unesp

CAMPUS DE SÃO JOSÉ DO RIO PRETO

Sociality, cognition and brain complexity in Neotropical cichlids

Manuela Lombardi Brandão

DOUTORADO

PÓS GRADUAÇÃO
EM BIOLOGIA ANIMAL

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

Manuela Lombardi Brandão

Sociality, cognition and brain complexity in Neotropical cichlids

São José do Rio Preto
2020

Manuela Lombardi Brandão

Sociality, cognition and brain complexity in Neotropical cichlids

Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Biologia Animal, junto ao Programa de Pós-Graduação em Biologia Animal, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto.

Financiadora: FAPESP – Proc. 2016/26160-2 e
2019/10808-1
CAPES / DS 001

Orientadora: Profª. Drª. Eliane Gonçalves de Freitas
Coorientador: Prof. Dr. Rui F. Oliveira

São José do Rio Preto
2020

B817s

Brandão, Manuela Lombardi
Sociality, cognition and brain complexity in Neotropical cichlids / Manuela Lombardi Brandão. -- São José do Rio Preto, 2020
127 p. : il., tabs., fotos

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto
Orientadora: Eliane Gonçalves-de-Freitas
Coorientador: Rui Filipe Oliveira

1. Comportamento animal. 2. Comportamento social. 3. Ciclídeos neotropicais. 4. Células cerebrais. 5. Cognição. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca do Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto. Dados fornecidos pelo autor(a).

Essa ficha não pode ser modificada.

Manuela Lombardi Brandão

Sociality, cognition and brain complexity in Neotropical cichlids

Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Biologia Animal, junto ao Programa de Pós-Graduação em Biologia Animal, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto.

Financiadora: FAPESP – Proc. 2016/26160-2 e
2019/10808-1
CAPES / DS 001

Comissão Examinadora

Profª. Drª. Eliane Gonçalves de Freitas
UNESP – Câmpus de São José do Rio Preto
Orientadora

Prof. Dr. Alexander Kotrschal
Stockholm University – Suécia

Profª. Drª. Ana Celia Silva Barbato
Universidad de la República – Uruguai

Profª. Drª. Marisa Fernandes de Castilho
UFPR – Câmpus de Assis

Prof. Dr. Redouan Bshary
Neuchâtel University – Suíça

São José do Rio Preto
28 de fevereiro de 2020

Aos meus pais, Manoel e Marcia,
meus maiores exemplos e melhores amigos

AGRADECIMENTOS

Não há palavras que possam ser ditas ou escritas para agradecer aos meus pais, Marcia e Manoel, por tudo o que fizeram e fazem por mim todos os dias. Vocês dois nunca mediram esforços para que eu pudesse ter as melhores condições, em todos os sentidos, fazendo de mim uma pessoa extremamente privilegiada. A Educação das suas duas filhas sempre veio em primeiro lugar, e vocês nunca pouparam esforços para garanti-la. Agradeço por terem dado à mim e à Marilia o melhor e mais completo lar, cheio de amor, risadas, cumplicidade e proteção. Agradeço por terem me ensinado a questionar, a ter pensamento crítico, a ser independente como mulher. Uma vida não é suficiente para agradecer e amar vocês. Obrigada do fundo do coração.

Agradeço à minha irmã Marilia, a caçula que precisava de uma irmã mais velha para dar o bom exemplo (espero ter conseguido, pelo menos na maior parte do tempo…). Por ser hoje uma recém mulher tão bonita, inteligente e independente, que com seu jeitinho nem sempre do mais "sutil" cuida da sua família com muito amor e afinco. Agradeço, enfim, por ser a minha amiga querida, que me ajuda a aliviar o peso das costas em dias não tão ensolarados, mesmo sem perceber.

Agradeço ao meu companheiro de vida, Vinicius, por ter estado ao meu lado, literalmente, em todas as etapas da minha vida acadêmica. Você tornou (e torna) tudo mais fácil. Obrigada por ter sido meu primeiro ajudante de laboratório. Obrigada por ter chorado de alegria quando eu passei no Mestrado. Obrigada por ter me confortado quando o desespero batia e eu não queria fazer mais nada. Obrigada por estar do meu lado em todos os momentos, na alegria e na tristeza, meu eterno namorado.

Agradeço à minha orientadora, Eliane, por todos esses 10 anos de convívio e por tudo o que você me ensinou durante eles. Não falo menos dos ensinamentos acadêmicos (que não caberíam nessas páginas!) mas mais da postura com a qual você nos ensina todos os dias. Por ser uma excelente Professora (com P maiúsculo), nos mostrando que devemos sempre dar o nosso melhor na profissão que escolhemos, embora o Brasil não seja o lugar mais cordial com os seus educadores. A sermos corretos e humildes com o conhecimento que produzimos, e que devemos retornar à sociedade da melhor forma possível. Agradeço por todas as correções e assinaturas, conselhos e conversas, projetos e incentivos, preocupações divididas; enfim, por toda a dedicação à minha formação durante todos esses anos. Agradeço, professora, a sua amizade e ensinamentos de uma década. Obrigada por acreditar e confiar em mim.

Agradeço aos meus amigos de laboratório (e fora dele), Ana Gauy, Felipe Dorigão e Marcela Bolognesi por todo o companheirismo dos últimos anos. Pela ajuda inestimável de vocês dentro do laboratório, sem a qual esse trabalho não existiria. E pela ajuda fora do laboratório, também. A amizade e companheirismo de vocês me fez existir durante esse doutorado.

Agradeço ao meu coorientador, professor Rui F. Oliveira, que mesmo com um oceano de distância esteve presente durante toda a construção dessa tese de doutorado, desde o dia um. Agradeço imensamente por ter me ajudado a ampliar meus horizontes, tanto como pesquisadora quanto como pessoa, me recebendo de braços abertos em seu laboratório em Portugal. Essa oportunidade foi ímpar na minha jornada, e com certeza mudou os rumos do meu futuro. Obrigada, Rui, pelos ensinamentos, discussões e confiança investidos em mim ao longo desses anos de doutorado. E, não menos importante, pelos sonhos que me ajudou a realizar nesse ínterim.

Agradeço à professora Thais Billalba de Carvalho, que me recebeu em seu laboratório na UFAM e me auxiliou da melhor forma possível durante toda a coleta de dados, providenciando todos os materiais e peixes e salas e equipamentos que eu poderia ter pedido, e mais um pouco.

Agradeço à minha querida amiga manauara Sheila Lima, minha companheira incansável de laboratório, que levava comigo uma jornada de quase 12 horas de laboratório sempre me

fazendo rir e esquecer que eu estava com fome, mas não podia parar. Obrigada, Sheilinha, por me apresentar o tacacá (sem goma!), o sorvete de cupuaçu, o MUSA embaixo de chuva e o açaí sem açúcar.

Agradeço aos amigos “estrangeiros” pela recepção maravilhosa que sempre me proporcionam no laboratório de Biologia Integrativa do Comportamento, em Portugal. Agradeço imensamente à Magda Teles, que me ajudou na burocracia infindável durante o transporte de cérebros Brasil/Portugal, me ensinou a técnica do fracionador isotrópico, e quebrou a cabeça comigo fazendo tudo funcionar da melhor forma possível. Agradeço ao meu companheiro de bancada, Miguel Correia, pelas conversas sobre política durante o almoço, e pelas conversas sobre comida durante o trabalho de bancada. E pela troca de lamúrias quando as coisas não saíam como o planejado. Agradeço à Rita Nunes por me apresentar pontos de Lisboa que eu não conhecia e que me fizeram amar ainda mais aquela cidade maravilhosa. Agradeço ao professor Kent Dunlap pela troca de experiências acadêmicas e pessoais, e pela convivência sempre tão alegre e entusiasmada que oferecia a todos em dias de trabalho cansativos. Agradeço, enfim, às todas as pessoas tão queridas que conheci no Instituto Gulbenkian de Ciência, e que me ajudaram a não me sentir tão estrangeira num país desconhecido: Ana Sofia, André Monteiro, Antonio Roleira, Claudia, Ibukun, João Solaris, Júlia, Kyriakos, Magda, Miguel, Rita, Sara, Susana. Obrigada por toda a ajuda com peixes, experimentos, construção de primers, análises de RNA, contagem de neurônios, discussões de projetos, etc. Muito obrigada pelo carinho de todos vocês, e pelos presentinhos de “farewell” mais lindos que já ganhei.

Agradeço aos “pescadores” professor Fernando Carvalho, ao seu Oscar Carvalho, ao seu Claudio e ao seu Mané Brandão (meu pai, de novo!) pela inestimável ajuda durante a coleta dos peixes utilizados nesse trabalho. Por terem parado suas próprias atividades e ajudado essa pescadora inexperiente a procurar cará, porquinho e papaterra pelos rios do estado de São Paulo. Sem vocês também não haveria esse trabalho. Obrigada!

Agradeço a todos os companheiros de laboratório que fizeram parte da minha jornada, desde os primórdios: Gauy, Dorigão, Ludmilla, Gisele, Marcela, Ana Paula, Ana Marina, André, Izabela, Kauã, Guilherme, Sara e Letícia, aos técnicos Carlos e Rose. Obrigada pela convivência no laboratório, nos congressos, nos *happy hours*, e obrigada pelas discussões durante nossas reuniões que tanto me ajudaram na construção dessa tese.

Agradeço à todos os funcionários da seção de Pós-Graduação do IBILCE / UNESP por terem sido sempre tão atenciosos e solícitos em solucionar todas as minhas dúvidas sobre todos os assuntos. Obrigada pelo bom humor e pela paciência infindáveis de vocês conosco, alunos. Obrigada por ajudarem a reduzir nossa ansiedade nesses momentos de “tensão”.

Agradeço ao Instituto de Biociências, Letras e Ciências Exatas, IBILCE / UNESP, por ter me dado a formação que tenho hoje, de forma gratuita e de excelente qualidade. Foram anos maravilhosos, nos quais aprendi a profissão que tanto amo. Agradeço aos anos que ali vivi, aos amigos que fiz, às oportunidades que tive, e aos excelentes professores que me ensinaram. Espero que um dia vivamos em um Brasil no qual seus cidadãos defendam, com garra, a Educação pública, gratuita e de qualidade, para todos os seus conterrâneos, enfrentando os governos que tanto almejam destruí-la.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001. Ademais, houve financiamento da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), relativo à categoria Projeto de Pesquisa Regular (processo nº 2016/26160-2) e à bolsa de treinamento técnico 3 conferida à aluna (processo nº 2019/10808-1).

“Dar as mãos simbolicamente. Penso muito nisso. Já se passaram tantos anos e ainda me imagino. Nós, juntos, diante da universidade. Ou aniquilavam todos, ou voltavam atrás. Permitimos. Não me conformo. Culpa que carrego. Ela me corrói. Nada pior que a memória do gesto não realizado.”

Ignácio de Loyola Brandão
(trecho da obra “Não Verás País Nenhum”, 1981, pp. 59*)*

“Fracassei em tudo o que tentei na vida.
Tentei alfabetizar as crianças brasileiras, não consegui.
Tentei salvar os índios, não consegui.
Tentei fazer uma universidade séria e fracassei.
Tentei fazer o Brasil desenvolver-se autonomamente e fracassei.
Mas os fracassos são minhas vitórias.
Eu detestaria estar no lugar de quem me venceu.”

Darcy Ribeiro
(Jornal “Estado de Minas”, 17 de fevereiro de 2007)

RESUMO

A organização cerebral em animais sociais pode ser explicada pelas relações filogenéticas – i.e., o cérebro evolui de acordo com uma linha de parentesco entre as espécies – e, também, pela seleção de estruturas direcionadas pela complexidade do ambiente social. O objetivo desta tese foi comparar a possível associação entre complexidade social, cerebral e cognitiva em espécies de peixes próximas filogeneticamente dentro da família Cichlidae que apresentam interações sociais consideradas mais complexas (cuidado biparental da prole em espécies monogâmicas) ou menos complexas (cuidado materno da prole em espécies poligínicas). As mesmas tiveram seus cérebros dissecados em macroáreas (telencéfalo, diencéfalo, teto óptico e cerebelo), posteriormente dissociadas através da técnica do fracionador isotrópico, permitindo a quantificação do número de neurônios e células não-neuronais que compõem essas macroáreas. Assim, em um primeiro estudo, comparamos os valores obtidos de neurônios e células não-neuronais com medidas cerebrais e morfométricas de cada espécie. Constatamos que, diferentemente do que é observado em estudos com mamíferos, espécies de peixes próximas filogeneticamente apresentam diferentes formas de organização cerebral, sugerindo que peixes são mais sujeitos às influências do ambiente comportamental. Após obtermos os dados sobre como os cérebros das espécies escolhidas são organizados, comparamos, em um segundo estudo, as espécies em seus tipos de cuidado parental e sistema de acasalamento. Peixes monogâmicos apresentaram mais neurônios apenas no teto óptico e cerebelo quando comparadas com espécies poligínicas. Indivíduos que cuidam da prole na boca apresentaram mais neurônios apenas no cerebelo. O ciclídeo anão, *A. agassizii*, foi a espécie que apresentou as maiores densidades de células cerebrais, destoando dos demais ciclídeos analisados, e revelando que as diferenças encontradas para esse peixe podem estar mais relacionadas à miniaturização sofrida por essa espécie do que a padrões sociais ligados à reprodução. Por fim, investigamos como o número de células cerebrais poderia estar associado à flexibilidade cognitiva e à socialidade apresentada pelos ciclídeos. Para isso, utilizamos o peixe monogâmico *G. brasiliensis,* espécie na qual ambos os pais cuidam da prole no substrato. Ao contrário do que esperávamos, o telencéfalo foi a única estrutura cerebral que não apresentou nenhuma associação entre os testes comportamentais aplicados e os valores de células cerebrais analisados. Já o diencéfalo, cerebelo e teto óptico mostraram correlações que parecem estar associadas a uma melhor avaliação do ambiente social por parte do animal. De forma geral, um maior número de células nessas regiões mostrou-se positivamente relacionado a indivíduos menos agressivos, que passam mais tempo próximos a coespecíficos e que são menos persistentes em tarefas irrelevantes, revelando animais mais cautelosos e avaliadores. Esse trabalho é inovador já que pouco se conhece sobre os mecanismos evolutivos e fisiológicos envolvidos com o controle do comportamento social em peixes neotropicais.

Palavras–chave: neurônios; células não-neuronais; *Geophagus brasiliensis*; *Geophagus sveni*; *Satanoperca pappaterra*; *Apistogramma agassizii*; fracionador isotrópico.

ABSTRACT

The cerebral organization in social animals can be explained by both, phylogenetic relationships (i.e., the brain evolves by a line of interrelationship between species) and by the selection of structures driven by the complexity of the social environment. The aim of this thesis was to compare the possible association between social behavior, brain complexity and cognitive ability in phylogenetically close fish species within the Cichlidae family, which present social interactions considered more complex (biparental brood care in monogamous species) or less complex (maternal brood care in polygynous species). Individuals had their brains dissected into macroareas (telencephalon, diencephalon, optic tectum and cerebellum), and subsequently dissociated using the isotropic fractionator technique, which allows the quantification of the number of neurons and nonneuronal cells that compose these macroareas. Therefore, in a first study, we compared the values obtained from neurons and nonneuronal cells with brain and morphometric measures of each species. We found that, differently from what is observed for mammals, phylogenetically close fish species have different brain organizations, suggesting that fish are more liable to the influences of the behavioral environment. After obtaining the data on how the brains of the chosen species are organized, in a second study we compared the species in their types of parental care and mating system. Monogamous fish presented more neurons only in the optic tectum and in the cerebellum when compared to polygynous species. Mouthbrooder individuals had more neurons in the cerebellum alone. The dwarf cichlid, *A. agassizii*, was the species that presented the highest brain cells densities, different from the other cichlids, revealing that the differences found for this fish may be more related to the miniaturization suffered by this species than to social patterns linked to reproduction. Finally, we investigated how the number of brain cells could be associated with cognitive flexibility and sociality presented by cichlid fish. For this, we used the monogamous fish *G. brasiliensis*, a species in which both parents take care of the brood in the substrate. Contrary to what we expected, the telencephalon was the only brain structure that showed no association between the applied behavioral tests and the brain cell values. The diencephalon, cerebellum and optic tectum showed correlations that seem to be associated with a better individual evaluation of the social environment. In general, a larger number of cells in these regions was positively related to individuals that were less aggressive, spent more time near conspecifics, and were less persistent in irrelevant tasks, revealing more cautious and evaluative animals. This work is innovative as little is known about the evolutionary and physiological mechanisms involved in social behavior control in Neotropical fish.

Keywords: neurons; nonneuronal cells; *Geophagus brasiliensis*; *Geophagus sveni*; *Satanoperca pappaterra*; *Apistogramma agassizii*; isotropic fractionator.

LIST OF FIGURES

LIST OF TABLES

Paper 3 – Neurons and nonneuronal cells are associate to sociality and cognition in fish

LIST OF ABBREVIATIONS AND ACRONYMS

AA	*Apistogramma agassizii*
SBH	Social Brain Hypothesis
cb	Cerebellum
di	Diencephalon
DAPI	4', 6-diamidino-2-phenylindole
GB	*Geophagus brasiliensis*
GS	*Geophagus sveni*
LM	Linear Models
LMM	Linear Mixed-Models
ot	Optic tectum
PBS	Phosphate-buffered saline
PCA	Principal Component analysis
SP	*Satanoperca pappaterra*
SL	Standard length (cm)
tl	Telencephalon

SUMMARY

GENERAL INTRODUCTION

The evolution of the animal behavior is associated to the evolution of the nervous system. Thus, it is logical to consider that animals with more complex brains will produce behaviors that are more complex. In this context, two models today try to explain the selection of anatomical variations in the brain that are related to the behavior. The mosaic evolution model proposes that the brain is composed by regions with distinct functions that control and select different behaviors (Barton and Harvey 2000; Gonzalez-Voyer et al. 2009) that will lead to a punctual selection of these particular brain areas (Gonzalez-Voyer et al. 2009; Gonzalez-Voyer and Kolm 2010). The concerted evolution model proposes that a single and simple structural change in the brain can modify the whole tissue as one single structure (Finlay and Darlington 1995). The both models are not excluding and use specific structural modifications of the brain to explain the evolutionary processes involved in the selection of individuals' brain and behavioral complexities (Herculano-Houzel et al. 2014).

The variation on brain complexity also depends of the environment in which the animal is found. Social animals, for example, present complex behaviors concerning conspecifics interactions that are frequently affected by the physical (Moberg et al. 2011; Salvanes et al. 2013) and social environment (Dunbar and Shultz 2007; Taborsky and Oliveira 2012). Nevertheless, abiotic alterations, such as seasonal changes and others climatic perturbations, are more predictable, enabling fast and standardized adjustments from an individual to respond properly to such changes (see Wingfield, 2013). On the other hand, an animal hardly will present standardized responses to deal with different individuals in a group, as such interactions are way to more unpredictable (Dunbar 1998; Dunbar and Shultz 2007; Bshary 2011; Ashton et al. 2018). This unpredictability requires a more complex appraisal and decision-making from an individual; therefore, it requires a more complex brain. Thus, the social stimuli are considered richer in information and will demand from an individual different behavioral responses, requiring a more complex neural framework (Dunbar and Shultz 2007).

