and relative eye size (eye and body size were both ln-transformed) and phototactic behavior were analyzed using linear mixed models via SPSS v.27 (IBM corporation). These analyses included the light treatment, clonal population, and the light x population interaction as fixed effects. Clone ID nested within mesocosm was included as a random effect. We included *Daphnia* age, parental clutch number, and rearing density as covariates for all analyses. *Daphnia* body size was included as an additional covariate in the analyses for relative eye size while trial light level was added as a covariate in the analyses of phototactic behavior. We did not control for the production of males in this experiment. As a result a small number of the replicates and resulting images were male *Daphnia* (<5%). We removed these images from the analyses of eye and body size, but the results are similar irrespective of their inclusion.

## RESULTS

*Selection experiment: Daphnia abundances.* The influence of the light treatments on the abundances of *Daphnia* depended upon day of the experiment (Fig. 1). This is because we observed a significant ( $p < 0.05$ ) light x day interaction ( $F_{8,37.9} = 24.5$ ,  $p < 0.001$ ). *Daphnia* abundances between the light treatments were similar during initial sampling events, but then *Daphnia* from the high light treatments attained abundances that were 2-3x greater during day 14-21 than the densities observed in the low light treatments (Fig. 1). Thereafter, the abundances of *Daphnia* in the high light treatments declined to level that was similar to the abundances observed in the low light treatments. Overall, effects due to light level ( $F_{1,91.5} = 53.7$ ,  $p < 0.001$ ) and day ( $F_{8,37.9} = 25.9$ ,  $p < 0.001$ ) were significant. The abundances of *Daphnia* also differed between the sampling locations ( $F_{1,91.5} = 9.34$ ,  $p = 0.003$ ). *Daphnia* were 23% more abundant at the bottom of the mesocosms (Average density of *Daphnia* per liter  $\pm$

1.0s.e.: Top=16.11± 0.84, Bottom=19.88 ±.91). The effects due to clonal population (F1,119.6=0.24, p=0.62), light x location (F1,91.5=0.3, p=0.59), and light x day x location (F16,37.9=0.51, p=0.93) were all non-significant.

*Selection experiment: Daphnia traits.* We observed a significant ( $p < 0.05$ ) light x day interaction for body size, absolute eye size, and relative eye size (Table 1). In general, the body size and absolute eye sizes of *Daphnia* from the low light treatments were larger than *Daphnia* from the high light treatments (Fig. 1). Such differences were especially apparent during the beginning and latter portions of the experiment but were reduced or disappeared during the middle of the experiment (days 21-29). The patterns were similar for relative eye size; *Daphnia* from the low light treatments exhibited a larger relative eye size for most sampling events but the differences between the light treatments were smaller than those observed for absolute eye size (Fig. 1). There was an overall effect of the light treatment for all three traits. *Daphnia* from the low light treatment were 34% larger with absolute and relative eyes that were 4% and 2% larger than *Daphnia* from the high light treatment, respectively (Fig. 1). We observed a significant effect due to sampling location for body size. *Daphnia* were 16% smaller at the bottom of mesocosms. The absolute and relative eye size also differed between the replicate populations (Table 1). All other terms were not significant ( $p > 0.05$ ) (Table 1).

*Selection experiment: Correlations between density and Daphnia traits.* For the high light treatment, we observed a significant negative correlation between *Daphnia* density and *Daphnia* body size (Fig. 2). The correlation between density and absolute and relative eye size were both significantly positive for the high light treatment (Fig. 2). In contrast, the relationship between density and body size was marginally positive in the low light treatments (Fig. 2;  $0.05 < p < 0.1$ ). The correlation between density and absolute eye size was significantly positive, while the relationship between density and relative eye was also positive but marginally non-significant in the low light treatment (Fig. 2).

*Common garden experiment 1.* We observed a significant ( $p < 0.05$ ) effect of light treatment on absolute and relative eye size but not body size (Table 2). The absolute and relative eye size of *Daphnia* from the high light treatment was 3.5% and 4.3% larger than individuals from the low light treatment, respectively (Fig. 3). We did not observe any overall differences between the clonal populations although the light x population interaction was significant for body size (Table 2) (Average body size  $\pm 1.0$ s.e.: Replicate 1: High light =  $0.463 \pm 0.02$ , Low light =  $0.423 \pm 0.021$ ; Replicate 2: High light =  $0.445 \pm 0.021$ , Low light =  $0.49 \pm 0.018$ ).

*Common garden experiment 2.* We observed a significant ( $p < 0.05$ ) influence of the light treatment on phototactic behavior (Table 3). *Daphnia* from the high light treatment exhibited a phototactic behavior that was 18% more negative than *Daphnia* from the low light treatment (Fig. 4). The influence of the light treatment on body size, absolute eye size, and relative eye size was non-significant (Table 4). The replicate clonal populations differed significantly in body size (Body size in mm  $\pm 1.0$ s.e.: replicate 1 =  $1.88 \pm 0.017$ , replicate 2 =  $1.94 \pm 0.016$ ) and absolute eye size (Absolute eye size in mm  $\pm 1.0$ s.e.: replicate 1 =  $0.119 \pm 0.001$ , replicate 2 =  $0.122 \pm 0.001$ ) but not for relative eye size and phototactic behavior (Table 4). The population x light interactions were all not-significant (Table 4).

## DISCUSSION

Here we used laboratory selection to experimentally test the connection between light availability and the evolution of *Daphnia* eye size and behavior. We observed clear shifts in the anti-predator behavior of *Daphnia* following 50 days of selection (Fig. 4). Laboratory populations of *Daphnia* that were exposed to lower levels of light availability exhibited a decreased phototactic response when compared with *Daphnia* from the high light treatments. Such differences are likely genetically based as they were maintained following two generations of common garden rearing. Our results also show that *Daphnia* from the low light treatments that were directly sampled from the mesocosms consistently exhibited a larger relative eye size



throughout the experiment (Fig. 1). These differences between the light treatments indicate that low light availability should favor the evolution of a larger eye. Yet, common garden experiments performed at the middle and end of the mesocosm experiment yielded results that were opposite of the phenotypic trends (Fig. 3) or did not find any differences between the light treatments (Fig. 4). Such results beg three questions: 1) Why did low light select for declines in anti-predator behavior? 2) Why did high light appear to favor a larger eye size at the mid-point of the selection experiment? 3) Why did we fail to observe evidence for evolutionary shifts in relative eye size by the end of the experiment (i.e., Fig. 4)?

The phototactic behavior of *Daphnia* is presumably an adaptive response that allows *Daphnia* to mitigate the negative impacts of visual fish predation (Lampert 1989). *Daphnia* migrate to the bottom of lakes during daylight but then return to the surface during the evening to forage. There are costs associated with *Daphnia* phototactic behavior (Loose & Dawidowicz 1994). The deep portion of lakes are colder and characterized by declines in food quantity and quality (Guisande et al. 1991). Research has indeed shown that shifts in predation intensity can drive evolutionary shifts in the phototactic behavior of *Daphnia* (Cousyn et al. 2001; Stoks et al. 2016; Landy et al. 2020). Typically, increased predation leads to the evolution of increasingly negative phototactic behavior (e.g., Landy et al. 2020). Research has also shown that light availability can modify the proximate expression of anti-predator behavior (Clarke 1983; Longland & Price 1991; Daly et al. 1992; Kotler et al. 1993). This includes research showing that the traits of *Daphnia* are phenotypically plastic in response to laboratory manipulations of light availability (Effertz & von Elert 2014, 2017; Talanda et al. 2018). Our results provide evidence that the intensity of the light environment can impose selection on the evolution of anti-predator behavior. Increased light availability favored the evolution of increased anti-predator behavior (Fig. 4). One logical interpretation is that increased light favors increasingly negative phototactic responses because such a response increases the likelihood that *Daphnia* will avoid visual

predators in environments that are characterized by high light. Conversely, low light may relax selection on anti-predator responses because strong phototactic responses are no longer necessary to avoid predators. Our results therefore provide new insights into how the light environment shapes the evolution of anti-predator behavior.