The structural organization of some brain areas can be explained by phylogenetic relationships (i.e., the brain evolves accordingly to a kinship line between species), and by selected structures provided by the complexity of the social environment. The Social Intelligence Hypothesis, for example, postulates that the brain complexity observed in several groups of social animals is a product of the complex social interactions selected for these individuals [see for fish: (Bshary 2011); primates: (Dunbar 1998); social insects: (Lihoreau et al. 2012); rodents: (Kverková et al. 2018). This hypothesis considers that the social complexity is associated to the nervous system complexity, and not just to the brain size, as individuals with smaller brains may yet present several social behaviors equally complexes [e.g., social insects (Lihoreau et al. 2012)]. Nevertheless, it is not easy to quantify brain complexity. The brain size and its volume are not

always associated to the neurons number present in such structures (Herculano-Houzel and Lent 2005; Herculano-Houzel et al. 2015). The number of neurons, in this case, proposes a higher probability of synaptic circuits. Thus, the association of the evolutionary mechanisms that modulate the social brain can be better understood if we look for neuron numbers in specific brain regions associated directly with social behavior [see the Social Decision-Making Network, (O'Connell and Hofmann 2012)].

Besides the anatomy of the brain, other parameter to be associated with brain complexity is the cognitive ability of the individuals. Cognition, in this case, can be considered as the "process in which an animal internalizes environmental information by perceiving this information in the nervous system, which will generates a behavioral change by learning and memory processes" (Braithwaite 2006). Environments that are rich in information may stimulate the formation of new neuronal connections linked to new memories and learning (Kandel 2001), making possible to an animal to promptly respond to different demands of the social environment. Therefore, when the animals conciliate a complex brain with cognitive systems, they can rapidly interpret the social environment in which they are inserted, learning to deal with different individuals of the group in a precisely form (Taborsky and Oliveira 2012). Thus, the cognitive ability may be considered as an expression of the brain complexity.

The social complexity can be evaluated by the social competence, which is the individual capacity to optimize its behavior in front of social information presented in the environment (Oliveira 2009; Taborsky and Oliveira 2012). Therefore, animals that live in groups and are more socially competent possibly will present a better individual fitness and, consequently, a higher rate of survivor in the nature. In this manner, the social competence is related to a reproductive efficiency of a species (Taborsky and Oliveira 2012), requiring different responses from the animals in the way they organize each other for mating (monogamy *vs.* polygyny) and for take care of their brood (maternal, paternal or biparental care). Thus, we can assume that mating systems and types of parental care that demand more interactions and conciliation between the parents (i.e., monogamous pairs that take care together of the offspring) will also demand a higher social competence from the individuals.

According to this rationale, the aim of this study was to test whether the social complexity is associated to brain complexity – here, measured by brain cells composition, brain volume and cognitive and social tests. Our study investigated fishes from the Cichlidae family, as the fish brain structure and physiology are well conserved along vertebrates, and they also present complexes social behaviors (Bshary et al. 2002). According to O'Connell and Hofmann (2012), there is a social decision-making network represented in the telencephalon and mesencephalon of birds and teleost fish that are homologous to mammals, which makes teleost fish excellent models in studies with neural structures and cognition. Indeed, Pollen et al. (2007) found association between telencephalon and hypothalamus size and social behavior in African cichlids.

Monogamous species present an enlarged telencephalon and a reduced hypothalamus when compared to polygynous cichlids. In Brazil, we have a huge diversity of fish that presents different habitats, feeding habits, mating systems, parental care types and, consequently, can present different strategies related to social competence. The Neotropical cichlids, like the African ones, are formed by species with a rich behavioral and ecological repertoire, although the evolutionary forces driving behavioral changes might be different among them (Barlow 2002).

In this study we compared Neotropical close related species, which present social interactions considered more or less complex – e.g., biparental brood care in monogamous species *vs.* maternal brood care in polygamous species, respectively – from a mating system and parental care point of view. Moreover, we compared the size of specific regions of the brain, and the brain cells composition between the selected species, to test whether there are associations between the anatomical factors and the ecological and social habits exhibited by these species, as preconized by the Social Intelligence Hypothesis: (Dunbar 1998). Our hypothesis was that species with similar social complexity would exhibit similar levels of brain and cognitive complexity, in spite of the phylogenetical proximity between them.

Predictions and innovation:

a. Species considered more socially complexes (i.e., monogamous individuals with biparental brood care) would present bigger brain regions related to social behaviors and with a bigger number of brain cells, specially neurons.
b. Species considered more socially complexes would present better cognitive and social abilities.

This was an innovative study since little is known about the evolutionary and physiological mechanisms involved in the social behavior of Neotropical fishes. Moreover, the knowledge about social behavior in Neotropical cichlids is very small, if we consider the number of fish species present in the Brazilian fauna and the number of papers published about this subject. The Amazonian cichlids, for example, encompass a huge number of species, but the number of specialists in animal behavior studying this area is reduced. Then, in this thesis we used the species *Geophagus brasiliensis, Geophagus sveni, Satanoperca pappaterra* and *Apistogramma agassizii,* and show 3 chapters (in paper formats) to test our hypothesis:

1. “Brain cells composition in Neotropical cichlid fishes” – the goal of this first chapter was to describe the brain composition of the above-mentioned Neotropical cichlid species, by using the isotropic fractionator technique. With this, we could inferred the number of brain’s neuronal and nonneuronal cells, comparing these numbers with individuals’ morphometric and brain measures.
2. “Neurons and glial cells are associated with parental care and mating systems in Neotropical cichlids” – in this chapter, our goal was to compare the four Neotropical

cichlids in their mating system and types of parental care, hypothesizing that more complex social species – i.e., monogamous substrate brooders – would present more neurons and nonneuronal cells in specific brain areas, mainly in those related to social behaviors.

3. “Neurons and nonneuronal cells are associate to sociality and cognition in fish” – in this last chapter, we aimed to observe whether brain complexity – inferred by number and density of neurons and nonneuronal cells – would be associated to cognitive flexibility and social behavior, measured by behavioral tests. These correlations were realized with the monogamous substrate brooder *Geophagus brasiliensis.*

This thesis was written in the form of three scientific articles, which will be submitted to international journals in line with their themes.

Paper 1. Brain cells composition in Neotropical cichlid fishes.

Paper 2. Neurons and glial cells are associated with parental care and mating systems in Neotropical cichlids

Paper 3. Neurons and nonneuronal cells are associate to sociality and cognition in fish

2 PAPER 1. Brain cells composition in Neotropical cichlid fishes

Manuela Lombardi Brandão[1], Eliane Gonçalves-de-Freitas[1,2] and Rui F. Oliveira[3,4,5].

[1]Departamento de Zoologia e Botânica, Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista (UNESP), Cristóvão Colombo, 2265, 15054-000, São José do Rio Preto, SP, Brazil.
[2]Centro de Aquicultura da UNESP, SP, Brazil.
[3]Instituto Gulbenkian de Ciência, Oeiras, Portugal.
[4]ISPA - Instituto Universitário, Lisbon, Portugal.
[3]Champalimaud Neuroscience Programme, Champalimaud Centre for the Unknown, Lisbon, Portugal.

ABSTRACT

The brain across the vertebrate clade presents several homologies that have been maintained during the evolution. Regardless that, the vertebrate brains show several differences in shape, structural organization and size, due to the different selective forces, such as social and reproductive repertoire, acting on them. In this context, it is expected that different numbers of cells, such as neurons and glial cells, are also variable in these different brains. However, the basic information about brain composition is necessary before test for hypothesis regarding ecological factor acting on brain shape. In this work, we describe for the first time, the brain composition of four Neotropical cichlid species, a neglect group regarding brain structure and behavior. We used four phylogenetic close species of southeast Brazilian rivers and one Amazonian miniaturized species. By using the isotropic fractionator technique, we inferred the number of brain's neuronal and nonneuronal cells, comparing these numbers with individuals' morphometric and brain measures. We observed that, different from mammals, closely related fish species may present very different forms of brain organization, which suggests that fishes' brain are more liable to the influences of the behavioral environment. Therefore, these species can be used as a model for testing theoretical framework regarding evolution of fish brain and behavior.

Key words: neurons, nonneuronal cells, *Geophagus brasiliensis*, *Geophagus sveni*, *Satanoperca pappaterra*, *Apistogramma agassizii*, dwarf cichlid, isotropic fractionator.

Introduction

Derived from a common ancestor, vertebrate brains have a continuity provided by the evolutionary process that allows them to present several homologies. Nevertheless, these

brains are also very different in their shapes, structure and sizes, as they are a result of distinct selective forces acting on particular regions and even in the all brain as a whole. Two different theories try to provide explanations on how brains changed along evolution. The concerted evolution hypothesis says that the brain evolves as a whole, no mattering the importance and specialization of a particular brain region in the survival of a giving individual (Finlay and Darlington 1995). On the other hand, the mosaic evolution hypothesis postulates that the brain is a tissue with a huge energy cost for individuals (Barton and Harvey 2000) and, therefore, selective forces target and favor the growth of specific regions of the brain instead of others. Nevertheless, these two hypothesis are not excluding, and have already been observed in fishes (see Gonzalez-Voyer et al. 2009). Along the years, the focus of these investigations have been the vertebrates with "more complex" nervous system and behaviors, such as mammals and birds (Gonzalez-Voyer et al. 2009). However, with the growing knowledge that fish group is represented by individuals with highly complex behavior and cognitive processes (Bshary et al. 2002; Brown and Laland 2003; Braithwaite 2006; Vila Pouca and Brown 2017; Maruska and Fernald 2018), it is necessary to look deeper into their "less complex" brain to better understand the behavioral forces driving vertebrate brain evolution.

Fish are the group with the largest radiation into the vertebrate clade, occupying virtually all aquatic habitats (Kotrschal et al. 1998) and exhibiting brains with several subdivisions typical of most vertebrates (Kotrschal et al. 1998; Shumway 2010). Nevertheless, different from other vertebrates, fish do not present a limited growth, neither in their body and, consequently, nor in the brain (Shumway 2010). Therefore, the allometric relationship between brain and body that applies to vertebrates in general – i.e., individual variation across a mean – is not applied to fishes – i.e., individual variation distributed along a continuous. For example, in most fish species, the brain is much smaller than the skull (Shumway 2010), revealing a distinct way of shaping across individual's life. Moreover, teleost fish exhibit the greatest neurogenesis observed in any adult vertebrate (Zupanc 2008), and therefore these allometries may account for changes in brain morphology along all individual's life (Brandstätter and Kotrschal 1989; Brandstätter and Kotrschal 1990). Additionally, processes such as body miniaturization is also a factor shaping fish brain morphology, with small individuals showing brains relatively more dense in number of cells (Marhounová et al. 2019). For last, the tremendous diversity of fish habitats leading to differential selective pressures may explain the extraordinary diversity of fish brains and behaviors that is known today (Shumway 2010). Nevertheless, the size and shape of the brain, as well as its structure composition, are not only shaped by the evolutionary history, but are also a result of the interrelationship between ecological and behavioral features. Habitat complexity and social interactions, for instance, are powerful factors that can affect brain's composition and influence its complexity (see Dunbar 1998; Dunbar and Shultz 2007; Bshary et al. 2014). In this sense, the cichlids, a family of teleost fishes, are an example of a group with

species showing several types of parental care, mating systems and a huge social complexity that probably selected most complex brains among fishes (Kotrschal et al. 1998).

Thus, cichlid fishes, particularly African ones, have been studied as good model for understanding fish brain and behavior evolution (Pollen et al. 2007; Gonzalez-Voyer and Kolm 2010; Gierszewski et al. 2013; Tsuboi et al. 2015; Reddon et al. 2016; Maruska and Fernald 2018). In this scenario, however, the Neotropical cichlids are a neglected group that can bring novelties to the comprehension of vertebrate brain evolution. Therefore, for testing any hypothesis about fish brain, we need firstly to know the basic architecture of these "neglected" species. Here we described the brain structure of four close related Neotropical cichlid species (Fig. 1) with different types of parental care and mating systems, including a miniaturized Amazonian species. We described, for the first time, the association between fish body mass and length with the brain mass, number of neurons and nonneuronal cells in the telencephalon, diencephalon, optic tectum, and cerebellum of these Neotropical cichlids. Afterwards, we checked if their general structure can be explained by concerted (Finlay and Darlington 1995) or mosaic evolution (Barton and Harvey 2000) hypothesis .

Methods

Fish housing

We used 6 male and 6 female individuals of each species, except in *S. pappaterra*, where we analyzed the brain of only 2 females. The species used in here were collected in natural water bodies in Brazil: *G. brasiliensis* and *S. pappaterra* were collected in the Rio Grande river; *G. sveni* were collected in in the Tietê river; and *A. agassizii* were collected in the Amazon-Solimões river. Rio Grande e Tietê are rivers from the southeast Brazil included in the Paraná River basin, while Amazon-Solimões river is located in the northwest of Brazil, and it is part of the Amazon River basin. Fish were transported and kept in the laboratory in polyethylene water tanks (ca. 500 L, 1 fish/10 L) in a regime of 27º C and light from 7AM to 7PM. They were fed once a day to apparent satiation with commercial food for tropical fish (28% protein). Water quality was maintained by using biological filters (400 L/h) and constant aeration until sacrifice for brain collection.

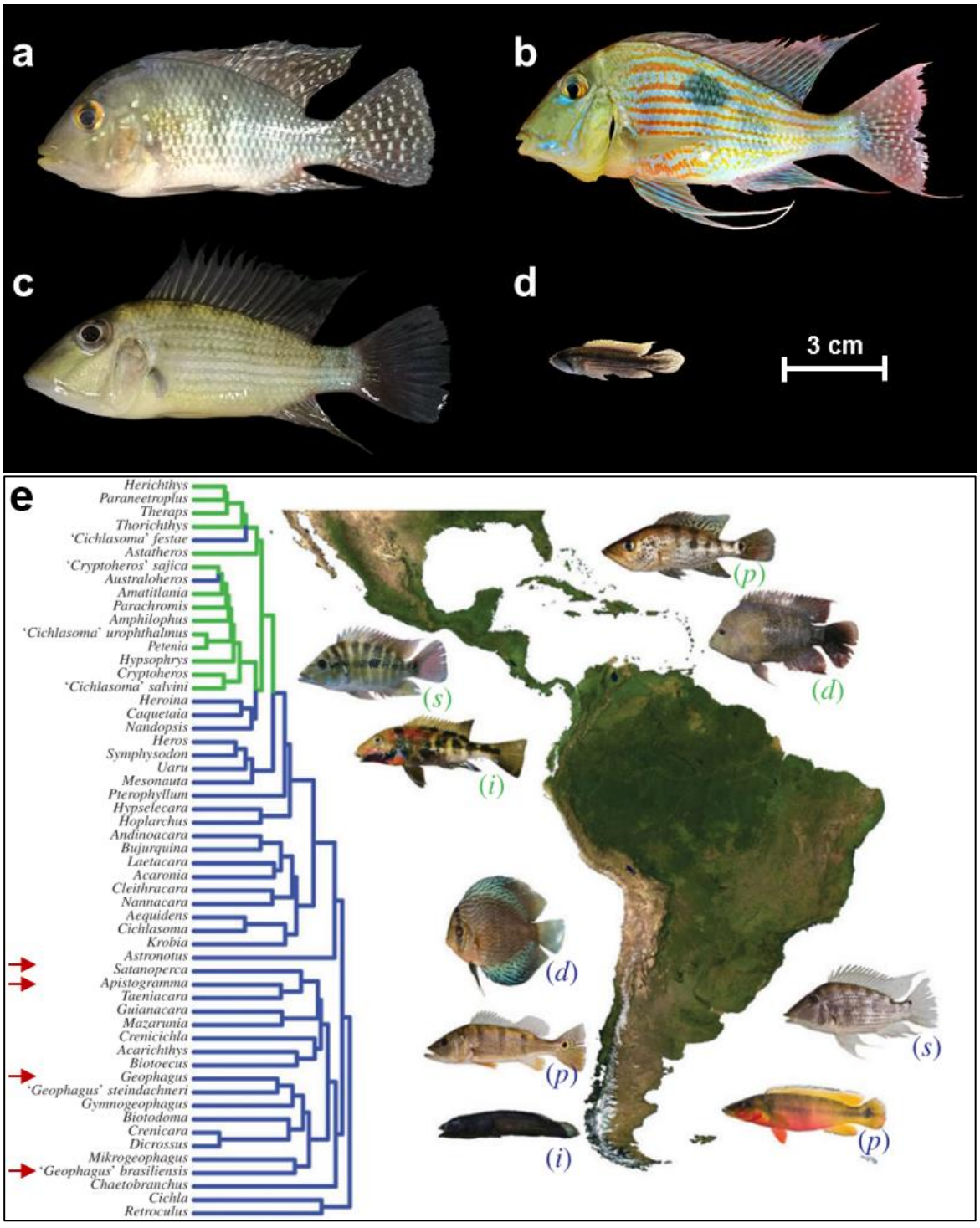

Figure 1. The four Neotropical cichlids used in this study, **a.** *Geophagus brasiliensis,* a monogamous species with biparental brood care in the substrate; **b.** *Geophagus sveni,* a monogamous species with biparental brood care and facultative mouthbrooding; **c.** *Satanoperca pappaterra,* a polygynous species with female mouthbrooding; **d.** the dwarf cichlid *Apistogramma agassizii*, a polygynous species with female brood care in the substrate. Photos from the author M.L. Brandão. **e.** Phylogeny of Neotropical cichlids, based on ecomorphological characters; from the work of Arbour and López-Fernández (2016).

Brain collection

Fish were euthanized with benzocaine (180 mg.L^{-1}) fallowed by skull removal and brain collection. The brain was immediately dissected into four structures of interest – telencephalon (tl), diencephalon (di), optic tectum (ot) and cerebellum (cb) – that were finally weighted and fixed individually in 4% paraformaldehyde for 24 hours. After that, each structure was transferred to a sucrose solution during overnight (4 °C) and stored in an antifreeze solution (-20 °C) until processing. Olfactory bulb and brainstem were also collected, but not analyzed in this work.

Isotropic fractionator

This step followed the protocol already used for several vertebrates, which consist in dissociate the brain structure in free nuclei of cells (Herculano-Houzel 2011). The structures were removed from the antifreeze solution and placed in a glass homogenizer containing 1 ml of buffer-detergent solution (1% Triton™ X-100 and 40 mM sodium citrate). After that, the region of interest is gently smashed with a pestle, using rotatory and up and down movements until no more cell' clustering is observed in the glass homogenizer with the full pestle inserted. This procedure enables highly anisotropic tissue to be transformed in a nuclei solution (Valério-Gomes et al. 2018). In the cichlids used here, the dissociation took about 15 minutes in the softer structures – i.e., telencephalon, diencephalon and cerebellum – and up to 1 hour in the optic tectum.

After tissue dissociation, we transferred the 1ml solution of free nuclei to 1.5 ml Eppendorf and added 1mg/ml fluorescent nuclear stain DAPI (4', 6-diamidino- 2-phenylindole, a dye with high affiliation to DNA) to afford us to count for the total free nuclei in the sample. After DAPI adding, the Eppendorf tube was inverted a few times to homogenized the sample. Then, four aliquots of 10 µl of the same sample were collected and placed in Neubauer chambers for counting DAPI stained nuclei. We counted 10 central squares of the Neubauer chamber in this study, for all the samples. The formula used to achieve the total number of free nuclei was the mean number of nuclei counted in the four aliquots multiplied by the volume expected in the 10 central squares in the Neubauer chamber multiplied, one more time, by the volume in ml of the initial sample (1 ml of solution with free nuclei).

For neurons and nonneuronal cells quantification, we collected a new aliquot of 500 µl from the original sample to realize an immunocytochemical protocol. In this phase, the original protocol suffered a few adjustments. The spare volume was stored in 4 °C for future use, when necessary. The aliquots were centrifuged, the supernatants were discarded and the pellets were washed twice in 0.1 M PBS (8000 rpm at 4 °C during 5 minutes, each wash). After that, the aliquots were incubated at 70 °C during one hour in 0.2 M boric acid, being washed again twice with 0.1 M PBS. After that, we added a blocking step (100 µl of 1% Albumin Bovine

Fraction V – BSA for each sample in a covered plate under agitation for 30 minutes) in the protocol to reduce fluorescence due to nonspecific antibody binding, what was observed in previous experiments. In the end of the 30 minutes, the samples were centrifuged, the supernatants were discarded and the samples were incubated overnight (350 rpm in a covered plate at 4 °C) with 100 µl of the anti-NeuN primary antibody diluted in 1% BSA at a 1:100 concentration.

Next day, the samples were centrifuged, the supernatants were discarded and the pellets were washed twice in 0.1 M PBS (8000 rpm at 4 °C during 5 minutes, each wash). The samples were suspended in final volumes of 50-400 µl depending on the pellet size, as there was a substantial loss of free nuclei during the immunocytochemical step. After that, we placed a new aliquot of this sample in the Neubauer chamber and counted the percentage of DAPI-labeled nuclei that was also labeled with the antibody (red colored, Fig. 2), and count a minimum of 500 DAPI-labeled cells to achieve statistical power. The formula to determine the total number of neurons (the NeuN-positive nuclei) is the percentage of NeuN-positive nuclei multiplied the total number of nuclei, with the result divided by 100. The nonneuronal cells number is the total number of cells minus the total number of neurons.

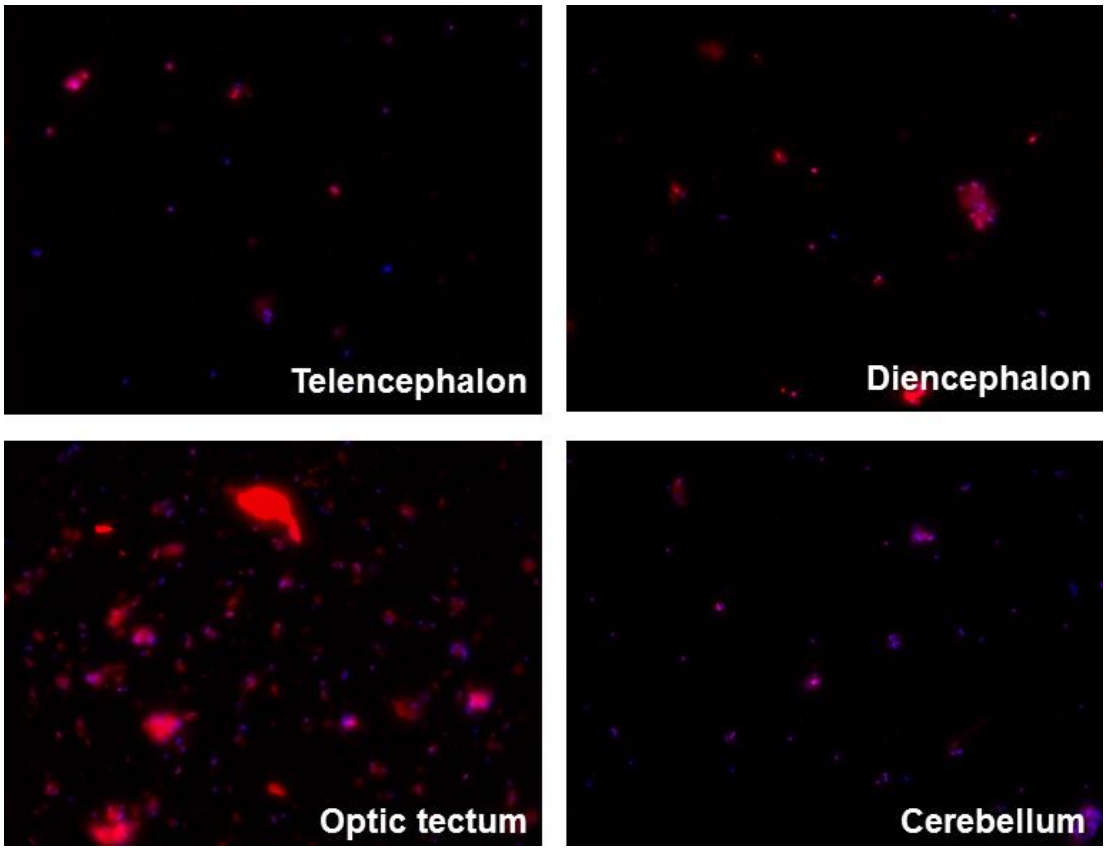

Figure 2. Immunocytochemical process for counting brain cells, with the neuronal cells appearing in red and blue colors overlapped and nonneuronal cells appearing only in blue color. The brain cells used as reference are from *G. brasiliensis*. Photos from the author M.L. Brandão.

Statistical analysis

Data analysis was done by using the free software R, version 3.5.1 (http://www.r-project.org). Normality and homoscedasticity were tested using Kolmogorov-Smirnov and Fmax test (Lehner 1998), respectively. Nevertheless, we used the nonparametric test of Spearman to check for correlation without miss nonlinear data. We checked for correlations between brain measures – i.e., density of cells (total number of cells divided by structure mass in milligrams) and total number of cells – and body measures – i.e., fish body mass and standard length – by using Spearman's correlation test, corrected with Bonferroni's ["psych" package (Revelle 2018)]. A principal component analysis (PCA) checked for the main variations and principal loading factors in our data in which we compared brain structure mass, number and density of brain cells (neurons and nonneuronal cells), total brain cells and ratio between nonneuronal cells and neurons. For last, we used Linear models completed with Tukey HSD post-hoc test ["glht" function of the "multcomp" package (Hothorn et al. 2008)] for comparing the ferets diameters between the four cichlid species. Statistical significance was set at $p \leq 0.05$. However, we considered a marginal trend towards significance when $p < 0.10$, based on Pollen et al. (2007).

Ethical statement

We conducted this study according to the ethical principles on animal experimentation adopted by the National Council for the Control of Animal Experimentation (CONCEA – Brazil). It was approved by the Committee on Ethics in Animal Use, UNESP, São José do Rio Preto, permit number 156/2016. This project was also registered in the Authorization and Information System in Biodiversity (SISBIO-ICMBio, Brazil, permit number 54287-1), which allowed the collection of biological material in natural environments for research purposes.

Results

We analyzed brain of males and females individuals in this study. Nevertheless, there was no correlation between sex and the other variables we measured (Appendix table 1 and 2). Therefore, we analyzed both male and female data together. As all the individuals we used in this study was caught in natural environments, the subjects' size were not always similar within species. Thus, for each species we checked for the variation in brain and body measures (Appendix Fig. 1-8). In *G. brasiliensis* individuals varied by 2.14-fold in body mass, and 1.21-fold in standard length (table 1). In the other monogamous species, *G. sveni,* the body mass varied 3.81-fold, and the standard length, 1.56-fold. The polygynous species, *S. pappaterra* and *A. agassizii* had a body mass variation and standard length variation of 8.24-fold and 2-fold, and 3.28-fold and 1.47-fold, respectively (table 1). In relation to brain structures mass, the biggest variations were at the cerebellum in all the four cichlids (except for the diencephalon in *A.*

agassizii, that varied 5.6-fold), ranging from 3.46-fold in *S. pappaterra* to 5.5-fold in *A. agassizii.* On the other hand, the smallest variations occurred in the optic tectum (from 1.39-fold in *S. pappaterra,* to 3.53-fold in *A. agassizii*), except in *G. sveni,* to whom it occurred in the telencephalon.

Principal component analysis shows that structure mass is not a limit for brain cells quantity

We run a principal component analysis (PCA) to compare the variation in structure mass, number and density of brain cells (neurons and nonneuronal cells), total brain cells and ratio between nonneuronal cells and neurons across all the four species' brain structures grouped together. This analysis revealed two main components that accounted for 75% of data variation. The first component was composed by total number of brain cells (loading factor of 0.519), followed by total number of neurons and nonneuronal cells (loading factors of 0.468, for both variables) and accounted for 49% of the variation found in our data (eigenvalue 3.455). The second component corresponded to structure mass (-0.673), and density of neurons and nonneuronal cells (0.503 and 0.439, respectively); and also accounts cumulatively for 75.4% of the variation (eigenvalue 1.825).

We also analyzed each species separately. The PCA reveals two main components that account for 81% of the variation in the monogamous *G. brasiliensis*. The first component corresponded to total number of cells (loading factor, 0.493), number of nonneuronal cells (0.468), followed by number of neurons (0.454), neurons density (0.425), and accounts for 52.9% of variation (eigenvalue, 3.706). The second component was composed by structure mass (-0.687), and nonneuronal cells density (0.507), and accounts cumulatively for 80.6% of the variation (eigenvalue, 1.939). For *G. sveni,* the other monogamous species of this study, the two components accounted for 76% of the variation. The PC1 corresponded to the number of total brain cells (loading factor of 0.478), total number of nonneuronal cells (0.453), density of neurons (0.437) and nonneuronal cells (0.456), accounting for 54% of the variation in this first component (eigenvalue 3.767). The PC2 was loaded by brain structure mass (0.728) and total of neurons (0.488), accounting for cumulative variation of 76.6% (eigenvalue 1.59).

For the polygynous mouthbrooder *S. pappaterra*, the two main component of the PCA accounted for 80% of the variation. The PC1 was loaded by the number of neurons (-0.491), total brain cells (-0.467), density of neurons (-0.469) and number nonneuronal cells (-0.449), with 45% of analysis variation (eigenvalue 3.182). PC2 was loaded by structure mass (0.595) and number of nonneuronal cells (0.514), which accounted for a cumulative variation of 79.6% (eigenvalue 2.393). The dwarf cichlid, the polygynous substrate brooder *A. agassizii*, showed a PCA that accounted for 78% of the observed variation (eigenvalue 3.655). The first component corresponded to total brain cells (-0.515), number of neurons (-0.502) and nonneuronal cells (-

0.466), accounting for 52% of the variation in this first component. The second component was composed by density of neurons (-0.608) and nonneuronal cells (-0.645), as well as by structure mass (0.453), which reflects in a cumulative variation of 78.1% (eigenvalue 1.811).

In all the four species, as well as their data are analyzed together, brain structure mass only presented significant influence in the second component of the PCA. As already pointed for mice (see Herculano-Houzel et al. 2015) this result suggests that the weight of brain structures is a consequence of the variation across individuals, and not a determinant of these variations, as it seems to be the case of the total number of brain cells, for example.

Table 1: Average body mass, standard length and brain structures mass in the four cichlid species.

G. brasiliensis	**Body measures**		**Brain structure**			
	Body mass (g)	**Standard length (cm)**	**tl**	**di**	**ot**	**cb**
Average	23.74 ± 1.68	8.87 ± 0.19	15.69 ± 0.83	8.97 ± 0.81	25.69 ± 0.88	5.84 ± 0.69
Minimum measured	15.96	8.0	10.3	4.9	20.1	2.3
Maximum measured	34.19	9.7	20.7	12.8	29.5	10.1
Variation	2.14×	1.21×	2.01×	2.61×	1.47×	4.39×
G. sveni	**Body measures**		**Brain structure**			
	Body mass (g)	**Standard length (cm)**	**tl**	**di**	**ot**	**cb**
Average	46.35 ± 4.99	11.32 ± 0.41	17.86 ± 1.04	15.54 ± 0.99	33.96 ± 2.32	7.54 ± 0.81
Minimum measured	20.27	8.6	12.9	10.0	21.3	3.1
Maximum measured	77.16	13.4	23.7	19.6	44.7	11.3
Variation	3.81×	1.56×	1.84×	1.96×	2.1×	3.65×
S. pappaterra	**Body measures**		**Brain structure**			
	Body mass (g)	**Standard length (cm)**	**tl**	**di**	**ot**	**cb**
Average	48.62 ± 11.29	10.74 ± 0.99	18.56 ± 2.31	15.07 ± 2.08	35.11 ± 3.76	5.51 ± 0.82
Minimum measured	11.43	7.5	12.1	10.2	20.4	2.6
Maximum measured	94.17	15.0	29.9	26.9	28.5	9.0
Variation	8.24×	2×	2.47×	2.64×	1.39×	3.46×
A. agassizii	**Body measures**		**Brain structure**			
	Body mass (g)	**Standard length (cm)**	**tl**	**di**	**ot**	**cb**
Average	0.37 ± 0.04	2.73 ± 0.09	1.19 ± 0.14	1.5 ± 0.24	3.08 ± 0.37	0.55 ± 0.11
Minimum measured	0.21	2.31	0.4	0.5	1.5	0.2
Maximum measured	0.69	3.40	1.8	2.8	5.3	1.1
Variation	3.28×	1.47×	4.5×	5.6×	3.53×	5.5×

Represented values are the means ± SD. Variation is the ratio between minimum and maximum values for each parameter. tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

Concerning total brain cells, the smallest variations were saw in *G. brasiliensis* (table 2), when compared to the other three cichlids. This probably occurred because it was the species with the smallest variation in body mass and standard length, as mentioned above. *A. agassizii* was the species that showed the greatest variation in the diencephalon between subjects, of 13.1-fold. This can be related to the importance of the diencephalic regions in reproduction responses that are differently modulated in males and females in this polygynous and highly dichromatic species.

Table 2: Average number (in millions) of neurons and nonneuronal cells in each brain structure of the four cichlid species.

G. brasiliensis	**Neurons**				**Nonneuronal cells**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Average	1.498 ± 0.192	0.769 ± 0.149	9.082 ± 0.395	7.132 ± 0.746	2.035 ± 0.187	1.769 ± 0.169	5.338 ± 0.687	6.115 ± 0.355
Minimum	0.294	0.328	7.588	3.186	1.170	0.962	2.818	4.531
Maximum	2.443	1.686	1.229	10.516	2.900	2.686	9.275	7.933
Variation	8.31×	5.14×	0.16×	3.3×	2.47×	2.79×	3.29×	1.75×
G. sveni	**Neurons**				**Nonneuronal cells**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Average	1.695 ± 0.262	1.829 ± 0.339	10.779 ± 0.457	9.623 ± 0.845	2.311 ± 0.159	2.134 ± 0.253	5.036 ± 0.520	11.057 ± 1.873
Minimum	0.505	0.841	8.245	4.867	1.519	1.065	2.061	4.563
Maximum	2.958	4.187	13.855	12.779	3.325	3.305	7.646	26.168
Variation	5.86×	4.98×	1.68×	2.62×	2.18×	3.10×	3.71×	5.73×
S. pappaterra	**Neurons**				**Nonneuronal cells**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Average	2.675 ± 0.479	2.358 ± 0.728	7.208 ± 1.264	9.614 ± 1.240	2.927 ± 0.445	2.135 ± 0.124	10.401 ± 1.216	6.945 ± 0.612
Minimum	0.897	0.801	4.593	4.073	1.379	1.606	4.211	4.36
Maximum	5.005	6.45	11.306	14.99	5.084	2.783	13.818	10.18
Variation	5.57×	8.05×	2.46×	3.68×	3.68×	1.73×	3.28×	2.33×
A. agassizii	**Neurons**				**Nonneuronal cells**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Average	0.257 ± 0.048	0.648 ± 0.144	1.634 ± 0.209	0.595 ± 0.117	0.218 ± 0.042	0.412 ± 0.066	0.656 ± 0.113	0.345 ± 0.069
Minimum	0.102	0.095	1.018	0.302	0.05	0.131	0.287	0.124
Maximum	0.542	1.245	2.828	1.018	0.403	0.901	1.212	0.614
Variation	5.31×	13.1×	2.77×	3.37×	8.06×	6.87×	4.22×	4.95×

Represented values are the means ± SE. Variation is the ratio between minimum and maximum values for each parameter. tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

For neurons densities, *A. agassizii* presented the higher variations in the cerebellum and in the diencephalon, as observed for total number of neurons in this brain structure (table 3). *G. brasiliensis*, although with the lowest variation in size, showed the highest variation in the density of cells in the telencephalon, evidencing a non-allometric relationship between number of cells in the telencephalon and its size. For nonneuronal cells, the variation was again higher for *A. agassizii* in the diencephalon and cerebellum (table 3).

Table 3. Average density (in millions) of neuronal and nonneuronal cells in the four cichlid species.

G. brasiliensis	**Neurons**				**Nonneuronal cells**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Average	0.094 ± 0.011	0.084 ± 0.014	0.354 ± 0.016	1.162 ± 0.111	0.130 ± 0.10	0.205 ± 0.018	0.206 ± 0.024	1.282 ± 0.196
Minimum	0.022	0.025	0.294	0.609	0.070	0.121	0.110	0.671
Maximum	0.153	0.173	0.444	1.923	0.200	0.275	0.359	2.836
Variation	6.95×	6.92×	1.51×	3.16×	2.86×	2.27×	3.26×	4.23×
G. sveni	**Neurons, density**				**Nonneuronal cells, density**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Average	0.082 ± 0.012	0.117 ± 0.018	0.341 ± 0.025	1.250 ± 0.136	0.142 ± 0.012	0.134 ± 0.010	0.154 ± 0.012	1.464 ± 0.138
Minimum	0.039	0.063	0.206	0.552	0.08	0.095	0.135	0.812
Maximum	0.138	0.226	0.478	1.887	0.242	0.173	0.205	2.516
Variation	3.54×	3.59×	2.32×	3.42×	3.03×	1.82×	1.52×	3.10×
S. pappaterra	**Neurons, density**				**Nonneuronal cells, density**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Average	0.147 ± 0.018	0.149 ± 0.308	0.229 ± 0.045	1.834 ± 0.184	0.169 ± 0.026	0.185 ± 0.027	0.314 ± 0.025	1.376 ± 0.134
Minimum	0.039	0.075	0.098	0.993	0.056	0.075	0.206	0.896
Maximum	0.19	0.289	0.419	2.736	0.269	0.299	0.442	1.844
Variation	4.87×	3.85×	4.28×	2.76×	4.80×	3.99×	2.15×	2.06×
A. agassizii	**Neurons, density**				**Nonneuronal cells, density**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Average	0.226 ± 0.038	0.337 ± 0.081	0.595 ± 0.058	1.058 ± 0.219	0.180 ± 0.025	0.303 ± 0.059	0.227 ± 0.029	0.630 ± 0.128
Minimum	0.116	0.082	0.343	0.343	0.072	0.109	0.13	0.162
Maximum	0.417	0.889	0.883	1.881	0.28	0.644	0.378	1.248
Variation	3.59×	10.84×	2.57×	5.48×	3.89×	5.91×	2.91×	7.70×

Represented values are the means ± SE. Variation is the ratio between minimum and maximum values for each parameter. tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

Correlations with body mass and standard length

Fish body mass and structure mass were positively and strongly correlated with each other across individuals within all brain structures (table 4). These correlations were also positive when compared fish standard length (SL) and structures mass. Thus, larger animals indeed seem to have heavier brains. Concerning neurons, fish body mass was negatively correlated with densities in the diencephalon and optic tectum, but positively correlated with number of neurons in the telencephalon, optic tectum and cerebellum (table 4). The SL followed the same results that body mass, except in density of neurons in the diencephalon, which did not show correlation. The nonneuronal cells presented positive correlations with body mass and SL in all brain structures, but just when total number of cells were compared (table 4). Nonneuronal cells densities only showed negative trends between telencephalon and body mass, and between diencephalon and both, body mass and SL. In general, the total number of cells in almost all brain structures followed a positive correlation between fish body size (table 4), as well as structure mass, this last one revealing an apparent concerted evolution of brain structures in these cichlids, at least when all species are analyzed together.