Much research has shown that low light likely favors the evolution of a larger relative eye size (Hiller-Adams & Case 1985; (Garamszegi et al. 2002; Lisney & Collin 2007; Ross & Kirk 2007; Hall 2008; Veilleux & Lewis 2011; Pearce & Dunbar 2012). We therefore expected that prolonged exposure to low light in the lab would lead to the evolution of a larger eye size. This expectation was generally supported by the direct sampling of live individuals throughout the mesocosm experiment (Fig. 1). But the results of the common garden experiments were either contradictory (Fig. 3) or failed to observe any differences in eye size (Fig. 4). Thus, the trends revealed by the common garden experiments (Fig. 3-4) did not follow the phenotypic trends revealed throughout the duration of the mesocosm experiment (Fig. 1). It was particularly surprising that high light appeared to favor the evolution of a larger eye at the approximate midpoint of the selection experiment (Fig. 3). Though, it is important to consider the timing of this experiment along with the differences in density observed between the light treatments (Fig. 1). We collected the lineages for the first common garden experiment when abundances of *Daphnia* were 2-3x greater in the high light vs. low light treatment (Fig. 1). We also found that increasing density consistently favored a larger (absolute and relative) eye size (Fig. 2). That is, increasing density also modified selection on the eye size. Furthermore, this connection between density and eye size was stronger in the high light treatments (Fig. 2). Thus, it is possible that the larger eye size observed in the high light treatments in the first common garden experiment is more of a response to the differences in density and not light *per se*. Regardless, our results highlight the need to further consider the role of density and selection on eye size (see also Brandon & Dudycha 2014).

Why did we fail to observe differences in relative eye size following ~50 days of selection? Given that the phenotypic trends revealed clear and consistent differences in eye size between the light treatments (Fig. 1), it seems likely that a 10x difference in light availability should exert sufficiently strong selection to drive an evolutionary response. We also started all mesocosms with a genetically diverse assemblage of clones and the mesocosms exhibited population sizes that were large enough to not constrain evolutionary processes (average population size from day 10-49=~2800 individuals). Though, we did not track clonal diversity over time. One possibility is that perhaps genetic variation rapidly declined in the experimental units. Further, our experiment began with *Daphnia* that were several days old (average age ~4d old) and *Daphnia pulicaria* mature at ~8 days old (at the experimental temperature). As a result, our experiment conservatively spanned five generations. But perhaps an increased number of generations is required to drive evolutionary shifts in eye size. Further experimental tests of the link between light and eye size are clearly needed.

## **Conclusions.**

We found that experimental manipulations of light availability led to consistent phenotypic divergence in eye size; the relative eye size of *Daphnia* was consistently larger in response to exposure to low light levels (Fig. 1). Yet, such differences were not maintained following multiple generations of common garden rearing (Fig. 3-4). This is potentially, in part, due to confounding effects of density on eye size (increased density favors a larger eye) (Fig. 2). We instead found clear shifts in the anti-predator behavior of *Daphnia* (Fig. 4). Low light favored the evolution of declines in phototactic behavior (Fig. 4). Such results indicate that the light environment will be an important selective force on the expression of anti-predator behavior in the wild. Our results provide new insights into the factors that influence selection on eye size and behavior and call for more experiments that test the drivers of eye size evolution.