Table 4: Brain cells densities and total numbers correlated with body mass and standard length in the four cichlid species.

Four cichlids	Neurons, density				Neurons, total			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Body mass (g)	r = -0.20	**r = -0.36**	**r = -0.67**	r = 0.001	**r = 0.59**	r = 0.22	**r = 0.48**	**r = 0.84**
	p = 0.28	**p = 0.05**	**p < 0.0001**	p = 0.99	**p < 0.001**	p = 0.24	**p = 0.007**	**p < 0.0001**
SL (cm)	r = -0.16	r = -0.29	**r = -0.66**	r = 0.04	**r = 0.62**	r = 0.30	**r = 0.48**	**r = 0.85**
	p = 0.38	p = 0.12	**p < 0.0001**	p = 0.83	**p < 0.001**	p = 0.11	**p = 0.007**	**p < 0.0001**

Four cichlids	Nonneuronal cells density				Nonneuronal cells, total				Structure mass (mg)			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Body mass (g)	***r = -0.31***	***r = -0.32***	r = -0.03	r = 0.06	**r = 0.46**	**r = 0.43**	**r = 0.61**	**r = 0.69**	**r = 0.92**	**r = 0.69**	**r = 0.89**	**r = 0.81**
	p = 0.09	***p = 0.08***	p = 0.85	p = 0.73	**p = 0.01**	**p = 0.01**	**p < 0.001**	**p < 0.0001**	**p < 0.0001**	**p < 0.0001**	**p < 0.0001**	**p < 0.0001**
SL (cm)	r = -0.24	***r = -0.33***	r = 0.04	r = 0.11	**r = 0.51**	**r = 0.45**	**r = 0.67**	**r = 0.74**	**r = 0.91**	**r = 0.72**	**r = 0.90**	**r = 0.78**
	p = 0.19	***p = 0.08***	p = 0.81	p = 0.55	**p = 0.004**	**p = 0.01**	**p < 0.0001**	**p < 0.0001**	**p < 0.0001**	**p < 0.0001**	**p < 0.0001**	**p < 0.0001**

Significance was settled at p ≤ 0.05. Significant correlations are highlighted in bold and gray. Trends (p < 0.10) are highlighted in bold and italic.
tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

Correlations with body mass and standard length by species

Looking to each species, *S. pappaterra* is the only species that maintained the tendency observed above to show a positive correlation between morphometric measures and structure mass in all structures (table 5). This was also observed for *G. brasiliensis* and *G. sveni*, but not for all brain regions. *G. sveni* did not show significant correlations between body mass and SL when compared to the diencephalic and cerebellar mass, although this last one showed a trend for a strong correlation (table 5; Appendix Fig. 1-8). For *G. brasiliensis*, correlation appeared only when compared body mass and SL with telencephalon and cerebellum mass.

Interestingly, several positive and negative correlations disappeared when we looked at each species separately (table 5). In *G. brasiliensis*, there was positive correlations between body mass and SL with number of neurons only in the cerebellum; for *G. sveni*, number of neurons in the telencephalon was positively correlated for both, body mass and SL. In *S. pappaterra*, there was a positive correlation between body mass and SL in the cerebellar neurons. For number of nonneuronal cells, there was significant correlations for *G. brasiliensis* (positive correlation between SL and optic tectum) and *S. pappaterra* (positive correlation between body mass and SL both with optic tectum cells), only (table 5).

Concerning the density of brain cells, *G. brasiliensis* showed negative correlations only between nonneuronal cells in the cerebellum and body mass and SL (table 5). *G. sveni* presented a strong positive correlation between both, body mass and SL, and density of neurons in the telencephalon, but a negative correlation between these morphometric measures and the neurons densities in the optic tectum (table 5). *S. pappaterra* presented only negative correlations between both morphometric measures and density of neurons in the optic tectum and cerebellum, and density of nonneuronal cells in the cerebellum (table 5). *A. agassizii* did not show any correlation between morphometric measures and any of the brain measurements realized, as cells density, number of cells and structure mass.

Table 5: Brain cells densities and total numbers correlated with body mass and standard length in each species separately.

G. brasiliensis	**Neurons, density**				**Neurons, total**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Body mass (g)	r = 0 p = 1	r = 0.35 p = 0.35	r = -0.41 p = 0.26	r = -0.06 p = 0.86	r = 0.35 p = 0.35	r = 0.31 p = 0.41	r = 0.28 p = 0.46	**r = 0.68** **p = 0.04**
SL (cm)	r = 0.04 p = 0.91	r = 0.57 p = 0.10	r = -0.25 p = 0.51	r = 0.10 p = 0.79	r = 0.54 p = 0.12	r = 0.48 p = 0.19	r = 0.40 p = 0.28	**r = 0.82** **p = 0.007**

G. brasiliensis	**Nonneuronal cells, density**				**Nonneuronal cells, total**				**Structure mass (mg)**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Body mass (g)	r = -0.20 p = 0.60	r = 0.11 p = 0.76	r = 0.36 p = 0.33	**r = -0.75** **p = 0.02**	r = 0.06 p = 0.86	r = -0.20 p = 0.60	***r = 0.61*** ***p = 0.07***	r = 0.18 p = 0.63	**r = 0.70** **p = 0.03**	r = -0.39 p = 0.29	r = 0.56 p = 0.11	**r = 0.80** **p = 0.009**
SL (cm)	r = -0.06 p = 0.88	r = 0.31 p = 0.41	***r = 0.60*** ***p = 0.09***	**r = -0.71** **p = 0.03**	r = 0.18 p = 0.63	r = -0.05 p = 0.89	**r = 0.79** **p = 0.01**	r = 0.34 p = 0.37	**r = 0.80** **p = 0.009**	r = 0.15 p = 0.27	r = 0.49 p = 0.18	**r = 0.83** **p = 0.005**

G. sveni	**Neurons, density**				**Neurons, total**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Body mass (g)	**r = 0.73** **p = 0.02**	r = -0.48 p = 0.19	**r = -0.81** **p = 0.007**	r = 0.16 p = 0.67	**r = 0.81** **p = 0.007**	r = -0.10 p = 0.79	r = 0.26 p = 0.49	r = 0.55 p = 0.12
SL (cm)	**r = 0.83** **p = 0.005**	r = -0.38 p = 0.31	**r = -0.78** **p = 0.01**	r = 0.13 p = 0.73	**r = 0.88** **p = 0.001**	r = 0.08 p = 0.83	r = 0.45 p = 0.22	r = 0.40 p = 0.28

G. sveni	**Nonneuronal cells, density**				**Nonneuronal cells, total**				**Structure mass (mg)**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Body mass (g)	r = -0.43 p = 0.24	r = 0.25 p = 0.51	r = -0.41 p = 0.26	r = -0.31 p = 0.40	r = 0.26 p = 0.49	***r = 0.61*** ***p = 0.07***	r = 0.38 p = 0.31	r = 0.51 p = 0.15	**r = 0.91** **p < 0.001**	r = 0.55 p = 0.12	**r = 0.82** **p = 0.007**	***r = 0.60*** ***p = 0.09***
SL (cm)	r = -0.31 p = 0.40	r = 0.11 p = 0.76	r = -0.28 p = 0.46	r = -0.13 p = 0.73	r = 0.36 p = 0.33	***r = 0.60*** ***p = 0.09***	r = 0.53 p = 0.14	r = 0.46 p = 0.20	**r = 0.90** **p < 0.001**	r = 0.46 p = 0.20	**r = 0.88** **p = 0.002**	***r = 0.60*** ***p = 0.09***

S. pappaterra	**Neurons, density**				**Neurons, total**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Body mass (g)	r = -0.33 p = 0.49	r = 0.02 p = 0.95	**r = -0.78** **p = 0.02**	**r = -0.76** **p = 0.03**	r = 0.40 p = 0.32	r = 0.12 p = 0.78	r = -0.21 p = 0.61	**r = 0.74** **p = 0.03**
SL (cm)	r = -0.40 p = 0.32	r = 0.05 p = 0.91	**r = -0.83** **p = 0.01**	**r = -0.74** **p = 0.03**	r = 0.24 p = 0.57	r = 0.14 p = 0.73	r = -0.36 p = 0.38	**r = 0.76** **p = 0.03**

S. pappaterra	**Nonneuronal cells, density**				**Nonneuronal cells, total**				**Structure mass (mg)**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Body mass (g)	r = -0.50 p = 0.21	r = -0.33 p = 0.42	r = 0.02 p = 0.95	**r = -0.66** **p = 0.07**	r = 0.05 p = 0.91	r = -0.23 p = 0.57	**r = 0.83** **p = 0.01**	***r = 0.69*** ***p = 0.06***	**r = 0.95** **p < 0.001**	**r = 0.83** **p = 0.01**	**r = 0.90** **p = 0.002**	**r = 0.88** **p = 0.004**
SL (cm)	r = -0.40 p = 0.32	r = -0.40 p = 0.32	r = 0.05 p = 0.91	**r = -0.74** **p = 0.03**	r = 0.12 p = 0.78	r = -0.33 p = 0.42	**r = 0.81** **p = 0.01**	***r = 0.66*** ***p = 0.07***	**r = 0.90** **p = 0.002**	**r = 0.74** **p = 0.03**	**r = 0.88** **p = 0.003**	**r = 0.90** **p = 0.002**

A. agassizii	**Neurons, density**				**Neurons, total**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Body mass (g)	r = -0.34 p = 0.36	r = 0.007 p = 0.98	r = -0.40 p = 0.29	r = 0.03 p = 0.94	r = -0.41 p = 0.26	r = -0.07 p = 0.85	r = 0.36 p = 0.34	r = 0.37 p = 0.37
SL (cm)	r = -0.33 p = 0.39	r = 0.03 p = 0.93	r = -0.39 p = 0.29	r = -0.02 p = 0.96	r = -0.39 p = 0.29	r = -0.06 p = 0.86	r = 0.37 p = 0.32	r = 0.31 p = 0.45

A. agassizii	**Nonneuronal cells, density**				**Nonneuronal cells, total**				**Structure mass (mg)**			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Body mass (g)	r = -0.40 p = 0.28	r = 0.18 p = 0.61	r = -0.17 p = 0.65	r = -0.14 p = 0.77	r = -0.34 p = 0.37	r = 0.13 p = 0.71	r = 0.39 p = 0.30	r = 0.37 p = 0.36	r = -0.26 p = 0.50	r = -0.11 p = 0.76	r = 0.08 p = 0.81	***r = 0.62*** ***p = 0.09***
SL (cm)	r = -0.35 p = 0.36	r = 0.22 p = 0.53	r = -0.15 p = 0.68	r = -0.22 p = 0.63	r = -0.29 p = 0.44	r = 0.14 p = 0.68	r = 0.40 p = 0.28	r = 0.33 p = 0.42	r = -0.21 p = 0.57	r = -0.16 p = 0.66	r = 0.11 p = 0.73	***r = 0.67*** ***p = 0.06***

Significance was settled at $p \leq 0.05$. Significant correlations are highlighted in bold and gray. Trends ($p < 0.10$) are highlighted in bold and italic. tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

Correlations across number and density of brain cells

In here, we found almost no correlations between neurons and nonneuronal cells when we use all cichlid species together (table 6), contrasting with the strong correlations found, in general, for structure mass. Density of neurons had no correlation with nonneuronal cells density. In total number of brain cells, neurons only correlated positively with nonneuronal cells in the telencephalon and cerebellum (table 6). Differently for what is observed for mammals (see Herculano-Houzel et al. 2015), increases or decreases in number of neurons in fish is not linked to increases or decreases in nonneuronal cells numbers, except in the telencephalon and cerebellum (with a mild and strong r value, respectively) when comparing total number of cells.

By making the correlations in each species, there was also few correlations between density of brain cells and total number of them in each structure. *G. brasiliensis* show no correlation, while *G. sveni,* showed two very strong positive correlation in the optic tectum, concerning both density of brain cells and total number of cells (table 6). The *S. pappaterra* presented three negative correlations, one concerning the total number of brain cells in the telencephalon and the other two concerning density of brain cells and total numbers, both in the diencephalon. This is interesting since it is the only species where the correlations were negative. For *A. agassizii,* we saw just one positive and strong correlation in the density of cells in the diencephalon, showing that the increase in density of neurons in the diencephalon is followed by an increase in the nonneuronal cells density in this region (table 6). Although we observed a positive allometric relation between morphometric characters and structure mass – i.e., showing heavier brain areas in bigger individuals –, this tendency does not seem to be strongly followed when we look at brain cells composition, in which neurons and nonneuronal cells do not follow the same rules that apply for mammals (see Mota and Herculano-Houzel 2014; Herculano-Houzel et al. 2015), as mentioned above.

Table 6. Density and number of neurons correlated to nonneuronal cells in each brain structure.

Four cichlids	Neurons, density			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, density	r = -0.08 p = 0.66	r = -0.19 p = 0.30	r = -0.22 p = 0.23	r = 0.21 p = 0.26
	Neurons, total			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, total	**r = 0.39** **p = 0.03**	r = 0.21 p = 0.26	r = 0.27 p = 0.14	**r = 0.68** **p < 0.0001**

Geophagus brasiliensis	Neurons, density			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, density	r = 0.38 p = 0.31	r = 0.33 p = 0.38	r = -0.06 p = 0.86	r = -0.06 p = 0.86
	Neurons, total			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, total	r = 0.18 p = 0.63	r = 0.13 p = 0.73	r = 0.18 p = 0.63	r = 0.35 p = 0.35

Satanoperca pappaterra	Neurons, density			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, density	r = -0.55 p = 0.16	**r = -0.88** **p = 0.004**	r = -0.24 p = 0.57	r = 0.14 p = 0.73
	Neurons, total			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, total	**r = -0.69** **p = 0.05**	**r = -0.78** **p = 0.02**	r = -0.19 p = 0.65	r = 0.52 p = 0.18

Geophagus sveni	Neurons, density			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, density	r = -0.51 p = 0.15	r = -0.46 p = 0.20	**r = 0.71** **p = 0.03**	r = 0.20 p = 0.60
	Neurons, total			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, total	r = 0.08 p = 0.83	r = 0.31 p = 0.40	**r = 0.95** **p < 0.0001**	r = 0.43 p = 0.24

Apistogramma agassizii	Neurons, density			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, density	r = -0.34 p = 0.65	**r = 0.96** **p = 0.03**	r = -0.31 p = 0.68	r = 0.36 p = 0.64
	Neurons, total			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, total	r = -0.32 p = 0.67	r = 0.90 p = 0.10	r = 0.74 p = 0.26	r = -0.34 p = 0.66

Significance was settled at $p \leq 0.05$. Significant correlations are highlighted in bold and gray. Trends ($p < 0.10$) are highlighted in bold and italic. tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

The strong correlations between neurons density and total number of this type of cells showed that the more the number of cells in the telencephalon and diencephalon, the higher will be the density of these when we grouped the four cichlids data (table 7). These results were maintained in *G. brasiliensis* and *G. sveni* (table 7). For *S. pappaterra,* the positive correlation between number of neurons and its density occurred in the diencephalon and in the optic tectum, but not in the telencephalon. The dwarf and polygynous cichlid, *A. agassizii*, was the only species in which a positive correlation between number and density of neurons occurred in the cerebellum. It was also the strongest correlation found, with an r-value of 0.99 (table 7).

The nonneuronal cells have two positive correlations between respective densities in the telencephalon and in the optic tectum, and the cerebellum showed a trend towards a positive correlation, although not very strong (table 7), when the four cichlids were putted together for analysis. *G. brasiliensis* presented two positive correlations also in the telencephalon and optic tectum. *G. sveni* followed this results only concerning telencephalon (table 7). For last, the both polygynous species, *S. pappaterra* and *A. agassizii,* presented positive and strong correlations between number of nonneuronal cells and densities in the telencephalon and diencephalon, only (table 7). These are interesting results, once that the increase of brain cells in number and density in a giving structure is not related, in the majority of cases, with other parameters, as morphometric measures (table 4 and 5). Therefore, the increase in fish size or in specifically brain structures of the brain are not reliable to infer number of brain cells.

Table 7. Density of brain cells correlated to its respective number of cells in each brain structure.

Four cichlids	Neurons, density			
	tl	**di**	**ot**	**cb**
Neurons, total	**r = 0.45** **p = 0.01**	**r = 0.53** **p = 0.002**	r = 0.02 p = 0.91	r = 0.25 p = 0.18
	Nonneuronal cells, density			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, total	**r = 0.48** **p = 0.006**	r = 0.19 p = 0.32	**r = 0.63** **p < 0.001**	***r = 0.38*** ***p = 0.07***

Geophagus brasiliensis	Neurons, density			
	tl	**di**	**ot**	**cb**
Neurons, total	**r = 0.73** **p = 0.02**	**r = 0.93** **p < 0.001**	r = 0.48 p = 0.19	r = 0.15 p = 0.70
	Nonneuronal cells, density			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, total	**r = 0.83** **p = 0.005**	r = 0.55 p = 0.12	**r = 0.88** **p = 0.001**	r = 0.08 p = 0.83

Satanoperca pappaterra	Neurons, density			
	tl	**di**	**ot**	**cb**
Neurons, total	r = 0.59 p = 0.12	**r = 0.95** **p < 0.001**	**r = 0.66** **p = 0.07**	r = -0.21 p = 0.61
	Nonneuronal cells, density			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, total	**r = 0.74** **p = 0.03**	**r = 0.90** **p = 0.002**	r = 0.38 p = 0.35	r = -0.24 p = 0.57

Geophagus sveni	Neurons, density			
	tl	**di**	**ot**	**cb**
Neurons, total	**r = 0.96** **p < 0.0001**	**r = 0.63** **p = 0.07**	r = 0.13 p = 0.73	r = 0.23 p = 0.54
	Nonneuronal cells, density			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, total	**r = 0.61** **p = 0.07**	r = 0.33 p = 0.38	r = 0.55 p = 0.12	r = 0 p = 1

Apistogramma agassizii	Neurons, density			
	tl	**di**	**ot**	**cb**
Neurons, total	***r = 0.94*** ***p = 0.06***	r = 0.90 p = 0.10	r = -0.24 p = 0.76	**r = 0.99** **p = 0.008**
	Nonneuronal cells, density			
	tl	**di**	**ot**	**cb**
Nonneuronal cells, total	**r = 0.92** **p = 0.08**	**r = 0.97** **p = 0.03**	r = 0.83 p = 0.17	r = 0.83 p = 0.16

Significance was settled at $p \leq 0.05$. Significant correlations are highlighted in bold and gray. Trends ($p < 0.10$) are highlighted in bold and italic. tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

Cell's sizes are independent from structure mass and number of cells, but in the dwarf cichlid

Ferets diameters (µm) – i.e., a measure for cell size – were obtained using a mean of 200 cells measurements for each brain structure and individual. Thus, the only brain structure where we found the most expressive correlations using this variable were in the cerebellum. The size of the cells in the cerebellum presented negative correlations between the fish body mass and SL, cerebellar mass, and total number of cells, both neuronal and nonneuronal (table 8). Other brain regions did not show any correlation between the variables measured, except the telencephalon, that cells size showed a positive trend with nonneuronal cells number, but with a low r-value of 0.32 (table 8). The fact that the total number of brain cells in the cerebellum is inversely related to cell size, but not to brain cells density, seems to be partially in agreement with the explanation provided by Herculano-Houzel et al. (2015). They state that an increase of number of cells accompanied by an increase in density of cells in a giving brain area, is follow by a decrease in the cell size. Nevertheless, the cerebellum mass also is negatively correlated with cell size, showing that the smaller these cells get, the heavier cerebellum becomes. When we look to these correlations by separating each species, however, the strong correlations observed concerning cerebellum seem to have been biased by the dwarf cichlid *A. agassizii*¸ and will be better discussed in the following section.

Table 8. Cells size measured by ferets diameters correlated with body and brain measures in each species.

Four cichlids	Ferets diameters (µm)			
	tl	**di**	**ot**	**cb**
Body mass (g)	r = 0.19 p = 0.31	r = -0.02 p = 0.89	r = -0.07 p = 0.72	**r = -0.56** **p = 0.002**
SL (cm)	r = 0.21 p = 0.27	r = -0.01 p = 0.96	r = -0.12 p = 0.52	**r = -0.59** **p < 0.001**
Structure mass (mg)	r = 0.14 p = 0.45	r = -0.06 p = 0.74	r = -0.15 p = 0.43	**r = -0.70** **p < 0.0001**
Neurons, total	r = 0.26 p = 0.16	r = -0.10 p = 0.60	r = 0.01 p = 0.94	**r = -0.71** **p < 0.0001**
Neurons, density	r = 0.09 p = 0.63	r = -0.19 p = 0.31	r = -0.01 p = 0.94	r = -0.26 p = 0.16
Nonneuronal cells, total	***r = 0.32*** ***p = 0.09***	r = -0.08 p = 0.66	r = -0.09 p = 0.65	**r = -0.50** **p = 0.005**
Nonneuronal cells, density	r = 0.12 p = 0.53	r = -0.21 p = 0.25	r = -0.18 p = 0.33	r = 0.05 p = 0.79

Significance was settled at $p \leq 0.05$. Significant correlations are highlighted in bold and gray. Trends ($p < 0.10$) are highlighted in bold and italic. tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

In three of the four species we tested, there was almost no correlation between feret diameters and the morphometric and brain variables tested (table 9). *S. pappaterra* and *G. sveni* showed strong negative correlations concerning cell size and number of cerebellar

neurons and cerebellum mass, respectively (table 9). The dwarf cichlid, *A. agassizii,* was the species that showed the majority of significant correlations concerning cell size. In this species, the size of the cells in the diencephalon were negatively correlated to both brain cells density and total numbers, showing that indeed a reduction in cell size are related to an increase in cells quantity in the diencephalon. In the cerebellum, cell size was strongly correlated to morphometric measures: body mass, SL and brain structure mass (table 9). Thus, bigger *A. agassizii* presented heavier cerebellums (significant trend observed in table 5) that were also composed by bigger cells, contrary to the results we found for the other three cichlids we tested. *A. agassizii* also presented a positive correlation between cell size and neurons density in the telencephalon, and two negative correlation between cell size in the optic tectum and total of nonneuronal cells and optic tectum mass (table 9). *G. brasiliensis* did not present any correlation.

Table 9. Cells size measured by ferets diameters correlated with body and brain measures in each species.

Geophagus brasiliensis	Ferets diameters (µm)			
	tl	**di**	**ot**	**cb**
Body mass (g)	r = -0.15 p = 0.70	r = 0.08 p = 0.83	r = 0.3 p = 0.43	r = -0.07 p = 0.86
SL (cm)	r = -0.08 p = 0.83	r = 0.18 p = 0.63	r = 0.06 p = 0.86	r = -0.33 p = 0.39
Structure mass (mg)	r = -0.06 p = 0.86	r = -0.01 p = 0.96	r = 0 p = 1	r = -0.035 p = 0.35
Neurons, total	r = 0.2 p = 0.60	r = 0.08 p = 0.83	r = 0.15 p = 0.70	r = -0.48 p = 0.18
Neurons, density	r = 0.46 p = 0.20	r = 0.21 p = 0.57	r = 0.23 p = 0.54	r = -0.53 p = 0.14
Nonneuronal cells, total	r = -0.13 p = 0.73	r = -0.41 p = 0.26	r = -0.38 p = 0.31	r = -0.03 p = 0.93
Nonneuronal cells, density	r = 0.23 p = 0.54	***r = -0.6*** ***p = 0.09***	r = -0.36 p = 0.33	r = 0.4 p = 0.28

Satanoperca pappaterra	Ferets diameters (µm)			
	tl	**di**	**ot**	**cb**
Body mass (g)	r = -0.19 p = 0.65	r = -0.59 p = 0.12	r = -0.38 p = 0.35	r = -0.33 p = 0.42
SL (cm)	r = -0.09 p = 0.82	r = -0.54 p = 0.16	r = -0.33 p = 0.42	r = -0.31 p = 0.45
Structure mass (mg)	r = -0.31 p = 0.45	r = -0.62 p = 0.10	r = -0.62 p = 0.10	r = -0.59 p = 0.12
Neurons, total	r = -0.26 p = 0.53	r = -0.42 p = 0.29	r = -0.19 p = 0.65	**r = -0.78** **p = 0.02**
Neurons, density	r = 0.14 p = 0.73	r = -0.36 p = 0.38	r = 0.12 p = 0.78	r = -0.14 p = 0.73
Nonneuronal cells, total	r = 0.05 p = 0.91	r = 0.31 p = 0.45	r = -0.62 p = 0.10	r = -0.26 p = 0.53
Nonneuronal cells, density	r = 0.36 p = 0.38	r = 0.40 p = 0.32	r = -0.26 p = 0.53	***r = 0.67*** ***p = 0.07***

Geophagus sveni	Ferets diameters (µm)			
	tl	**di**	**ot**	**cb**
Body mass (g)	r = 0.14 p = 0.76	r = 0.5 p = 0.25	r = -0.32 p = 0.48	r = -0.43 p = 0.53
SL (cm)	r = 0 p = 1	r = 0.35 p = 0.43	r = -0.43 p = 0.34	r = -0.28 p = 0.53
Structure mass (mg)	r = -0.14 p = 0.76	r = 0.11 p = 0.82	r = -0.38 p = 0.40	**r = -0.78** **p = 0.03**
Neurons, total	r = -0.28 p = 0.53	r = -0.46 p = 0.29	***r = -0.71*** ***p = 0.07***	r = -0.35 p = 0.43
Neurons, density	r = -0.18 p = 0.70	r = -0.21 p = 0.64	r = -0.11 p = 0.82	r = 0.11 p = 0.82
Nonneuronal cells, total	r = 0.64 p = 0.12	r = -0.07 p = 0.88	***r = -0.71*** ***p = 0.07***	r = -0.14 p = 0.76
Nonneuronal cells, density	r = 0.21 p = 0.64	r = -0.21 p = 0.64	p = -0.43 p = 0.34	p = 0.46 p = 0.29

Apistogramma agassizii	Ferets diameters (µm)			
	tl	**di**	**ot**	**cb**
Body mass (g)	***r = 0.94*** ***p = 0.06***	r = -0.59 p = 0.41	r = -0.89 p = 0.10	**r = 0.95** **p = 0.05**
SL (cm)	***r = 0.94*** ***p = 0.06***	r = -0.58 p = 0.41	r = -0.89 p = 0.10	**r = 0.95** **p = 0.05**
Structure mass (mg)	r = 0.78 p = 0.22	r = -0.17 p = 0.82	**r = -0.95** **p = 0.05**	**r = 0.99** **p = 0.007**
Neurons, total	r = 0.82 p = 0.18	**r = -0.99** **p = 0.003**	r = -0.76 p = 0.23	r = -0.58 p = 0.41
Neurons, density	**r = 0.98** **p = 0.02**	**r = -0.94** **p = 0.05**	r = -0.61 p = 0.39	r = -0.70 p = 0.29
Nonneuronal cells, total	r = -0.21 p = 0.78	**r = -0.99** **p < 0.001**	**r = -0.94** **p = 0.05**	r = -0.61 p = 0.38
Nonneuronal cells, density	r = -0.57 p = 0.42	**r = -0.98** **p = 0.01**	***r = -0.93*** ***p = 0.06***	***r = -0.91*** ***p = 0.09***

Significance was settled at p ≤ 0.05. Significant correlations are highlighted in bold and gray. Trends (p < 0.10) are highlighted in bold and italic. tl: telencephalon; di: diencephalon; ot: optic tectum; cb: cerebellum.

As no other study concerning brain architecture have already checked for cells size, we wanted to explore in more details this variable between species using Linear Models (LM). As for correlation data, there was no difference between males and females concerning cell size ($F_{(3,151)} = 0.33$, p = 0.79); thus, we analyzed both sexes together. The diencephalon was smaller in *A. agassizii* ($F_{(3.35)} = 9.39$, p = 0.0001; Fig. 4b) when compared to the other three species, *G. brasiliensis* (p < 0.001), *G. sveni* (p = 0.01) and *S. pappaterra* (p = 0.003). In the optic tectum, cell size was again smaller in *A. agassizii* ($F_{(3.36)} = 8.19$, p = 0.0003; Fig. 4c) than in the monogamous species, *G. brasiliensis* (p < 0.001), *G. sveni* (p = 0.02), and the polygynous *S. pappaterra* (p = 0.008). The cerebellum was the only brain structure where the cell size was bigger in the dwarf cichlid *A. agassizii* ($F_{(3.36)} = 7.59$, p = 0.0004; Fig. 4d) than in *G. brasiliensis* (p = 0.01), *G. sveni* (p < 0.001) and *S. pappaterra* (p = 0.02). This shows a

possible compensation for this area to be the one with the smallest number of neurons when compared with the other cichlids studied here, as bigger nervous cells are faster in synaptic transmissions. We did not find any difference between species concerning cells size in the telencephalon.

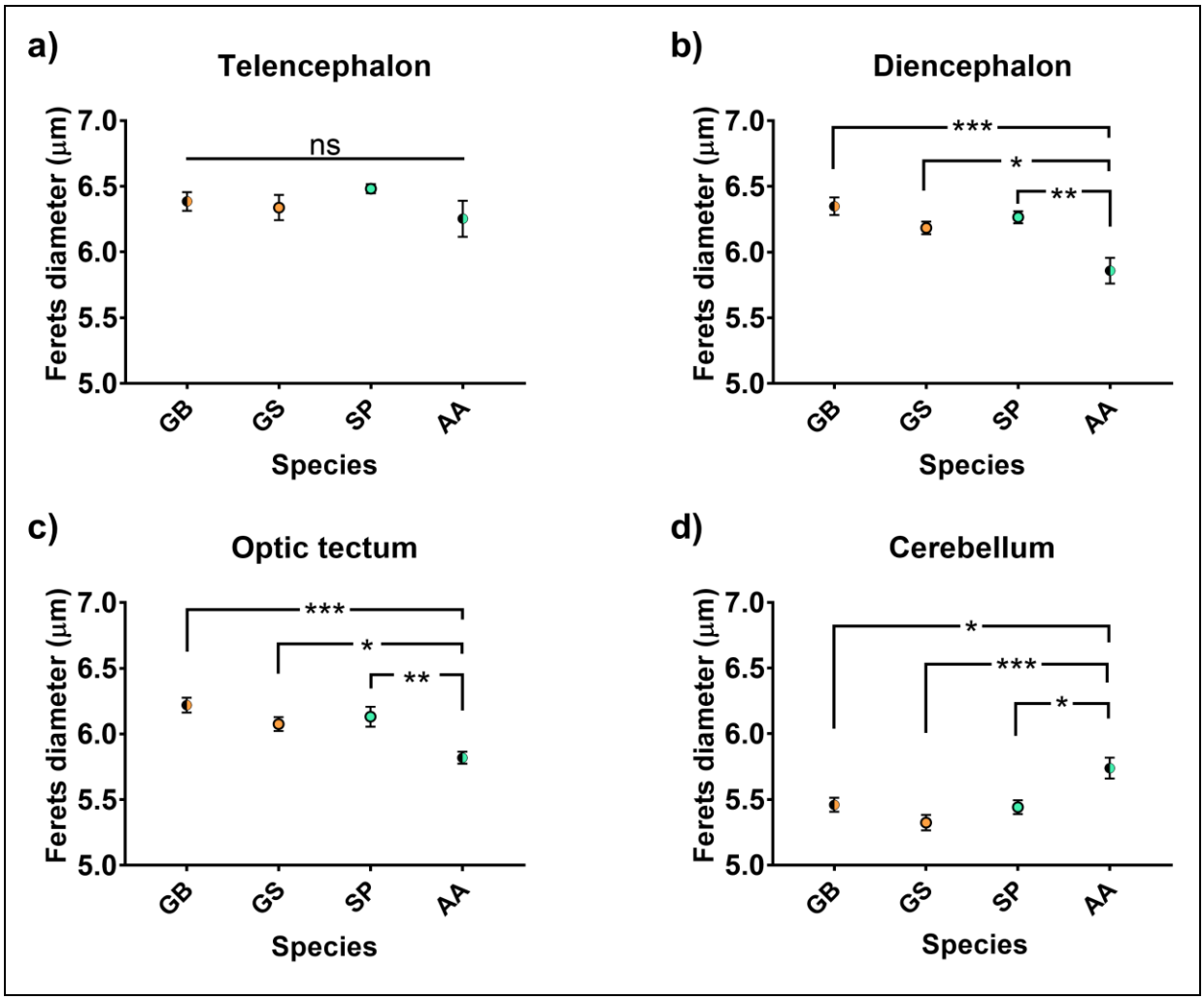

Figure 3. Cells size, measure by ferets diameters, in the **a.** telencephalon, **b.** diencephalon, **c.** optic tectum and **d.** cerebellum compared between cichlid species. GB: *Geophagus brasiliensis*; GS: *Geophagus sveni*; SP: *Satanoperca pappaterra*; AA: *Apistogramma agassizii*.

Discussion

This is the first descriptive study regarding number of brain cells and their correlations with morphometric measures, as body mass and standard length, in Neotropical cichlid species. Moreover, in here we used four cichlid species differing between each other in reproductive mating system and type of parental care, as these factors are behind selection of specific brain regions. Except in the dwarf cichlid, *Apistogramma agassizii,* structure mass of brain areas seems to follow an allometric relation with fish body mass and standard length, in which larger fish indeed present heavier brains. Number and density of brain cells were also

positively correlated. Therefore, in cichlid fish it seems that bigger brains in fact have more neurons, as well as nonneuronal cells. For last, individual sex was not correlated to any variable we used in our study, neither morphometric nor cerebral, therefore, does not seem to be a strong sex influence in cichlid brain composition.

The interesting find of our work is that the number of brain cells was related to larger brain areas, as recently observed for other teleost fish, the guppy *Poecilia reticulata* (Marhounová et al. 2019). Moreover, number of brain cells and the density of them are positively related. In the literature concerning mammals' brains, it would be expected that an increase in number of cells followed by an increase in cells density would be reflected in a reduction of cell size (Mota and Herculano-Houzel 2014; Herculano-Houzel et al. 2015). To have bigger brains is very expensive (Aiello and Wheeler 1995; Tsuboi et al. 2015), and the trade-offs between cells density and cell size somewhat could compensate the energy spent. Indeed, teleost fishes present the smallest brains in the vertebrate group, with an extreme miniaturization of brain cells when compared to other vertebrates; moreover, teleost brains develop from a massive accumulation of embryonic cells, which may represent another adaptation responsible for the small cell sizes observed in this fish group (Kotrschal et al. 1998). Three cichlid species used in our study, *Geophagus brasiliensis, Geophagus sveni* and *Satanoperca pappaterra,* presented, in general, bigger brain structures formed by increased number and density of cells. On the contrary, the dwarf cichlid, *A. agassizii*, was the species with the most different correlations concerning brain cells' composition. In this species, structure mass did not show any correlation to morphometric measures, as the other three cichlids. Nevertheless, it is interesting to point that total number of cells in the cerebellum, for example, showed the strongest – and positive – correlation between density of neurons in the same region. In addition, the average cells size in the cerebellum of *A. agassizii* was bigger than in the other three cichlids, even though the first one is a dwarf cichlid. In this species, the cerebellum seems to have a very important role, maybe linked to the environmental complexity of Amazonian rivers in which the individuals live, also provided by the miniaturization suffered by this dwarf cichlid, that will be better discussed later.

Investigating the correlations concerning the morphometric measures – i.e., fish body mass and SL –, *G. brasiliensis* only showed a positive correlation regarding neuronal cells in the cerebellum. Thus, it seems that bigger fishes indeed present more cells in this area, maybe due to a motor compensation provided by the cerebellum for larger individuals, which was not followed by an increase in cells density. Regarding nonneuronal cells, there was a negative correlation between the density of these cells in the cerebellum and the morphometric measures. For total number of nonneuronal cells, a positive correlation appeared in the optic tectum. The structure mass was positively correlated to body mass and SL in the telencephalon and cerebellum. These results are not in accordance with Northcutt et al. (1978), that states that fish brains present negative allometric correlations with body

size, with small species presenting relatively larger brains and vice-versa (Brandstätter and Kotrschal 1990). In here, we observed the opposite, with bigger fishes presenting bigger brain structures, except in the dwarf cichlid, *A. agassizii,* where these rules of allometry were not apply at all.

Other novelty of our work was the quantification of cells size. Interestingly, the cerebellum was the only brain region that showed correlation between morphometric and brain measures, all of them negative. Thus, when grouped together, bigger cichlid fish, with heavier cerebellums composed by more cells, have smaller average cell size in this region. The densities of neurons and nonneuronal cells were the only two variables that were not associated to cell size in the cerebellum. When analyzed in separately, several correlations disappear. While average cell size in *G. brasiliensis* is not related to any variable we tested, the mouthbrooders *G. sveni* and *S. pappaterra* presented smaller cells in the cerebellum when cerebellum mass and number of cerebellar neurons increase. *A. agassizii*, however, presented several correlations between cell size and their respective brain structures. Larger individuals have bigger cells in the telencephalon (a trend with strong r-value) and in the cerebellum. Bigger cells are also associated to higher density of neurons in the telencephalon and a heavier cerebellum in *A. agassizii.* Average cell size in the diencephalon was negatively correlated to number and density of cells, both in neuronal and nonneuronal cells. Optic tectum mass was also negatively correlated with cell size in this region, as well and nonneuronal cells number and density (a trend wih strong r-value in this last measure).