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## TABLES

**Table 1.** Analyses for the live caught individuals sampled during the selection experiment.

	Num. df	Body Size			Absolute Eye Size			Relative Eye size		
		F	p-value	denom df.	F	p-value	denom df.	F	p-value	denom df.
<b>Day</b>	8	<b>24.24</b>	<b>&lt;0.001</b>	33.59	<b>79.9</b>	<b>&lt;0.001</b>	35.6	<b>262.3</b>	<b>&lt;0.001</b>	36.3
<b>Light</b>	1	<b>21.41</b>	<b>&lt;0.001</b>	124.5	<b>27.7</b>	<b>&lt;0.001</b>	66.4	<b>8.75</b>	<b>0.006</b>	29.2
<b>Population</b>	1	1.08	0.3	142.2	<b>8.46</b>	<b>0.004</b>	149	<b>11.01</b>	<b>0.001</b>	110.8
<b>Location</b>	1	<b>5.51</b>	<b>0.02</b>	124.5	2.09	0.15	66.4	0.03	0.86	28.5
<b>Day x Light</b>	8	<b>3.04</b>	<b>0.011</b>	33.59	<b>2.28</b>	<b>0.044</b>	35.61	<b>2.52</b>	<b>0.03</b>	32.9
<b>Light x location</b>	1	1.08	0.3	124.5	0.32	0.57	66.4	0.035	0.85	28.4
<b>Day x Light x Location</b>	16	0.63	0.84	33.54	0.57	0.89	35.9	0.26	0.998	32.6
<b>Body size</b>								<b>1110.4</b>	<b>&lt;0.001</b>	92.7



**Table 2.** Analyses from the first common garden experiment performed at the midpoint of the selection experiment.

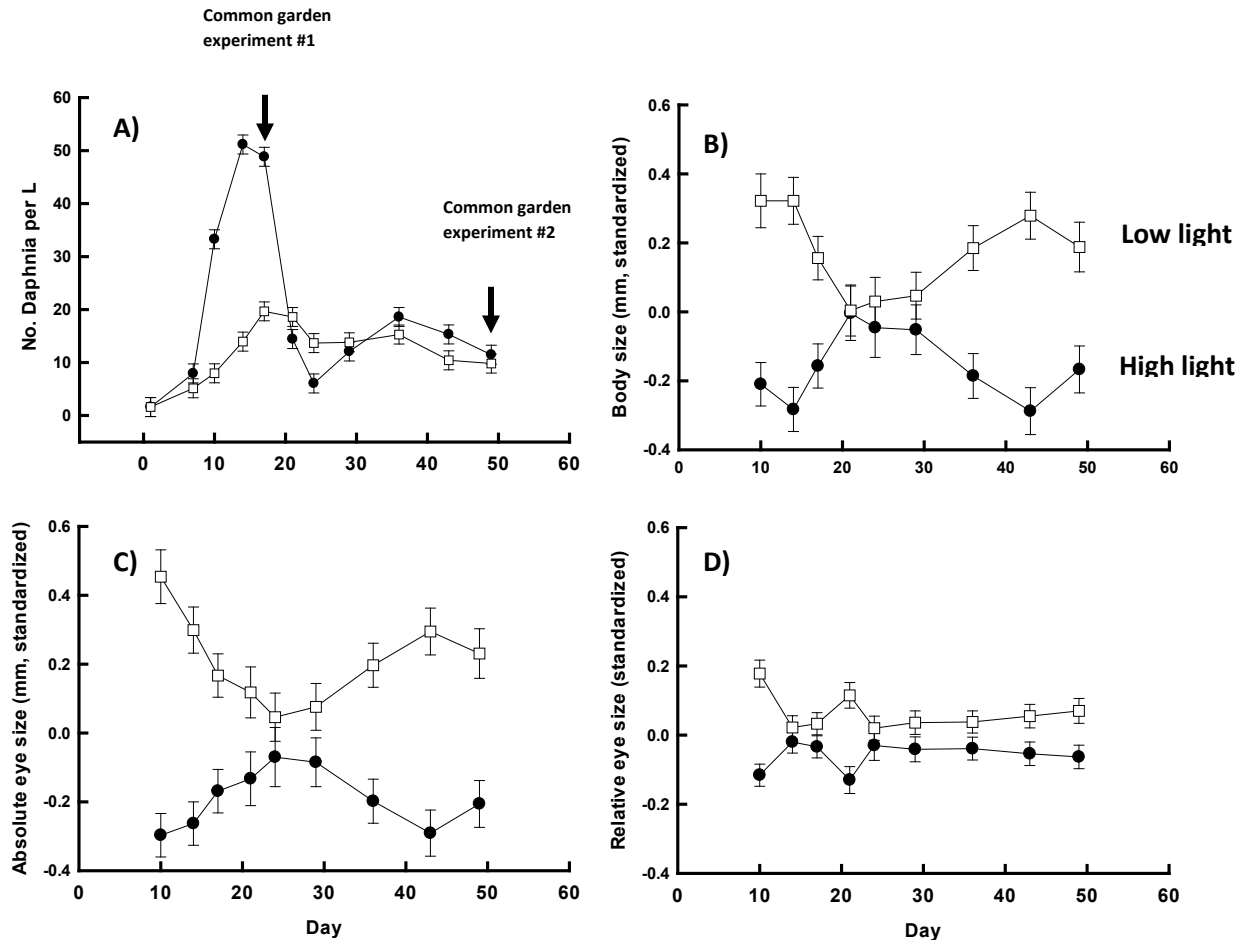
	Num df	Body Size			Absolute eye size			Relative eye size		
		F	p-value	denom df	F	p-value	denom df	F	p-value	denom df
<i>Covariates:</i>										
<b>Age</b>	1	<b>33.24</b>	<b>&lt;0.001</b>	244.8	<b>45.2</b>	<b>&lt;0.001</b>	208.4	<b>19</b>	<b>&lt;0.001</b>	209
<b>Body size</b>	1	.....	.....	.....	.....	.....	.....	<b>252.9</b>	<b>&lt;0.001</b>	497.4
<i>Fixed effects:</i>										
<b>Light</b>	1	0.05	0.82	172.9	<b>12.1</b>	<b>0.001</b>	174	<b>19.7</b>	<b>&lt;0.001</b>	172.1
<b>Population</b>	1	1.5	0.22	170	0.05	0.83	170.3	1.76	0.19	169
<b>Light x population</b>	1	<b>4.17</b>	<b>0.043</b>	171.6	3.3	0.071	170.5	0.15	0.7	170.7
<i>Random effects:</i>										
<b>Clone(Mesocosm)</b>	1	<b>7.49</b>	<b>&lt;0.001</b>		<b>5.47</b>	<b>&lt;0.001</b>		<b>5.15</b>	<b>&lt;0.001</b>	