Differently from what is observed for mice (see Herculano-Houzel et al. 2015), number of neurons are not linked to number of nonneuronal cells in cichlid fish. The same is true for brain cells' densities. When the four species of cichlids had their data analyzed together, we observed that number of brain cells were only correlated in the telencephalon and in the cerebellum, and that the correlation of telencephalon was indeed weak. *G. brasiliensis* did not show any correlation of this kind. For *G. sveni,* density and number of cells were only positively linked to one another in the telencephalon. In *S. pappaterra* and *A. agassizii*; these links appeared in the diencephalon; nevertheless, it was a negative correlation in *S. pappaterra,* and a positive one in the *A. agassizii*. For *S. pappaterra,* there was also a negative correlation in the number of cells in the telencephalon. Therefore, we can observe that even in closely related species as the ones we chose in our study, there was no pattern or relation between neurons and nonneuronal cells. It is interesting, although, that only *S. pappaterra* presented negative correlations between neurons and nonneuronal cells in the forebrain.

In three species of this study, *G. brasiliensis, G. sveni* and *S. pappaterra*, larger individuals presented heavier brain structures. Nevertheless, the principal component analysis made for each species, including *A. agassizii,* reveals that structure mass of brain areas was only loaded in the second component, after number and density of brain cells. A

study of Herculano-Houzel et al. (2015) with mice presented this same result, with structure mass being loaded only in the second component of analysis. For this reason, they states that structure mass cannot be considered a determinant factor driving number of brain cells, but a consequence of the number of brain cells and their size together (Herculano-Houzel et al. 2015). Different from what happens for the rodents, nevertheless, each brain structure in fish show a specific relation between the variables we compared. Moreover, increased number of cells in the brain is always linked to increased density of these cells, showing that cell size does not seems to be decreasing in fish. Thus, increases in structure mass may be, indeed, a result of increased number of cells without cell size reduction in fish, as occurred in mice (but see Haug (1987) and Herculano-Houzel (2014), with other mammals, in which larger number of neurons are accompanied by a small density of these cells).

The most different patterns of brain organization in our study was provided by *A. agassizii.* This is a species phylogenetically close to the other three species we use in our work. Moreover, *A. agassizii* is similar to *S. pappaterra* since it is the female that takes care of the brood in both species, while male invest in reproductive partners (Kullander 2003; and FishBase.org). *A. agassizii* is also similar to *G. brasiliensis* as in the both species parents provide the parental care of the brood in the substrate. These similarities between the two cichlids and *A. agassizii* may have selected their brains for specific behaviors concerning these reproductive traits, as already saw for several cichlid species (see Pollen et al. 2007; Shumway 2010; Tsuboi et al. 2015). Nevertheless, some differences must be pointed. *A. agassizii* is the only species in here that suffered a miniaturization process. Miniaturization regards not only a small body, but affects individuals' physiology, ecology, life history and behavior: the costs of being a bigger individual decreases, as well as its gains (Hanken and Wake 1993). What evolutionary forces drove fishes' miniaturization are, even today, speculative. One possibility is that acidic waters may result in reduced poring of the fish's laterosensory system, which may reflects in overall body size (Collette 1962). Other studies mention that a decrease in body size is due to a reduction in individual fecundity and increase in egg size (Schultz et al. 1991; Shine and Greer 1991). Weitzman and Vari (1988) state that miniaturized species are generally find in slow-flowing shallow waters. All these hypotheses are the case of *A. agassizii*, a fish commonly found in shallow and acidic waters in the Amazon River basin in igarapés' regions (Kochhann et al. 2015; Kochhann and Val 2017), that presents low fecundity and short spawning periods (de Oliveira and de Queiroz 2017), being found exclusively in the northwest region of Brazil (Kullander 2003). Meanwhile, *G. brasiliensis, G. sveni* and *S. pappaterra* are species that cohabitate water bodies in south central Brazilian region (Kullander 2003; Lucinda et al. 2010), occupying environments very similar in their composition – i.e., temperature, water flow regime, oxygen concentration, acidification, concentration of organic material, etc. – and very different from the Amazonian body waters. Therefore, although presenting similarities in their reproductive behaviors, *A.*

agassizii seems to be under selection of other ecological and social factors provided by the peculiarities of Amazonian Rainforest than the reproductive ones. Further analysis; however, are necessary to understand the associations between brain and reproductive repertoire.

Regarding the both theories about brain evolution – e.g., the mosaic (Barton and Harvey 2000) and the concerted evolution hypothesis (Finlay and Darlington 1995) –, it is interesting to note that indeed they are not exclusive, since our results was in accordance with both of them depending on the variable analyzed. In relation to structure mass, for example, we could observe brain development occurring in a concerted fashion, with bigger individuals indeed presenting heavier brain structures in the four regions studied, at least when all species are analyzed together. On the other hand, number of brain cells follow the mosaic evolutionary model, as each brain region presented negative or positive correlations between our tested variables in a same individual, showing that these brain areas are very independent from one another to vary in number of neurons and nonneuronal cells. Moreover, the species choose for this study were better or worse models of each theory in particular – e.g., bigger individuals of *S. pappaterra* also presented bigger brain structures in all the areas analyzed – showing once again how diverse fishes can be in their physiology and anatomy, which for sure will reflects in their ecology and behavior.

Acknowledgements

We thank Ana Carolina dos Santos Gauy, Marcela Cesar Bolognesi and Magda Teles for technical assistance during data collection, and Nuno Pimpão Martins for the development of the macro used in brain cells' counting. This research was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), research grant #2016/26160-2, to EGF. MLB was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES #001) – DS for PhD Student and by FAPESP (scholarship #2019/10808-1 – TT-3).

References

Aiello LC, Wheeler P (1995) The Expensive-Tissue Hypothesis: The Brain and the Digestive System in Human and Primate Evolution.

Arbour JH, López-Fernández H (2016) Continental cichlid radiations: functional diversity reveals the role of changing ecological opportunity in the Neotropics. Proc R Soc B Biol Sci 283:20160556. doi: 10.1098/rspb.2016.0556

Barton RA, Harvey PH (2000) Mosaic evolution of brain structure in mammals. Nature 405:1055–1058. doi: 10.1038/35016580

Braithwaite VA (2006) Cognitive Ability in Fish. In: Sloman K, Balshine S, Wilson R (eds) Behaviour and Physiology of Fish, 1st edn. Elsevier Academic Press, London, UK, pp 1–37

Brandstätter R, Kotrschal K (1990) Brain Growth Patterns in Four European Cyprinid Fish Species (Cyprinidae, Teleostei): Roach *(Rutilus rutilus),* Bream *(Abramis brama),* Common

Carp *(Cyprinus carpio)* and Sabre Carp *(Pelecus cultratus)*. Brain Behav Evol 35:195–211. doi: 10.1159/000115867
Brandstätter R, Kotrschal K (1989) Life History of Roach, *Rutilus rutilus* (Cyprinidae, Teleostei). Brain Behav Evol 34:35–42. doi: 10.1159/000116489
Brown C, Laland KN (2003) Social learning in fishes : a review. Fish Fish 4:280–288.
Bshary R, Gingins S, Vail AL (2014) Social cognition in fishes. Trends Cogn Sci 18:465–471. doi: 10.1016/j.tics.2014.04.005
Bshary R, Wickler W, Fricke H (2002) Fish cognition: a primate's eye view. Anim Cogn 5:1–13. doi: 10.1007/s10071-001-0116-5
Collette BB (1962) The swamp darters of the subgenus *Hololepis* (Pisces, Percidae). Tulane Stud Zool 9:115–211.
de Oliveira JC, de Queiroz HL (2017) Life history traits of two dwarf cichlids species in the white waters of the Amazonian floodplain. Environ Biol Fishes 100:1497–1505. doi: 10.1007/s10641-017-0660-8
Dunbar RIM (1998) The social brain hypothesis. Evol Anthropol Issues, News, Rev 6:178–190. doi: 10.1002/(SICI)1520-6505(1998)6:5<178::AID-EVAN5>3.3.CO;2-P
Dunbar RIM, Shultz S (2007) Evolution in the Social Brain. Science (80-) 317:1344–1347. doi: 10.1126/science.1145463
Finlay B, Darlington R (1995) Linked regularities in the development and evolution of mammalian brains. Science (80-) 268:1578–1584. doi: 10.1126/science.7777856
Gierszewski S, Bleckmann H, Schluessel V, et al (2013) Cognitive Abilities in Malawi Cichlids (Pseudotropheus sp.): Matching-to-Sample and Image/Mirror-Image Discriminations. PLoS One 8:e57363. doi: 10.1371/journal.pone.0057363
Gonzalez-Voyer A, Kolm N (2010) Sex, Ecology and the Brain: Evolutionary Correlates of Brain Structure Volumes in Tanganyikan Cichlids. PLoS One 5:e14355. doi: 10.1371/journal.pone.0014355
Gonzalez-Voyer A, Winberg S, Kolm N (2009) Brain structure evolution in a basal vertebrate clade: evidence from phylogenetic comparative analysis of cichlid fishes. BMC Evol Biol 9:238. doi: 10.1186/1471-2148-9-238
Hanken J, Wake DB (1993) Miniaturization of Body Size: Organismal Consequences and Evolutionary Significance. Annu Rev Ecol Syst 24:501–519. doi: 10.1146/annurev.es.24.110193.002441
Haug H (1987) Brain sizes, surfaces, and neuronal sizes of the cortex cerebri: A stereological investigation of man and his variability and a comparison with some mammals (primates, whales, marsupials, insectivores, and one elephant). Am J Anat 180:126–142. doi: 10.1002/aja.1001800203
Herculano-Houzel S (2011) The Isotropic Fractionator: A Fast, Reliable Method to Determine Numbers of Cells in the Brain or Other Tissues. 391–403. doi: 10.1007/7657_2011_13

Herculano-Houzel S (2014) The glia/neuron ratio: How it varies uniformly across brain structures and species and what that means for brain physiology and evolution. Glia 62:1377–1391. doi: 10.1002/glia.22683
Herculano-Houzel S, Messeder DJ, Fonseca-Azevedo K, Pantoja NA (2015) When larger brains do not have more neurons: increased numbers of cells are compensated by decreased average cell size across mouse individuals. Front Neuroanat 9:64. doi: 10.3389/fnana.2015.00064
Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models. Biometrical J 50:346–363. doi: 10.1002/bimj.200810425
Kochhann D, Campos DF, Val AL (2015) Experimentally increased temperature and hypoxia affect stability of social hierarchy and metabolism of the Amazonian cichlid *Apistogramma agassizii*. Comp Biochem Physiol -Part A Mol Integr Physiol 190:54–60. doi: 10.1016/j.cbpa.2015.09.006
Kochhann D, Val AL (2017) Social hierarchy and resting metabolic rate in the dwarf cichlid *Apistogramma agassizii*: the role of habitat enrichment. Hydrobiologia 789:123–131. doi: 10.1007/s10750-016-2806-7
Kotrschal K, Van Staaden MJ, Huber R (1998) Fish brains: evolution and environmental relationships.
Kullander SO (2003) Family Cichlidae. In: Reis RE, Kullander SO, Ferraris Jr. CJ (eds) Check List of the Freshwater Fishes of South and Central America. EdiPUCRS, Porto Alegre, RS, pp 605–654
Lehner PN (1998) Handbook of Ethological Methods, 2nd edn. Cambridge University Press
Lucinda PHF, Lucena CAS, Assis NC (2010) Two new species of cichlid fish genus *Geophagus* Heckel from the Rio Tocantins drainage (Perciformes: Cichlidae). Zootaxa 29–42. doi: 10.5281/zenodo.194678
Marhounová L, Kotrschal A, Kverková K, et al (2019) Artificial selection on brain size leads to matching changes in overall number of neurons. Evolution (N Y) 73:2003–2012. doi: 10.1111/evo.13805
Maruska KP, Fernald RD (2018) *Astatotilapia burtoni*: A Model System for Analyzing the Neurobiology of Behavior. ACS Chem Neurosci 9:1951–1962. doi: 10.1021/acschemneuro.7b00496
Mota B, Herculano-Houzel S (2014) All brains are made of this: a fundamental building block of brain matter with matching neuronal and glial masses. Front Neuroanat 8:1–21. doi: 10.3389/fnana.2014.00127
Northcutt RG, Neary TJ, Senn DG (1978) Observations on the brain of the coelacanth *Latimeria chalumnae*: External anatomy and quantitative analysis. J Morphol 155:181–192. doi: 10.1002/jmor.1051550205
Pollen AA, Dobberfuhl AP, Scace J, et al (2007) Environmental complexity and social

organization sculpt the brain in Lake Tanganyikan cichlid fish. Brain Behav Evol 70:21–39. doi: 10.1159/000101067
Reddon AR, O'Connor CM, Ligocki IY, et al (2016) No evidence for larger brains in cooperatively breeding cichlid fishes. Can J Zool 94:373–378. doi: 10.1139/cjz-2015-0118
Revelle W (2018) psych: Procedures for Psychological, Psychometric, and Personality Research. Northwestern University, Evanston, Illinois
Schultz ET, Clifton LM, Warner RR (1991) Energetic constraints and size-based tactics: the adaptive significance of breeding-schedule variation in a marine fish (Embiotocidae: *Micrometrus minimus*). Am Nat 138:1408–1430. doi: 10.1086/285294
Shine R, Greer AE (1991) Why are clutch sizes more variable in some species than in others? Evolution (N Y) 45:1696–1706. doi: 10.1111/j.1558-5646.1991.tb02675.x
Shumway C a. (2010) The evolution of complex brains and behaviors in African cichlid fishes. Curr Zool 56:144–156.
Tsuboi M, Husby A, Kotrschal A, et al (2015) Comparative support for the expensive tissue hypothesis: Big brains are correlated with smaller gut and greater parental investment in Lake Tanganyika cichlids. Evolution (N Y) 69:190–200. doi: 10.1111/evo.12556
Valério-Gomes B, Guimarães DM, Szczupak D, Lent R (2018) The Absolute Number of Oligodendrocytes in the Adult Mouse Brain. Front Neuroanat 12:90. doi: 10.3389/fnana.2018.00090
Vila Pouca C, Brown C (2017) Contemporary topics in fish cognition and behaviour. Curr Opin Behav Sci 16:46–52. doi: 10.1016/j.cobeha.2017.03.002
Weitzman SH, Vari RP (1988) Miniaturization in South American freshwater fishes; an overview and discussion. Proc Biol Soc Washingt 101:444–465.
Zupanc GKHH (2008) Adult neurogenesis and neuronal regeneration in the brain of teleost fish. J Physiol Paris 102:357–373. doi: 10.1016/j.jphysparis.2008.10.007

APPENDIX

Table 1. Correlations between individual sex and body and brain measures in all the four cichlids tested.

Four cichlids	p	r
Standard length (cm)	0.25	0.22
Body mass (g)	0.11	0.31
Cell size - tl	***0.08***	0.33
Cell size - di	0.96	-0.01
Cell size - ot	1.00	0.00
Cell size - cb	0.92	0.02
Neurons, density - tl	0.54	0.12
Neurons, density - di	0.36	-0.18
Neurons, density - ot	0.51	-0.13
Neurons, density - cb	0.22	-0.24
Nonneuronal cells, density - tl	0.33	-0.19
Nonneuronal cells, density - di	0.48	-0.14
Nonneuronal cells, density - ot	0.96	-0.01
Nonneuronal cells, density - cb	0.65	-0.09
Neurons, total - tl	0.36	0.18
Neurons, total - di	0.61	-0.10
Neurons, total - ot	0.88	0.03
Neurons, total - cb	0.48	0.14
Nonneuronal cells, total - tl	0.72	0.07
Nonneuronal cells, total - di	0.76	-0.06
Nonneuronal cells, total - ot	0.80	0.05
Nonneuronal cells, total - cb	0.51	0.13
Structure mass (mg) - tl	***0.08***	0.33
Structure mass (mg) - di	0.96	0.01
Structure mass (mg) - ot	0.10	0.32
Structure mass (mg) - cb	0.15	0.28

Significance was settled at $p \leq 0.05$. Significant correlations are highlighted in bold and gray. Trends ($p < 0.10$) are highlighted in bold and italic. tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

Table 2. Individual sex correlated with body and brain measures in each cichlid species.

Geophagus brasiliensis	p	r
Standard length (cm)	0.29	0.39
Body mass (g)	0.15	0.52
Cell size - tl	***0.08***	***0.61***
Cell size - di	1.00	0.00
Cell size - ot	0.15	0.52
Cell size - cb	***0.08***	***0.61***
Neurons, density - tl	0.36	0.35
Neurons, density - di	0.66	-0.17
Neurons, density - ot	0.82	0.09
Neurons, density - cb	0.82	-0.09
Nonneuronal cells, density - tl	0.82	0.09
Nonneuronal cells, density - di	0.50	-0.26
Nonneuronal cells, density - ot	1.00	0.00
Nonneuronal cells, density - cb	0.82	-0.09
Neurons, total - tl	0.50	0.26
Neurons, total - di	1.00	0.00
Neurons, total - ot	0.15	0.52
Neurons, total - cb	0.66	0.17

Geophagus sveni	p	r
Standard length (cm)	0.69	-0.17
Body mass (g)	0.89	-0.06
Cell size - tl	0.69	0.17
Cell size - di	0.89	0.06
Cell size - ot	0.33	0.39
Cell size - cb	0.33	-0.39
Neurons, density - tl	0.69	-0.17
Neurons, density - di	0.20	-0.51
Neurons, density - ot	0.50	-0.28
Neurons, density - cb	0.69	-0.17
Nonneuronal cells, density - tl	0.50	-0.28
Nonneuronal cells, density - di	0.50	0.28
Nonneuronal cells, density - ot	0.20	-0.51
Nonneuronal cells, density - cb	0.89	-0.06
Neurons, total - tl	0.89	-0.06
Neurons, total - di	0.10	-0.62
Neurons, total - ot	0.33	-0.39
Neurons, total - cb	0.50	-0.28

Nonneuronal cells, total - tl	1.00	0.00
Nonneuronal cells, total - di	0.66	-0.17
Nonneuronal cells, total - ot	0.66	0.17
Nonneuronal cells, total - cb	0.36	0.35
Structure mass (mg) - tl	0.36	0.35
Structure mass (mg) - di	0.91	-0.04
Structure mass (mg) - ot	0.15	0.52
Structure mass (mg) - cb	0.66	0.17

Nonneuronal cells, total - tl	0.89	0.06
Nonneuronal cells, total - di	0.89	0.06
Nonneuronal cells, total - ot	0.33	-0.39
Nonneuronal cells, total - cb	0.89	0.06
Structure mass (mg) - tl	0.89	0.06
Structure mass (mg) - di	0.33	-0.39
Structure mass (mg) - ot	1.00	0.00
Structure mass (mg) - cb	0.20	0.51

Satanoperca pappaterra	**p**	**r**
Standard length (cm)	0.20	0.50
Body mass (g)	0.20	0.50
Cell size - tl	***0.09***	***-0.63***
Cell size - di	0.77	-0.13
Cell size - ot	0.36	-0.38
Cell size - cb	0.55	-0.25
Neurons, density - tl	0.55	-0.25
Neurons, density - di	1.00	0.00
Neurons, density - ot	0.77	-0.13
Neurons, density - cb	0.20	-0.50
Nonneuronal cells, density - tl	0.55	-0.25
Nonneuronal cells, density - di	0.55	-0.25
Nonneuronal cells, density - ot	0.20	-0.50
Nonneuronal cells, density - cb	0.36	-0.38
Neurons, total - tl	0.77	0.13
Neurons, total - di	0.77	0.13
Neurons, total - ot	1.00	0.00
Neurons, total - cb	0.55	0.25
Nonneuronal cells, total - tl	0.77	0.13
Nonneuronal cells, total - di	0.36	-0.38
Nonneuronal cells, total - ot	0.55	0.25
Nonneuronal cells, total - cb	0.55	0.25
Structure mass (mg) - tl	0.20	0.50
Structure mass (mg) - di	0.36	0.38
Structure mass (mg) - ot	0.20	0.50
Structure mass (mg) - cb	0.20	0.50