**Table 3.** Analyses for the second common garden experiment performed at the end of the selection experiment.

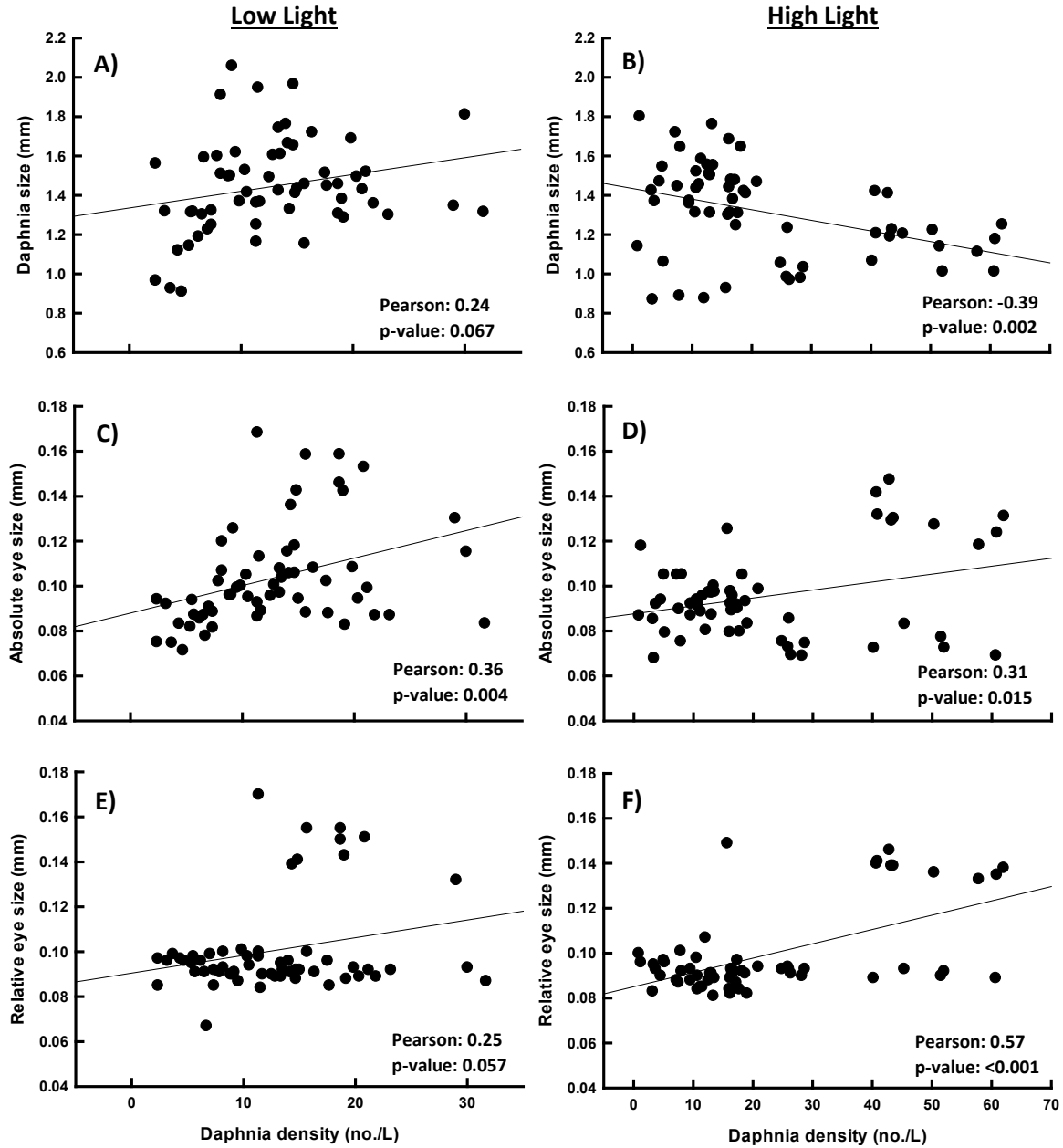
	Num df	Body Size			Absolute eye size			Relative eye size			Phototactic behavior		
		F	p-value	denom df	F	p-value	denom df	F	p-value	denom df	F	p-value	denom df
<i>Covariates:</i>													
<b>Age</b>	1	<b>79.14</b>	<b>&lt;0.001</b>	503.9	<b>52.68</b>	<b>&lt;0.001</b>	443.6	<b>6.25</b>	<b>0.013</b>	417.3	3.51	0.062	542
<b>No. of Daphnia</b>	1	2.41	0.12	499.8	1.35	0.25	516.6	0.45	0.51	508.4	3.36	0.067	497.7
<b>Clutch number</b>	1	9.73	0.002	484.1	2.14	0.14	406.9	0.02	0.9	365.1	2.26	0.13	532.5
<b>Lux level</b>	1										0.75	0.39	483.9
<b>Body size</b>	1							<b>226.73</b>	<b>&lt;0.001</b>	462.5			
<i>Fixed effects:</i>													
<b>Light</b>	1	0.06	0.81	196.7	0.14	0.71	192	0.06	0.8	193.5	<b>7.99</b>	<b>0.005</b>	204
<b>Population</b>	1	<b>7.3</b>	<b>0.008</b>	204.3	<b>3.97</b>	<b>0.048</b>	200.4	0.24	0.62	205.3	2.31	0.13	209.5
<b>Light x population</b>	1	0.26	0.61	199.2	1.28	0.26	194.3	1.08	0.3	196	0.85	0.36	206.6
<i>Random effects:</i>													
<b>Clone(Mesocosm)</b>	1	<b>5.36</b>	<b>&lt;0.001</b>		<b>2.86</b>	<b>0.004</b>		1.35	0.18		<b>6.81</b>	<b>&lt;0.001</b>	