Apistogramma agassizii	**p**	**r**
Standard length (cm)	0.37	0.63
Body mass (g)	0.36	0.64
Cell size - tl	0.30	0.70
Cell size - di	0.92	0.08
Cell size - ot	0.32	-0.68
Cell size - cb	0.25	0.75
Neurons, density - tl	0.45	-0.55
Neurons, density - di	0.62	0.38
Neurons, density - ot	0.83	-0.17
Neurons, density - cb	0.11	-0.89
Nonneuronal cells, density - tl	0.64	-0.36
Nonneuronal cells, density - di	0.63	0.37
Nonneuronal cells, density - ot	0.57	0.43
Nonneuronal cells, density - cb	0.68	-0.32
Neurons, total - tl	0.68	-0.32
Neurons, total - di	0.96	-0.04
Neurons, total - ot	0.11	0.89
Neurons, total - cb	0.11	-0.89
Nonneuronal cells, total - tl	1.00	0.00
Nonneuronal cells, total - di	0.87	0.13
Nonneuronal cells, total - ot	0.39	0.61
Nonneuronal cells, total - cb	0.75	0.25
Structure mass (mg) - tl	0.22	0.78
Structure mass (mg) - di	0.11	-0.89
Structure mass (mg) - ot	0.40	0.60
Structure mass (mg) - cb	0.18	0.82

Significance was settled at $p \leq 0.05$. Significant correlations are highlighted in bold and gray. Trends ($p < 0.10$) are highlighted in bold and italic. tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

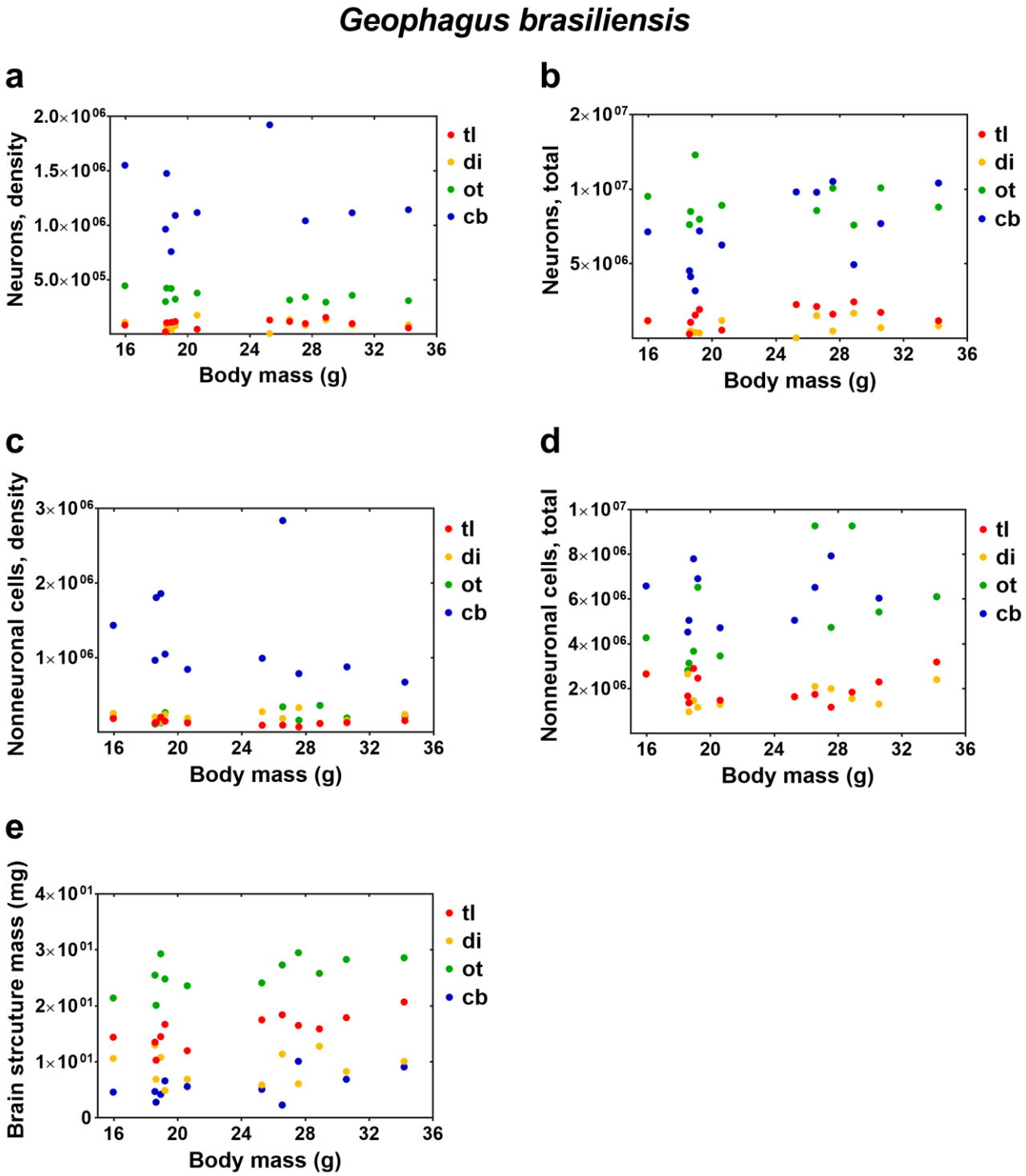

Figure 1. Variation in density and number of **(a,b)** neurons, **(c,d)** nonneuronal cells and **(e)** brain structure mass with individuals body mass in *Geophagus brasiliensis*. tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

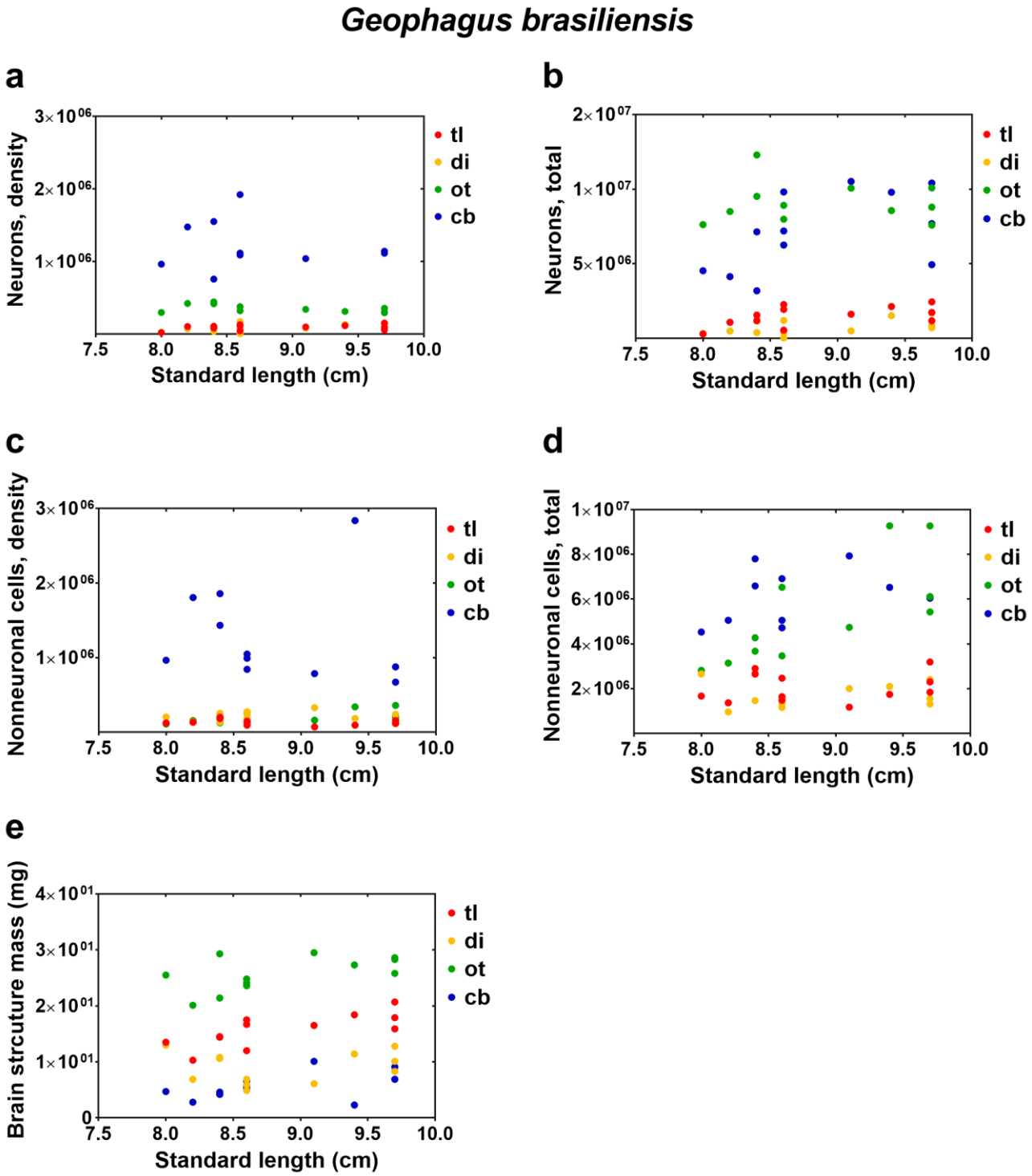

Figure 2. Variation in density and number of **(a,b)** neurons, **(c,d)** nonneuronal cells and **(e)** brain structure mass with individuals standard length in *Geophagus brasiliensis*. tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

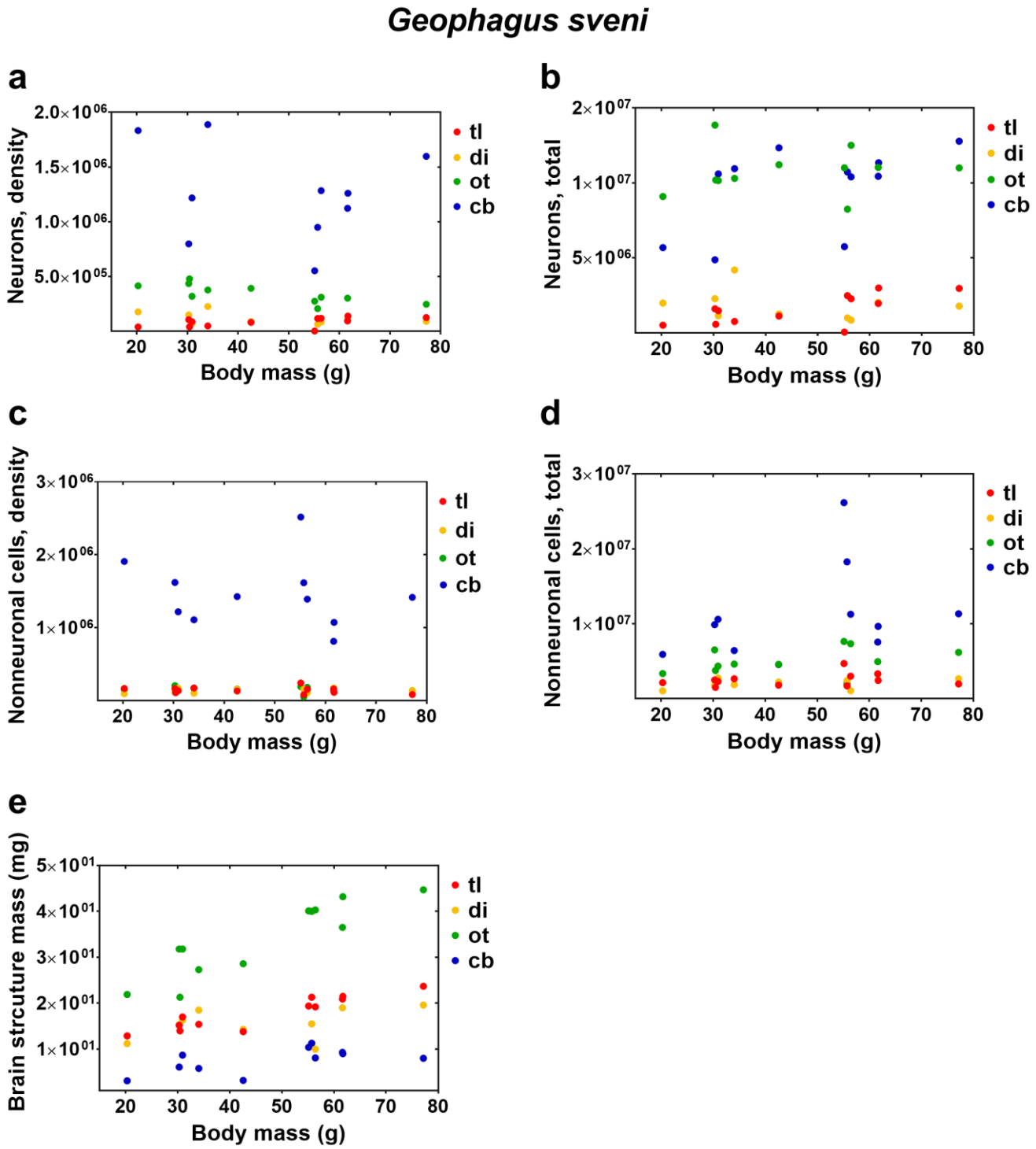

Figure 3. Variation in density and number of **(a,b)** neurons, **(c,d)** nonneuronal cells and **(e)** brain structure mass with individuals body mass in *Geophagus sveni*. tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

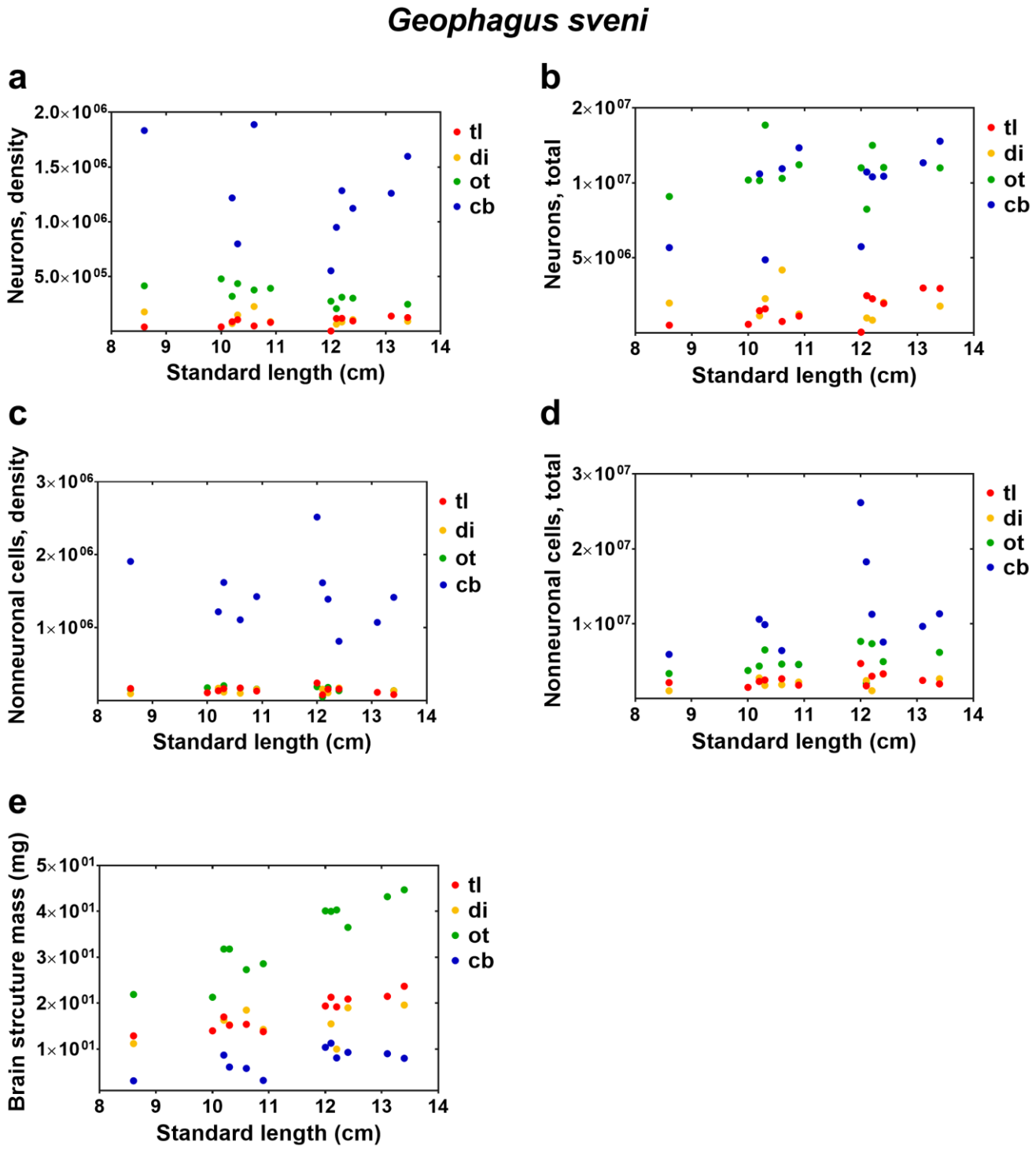

Figure 4. Variation in density and number of **(a,b)** neurons, **(c,d)** nonneuronal cells and **(e)** brain structure mass with individuals standard length in *Geophagus sveni*. tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