## FIGURES

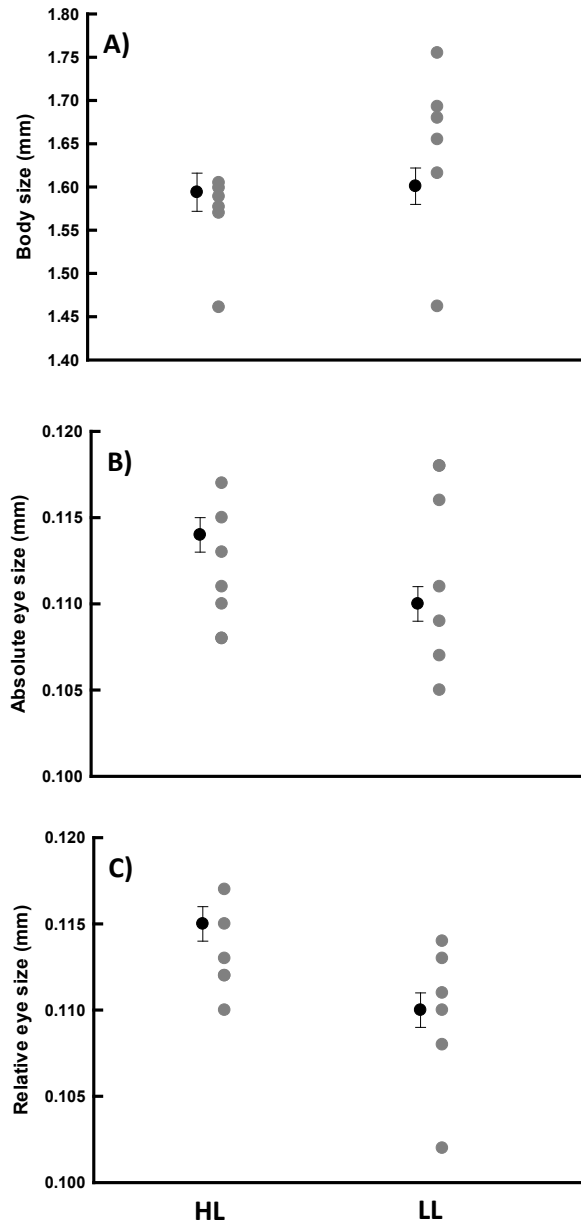
**Fig. 1. Variation in *Daphnia* abundances and eye size over time.** (A) *Daphnia* abundances, (B) body size, (C) absolute eye size, and (D) relative eye size. Open squares = low light. Closed circles = high light. Estimates of eye and body size are based upon images taken from live wild caught individuals. Error= $\pm 1.0$ s.e.



**Fig. 2. Correlations between Daphnia density and (A-B) body size, (C-D) absolute eye size, and (E-F) relative eye size. Low light = panels A, C, E. high light = panels B, D, F.**



**Fig. 3. Variation in (A) body size, (B) absolute eye size, and (C) and relative eye size stemming from common garden reared lineages that were sampled at the ~midpoint (day 21) of the experiment. HL - high light, LL - low light. Closed circles = average treatment mean. Grey circles = replicate mesocosm means. Differences in absolute and relative eye size were significant. Error= $\pm 1.0$ s.e.**



**Fig. 4. Variation in (A) body size, (B) absolute eye size, (C) relative eye size, and (D) phototactic behavior stemming from common garden reared lineages that were sampled at the end of the mesocosm experiment. HL - high light, LL - low light. Closed circles = average treatment mean. Grey circles = replicate mesocosm means. Differences in phototactic behavior were significant. Error= $\pm 1.0$ s.e.**