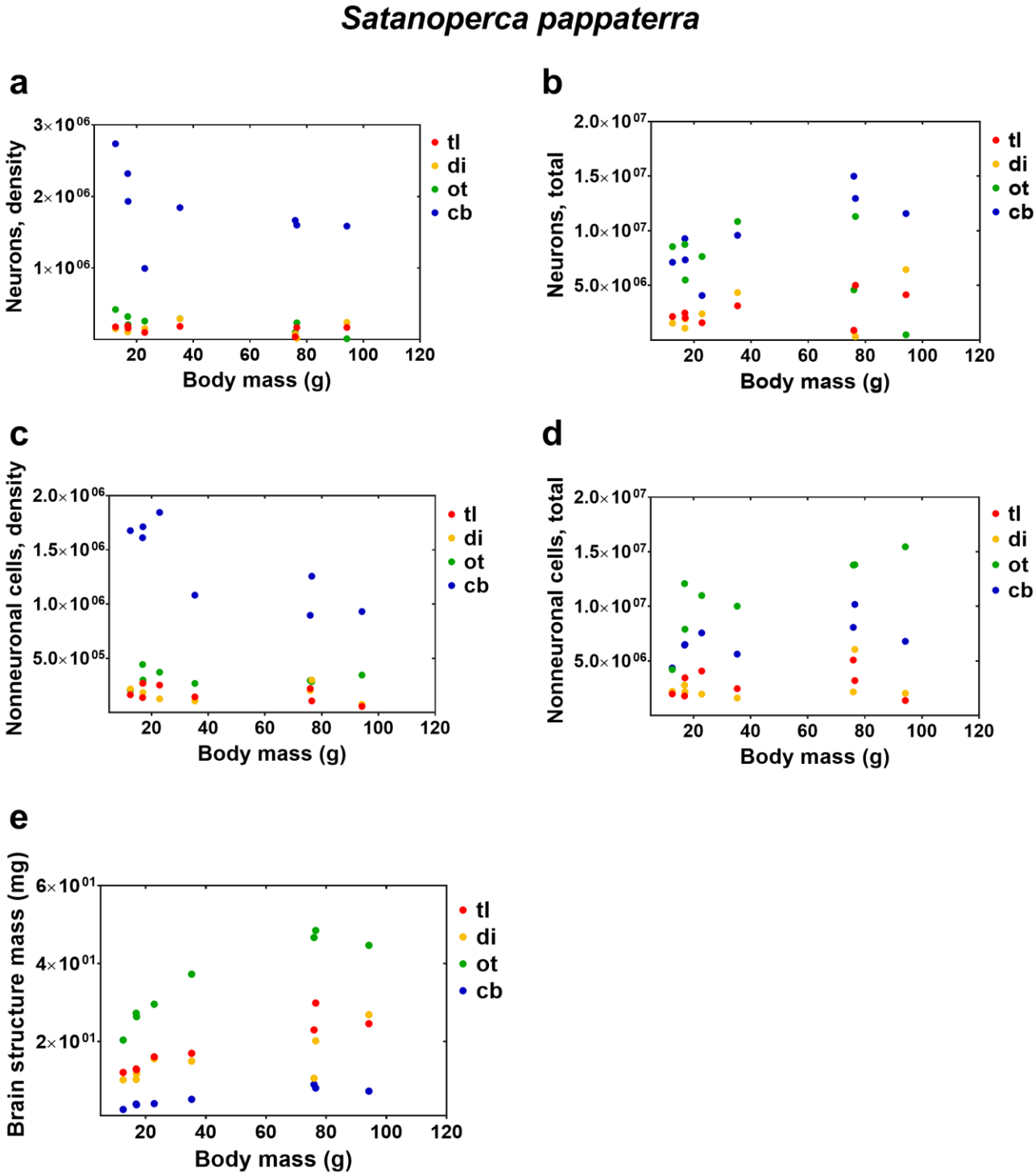

Figure 5. Variation in density and number of **(a,b)** neurons, **(c,d)** nonneuronal cells and **(e)** brain structure mass with individuals body mass in *Satanoperca pappaterra.* tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

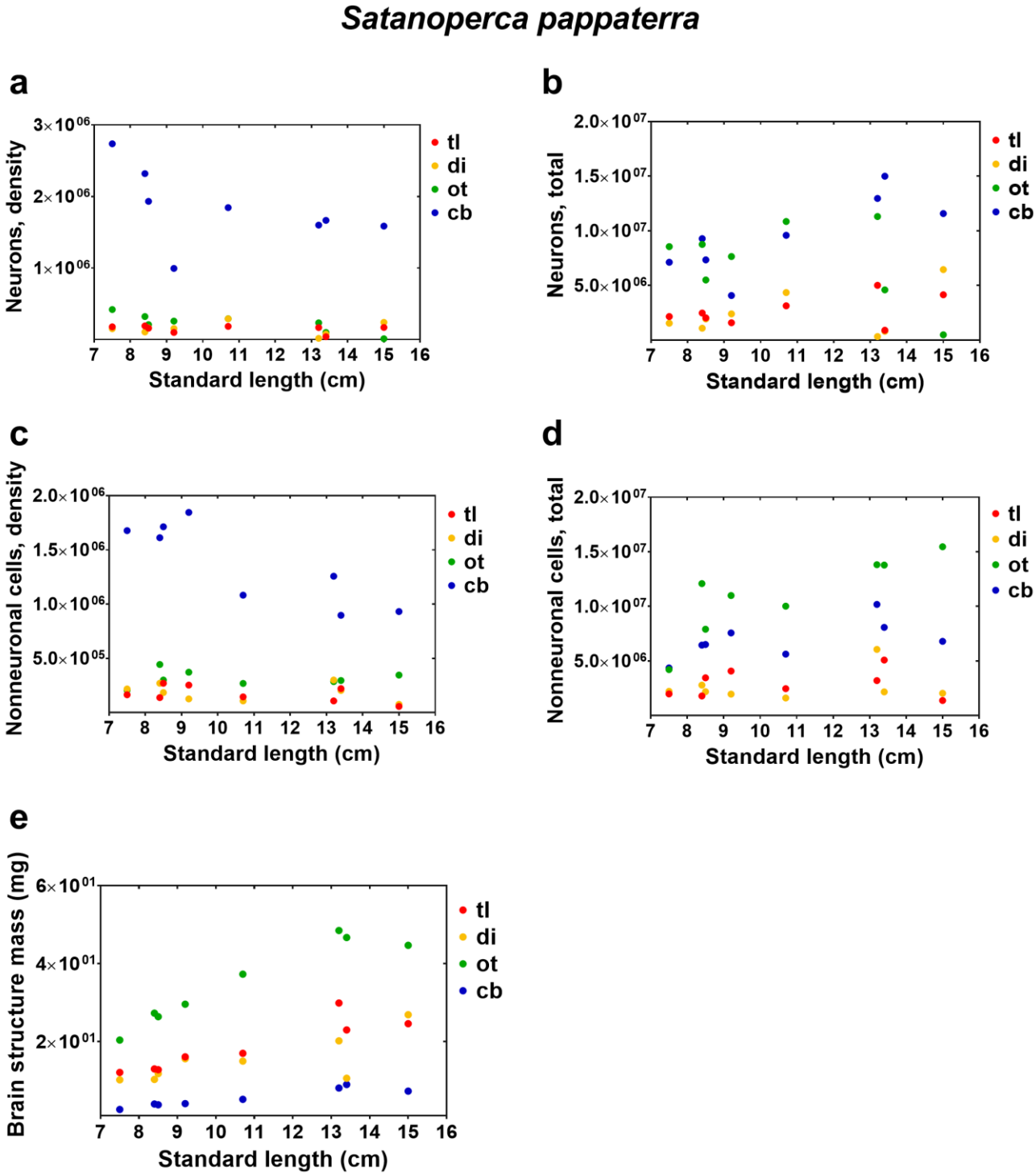

Figure 6. Variation in density and number of **(a,b)** neurons, **(c,d)** nonneuronal cells and **(e)** brain structure mass with individuals standard length in *Satanoperca pappaterra.* tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

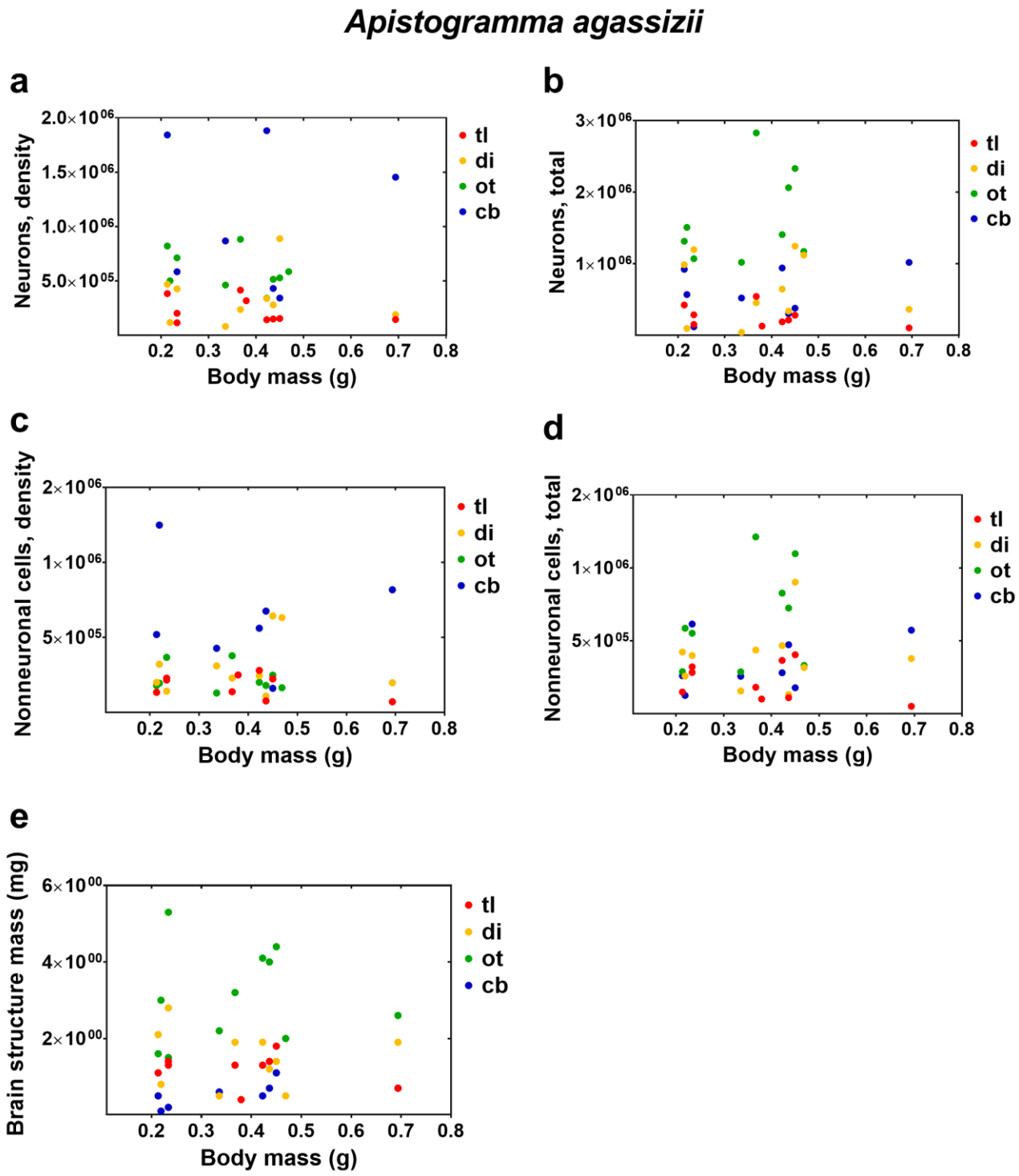

Figure 7. Variation in density and number of **(a,b)** neurons, **(c,d)** nonneuronal cells and **(e)** brain structure mass with individuals body mass in *Apistogramma agassizii.* tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

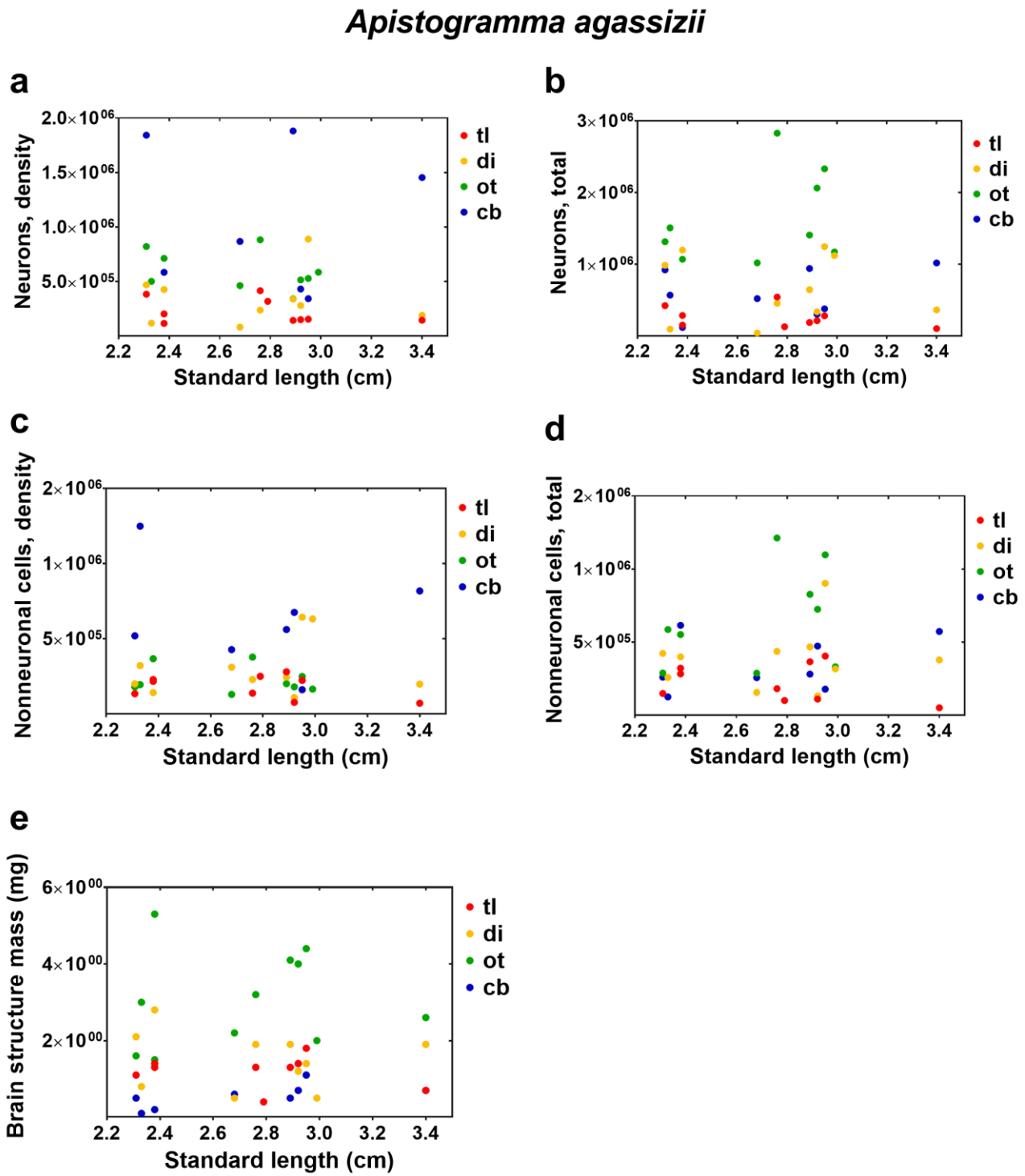

Figure 8. Variation in density and number of **(a,b)** neurons, **(c,d)** nonneuronal cells and **(e)** brain structure mass with individuals standard length in *Apistogramma agassizii.* tl: telencephalon, di: diencephalon; ot: optic tectum; cb: cerebellum.

3 PAPER 2. Neurons and glial cells are associated with parental care and mating systems in Neotropical cichlids

Manuela Lombardi Brandão[1], Eliane Gonçalves-de-Freitas[1,2] and Rui F. Oliveira[3,4,5].

[1]Departamento de Zoologia e Botânica, Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista (UNESP), Cristóvão Colombo, 2265, 15054-000, São José do Rio Preto, SP, Brazil.
[2]Centro de Aquicultura da UNESP, SP, Brazil.
[3]Instituto Gulbenkian de Ciência, Oeiras, Portugal.
[4]ISPA - Instituto Universitário, Lisbon, Portugal.
[3]Champalimaud Neuroscience Programme, Champalimaud Centre for the Unknown, Lisbon, Portugal.

ABSTRACT

The Social Brain Hypothesis (SBH) postulates that complex social interactions in animals could drive the evolution of brain's size, which has been corroborated by associations between social complexity and the volume of specific vertebrate brain structures. Volume, however, do not consider the variations neither in neurons, the core cells controlling behavior, nor in nonneuronal cells that supports neurons. Here, we used the isotropic fractionator method to reach the number and density of both, neuronal and nonneuronal brain cells in four Neotropical cichlid fish with different mating system and parental care, hypothesizing that more complex social species – i.e., monogamous substrate brooders – would present more brain cells in specific areas, thus supporting the SBH. We used four phylogenetically very closed species, combining monogamous and polygynous species with mouthbrooding and substrate brooding type of parental care. We found positive associations regarding social complexity and brain cells. The total number of neurons were higher for monogamous species in the optic tectum and cerebellum. The telencephalon and cerebellum had more nonneuronal cells in monogamous individuals. Mouthbrooders showed more neurons in the cerebellum and more nonneuronal cells in the telencephalon, optic tectum and also in the cerebellum. *A. agassizii,* a polygynous dwarf cichlid, showed the highest densities of neurons and nonneuronal cells. Interestingly, the density of nonneuronal cells in the *A. agassizii*'s cerebellum was not different from other cichlids but was the only brain region wherein the cells' diameter was bigger than the other fish species. Volume of brain structures did not show any relevant association with species, sex, reproductive behaviors or brain cells. Although not entirely agreeing with the SBH, our results suggest that social complexity represented by mating system and types of parental care are related to the number of brain cells. Nevertheless, this seems to follow a mosaic evolutionary fashion, with different brain structures being influenced by species' life history and other social and

ecological habits. This work brings, for the first time, interesting insights about the brain cells' composition in cichlids species with different social organizations, as mating system and the type of parental care, both considered as strong forces driving brain evolution.

Key words: social species; substrate brooding; mouthbrooding; monogamy; polygamy; *Geophagus brasiliensis*; *Geophagus sveni*; *Satanoperca pappaterra*; *Apistogramma agassizii*; isotropic fractionator.

Introduction

Complex animal behavior has been largely explained by the animal's brain complexity. The Social Brain Hypothesis (SBH), for instance, postulates that the more complex the social relations of a giving species, the bigger the brain of an individual belonging to that group, as well as its cognitive abilities (Dunbar 1998; Ashton et al. 2018). For two decades, this theory was the base of many studies comparing several vertebrate (MacLean et al. 2012; MacLean et al. 2014; Holekamp et al. 2015; Lucon-Xiccato et al. 2017) and invertebrate groups (Lihoreau et al. 2012), in order to find correlations between their social complexity with the evolution of enlarged brains.

The way species deal with sexual partners and take care of the offspring are considered a huge evolutionary driver in social groups. Several studies suggested that the mating systems guides the brain size evolution in which monogamous species – where a male and a female take care of the offspring together – will present larger relative brain sizes than polygamous ones – species with male or female-only parental care (Pitnick et al. 2006; Schillaci 2006; Dunbar 2009). These differences are also observed in particular brain structures. For instance, some studies showed that monogamous species of African cichlid fishes present some brain regions, such as the telencephalon, 15%-20% larger than polygynic species (Pollen et al. 2007); in mammals and birds, telencephalon is also enlarged in monogamous species (Dunbar and Shultz 2007); frogs show this correlation only in the olfactory bulbs, also larger in monogamous species then in polygynic ones (Zeng et al. 2016). Besides differences in brain size, these examples follow the mosaic brain evolution theory (Barton and Harvey 2000), in which mating systems and parental care types are associated with the complexity of a specific brain structure. Nevertheless, all of these studies point to an association between monogamous mating systems and the enlargement of some brain regions, suggesting that monogamy requires greater cognitive abilities and acuity (Pitnick et al. 2006; Schillaci 2006) than other types of mating systems.

Nevertheless, the brain size itself is not considered the whole answer for behavioral complexity anymore. There are several physiological processes linked to changes in individuals' social behavior that can influence the coordination of social responses in a group, from hormonal modulations altering the way individuals interact with conspecifics (Oliveira 2009; Huffman et al. 2012; Garcia et al. 2017; Munley et al. 2018) to changes in brain' immediate early genes, specific brain cells, receptors and circuits (Maruska et al. 2013; Fernald 2015; Garcia et al. 2017; Fernald

2017; Bludau et al. 2019). All these factors may, therefore, influence the structure in specific areas of the brain or even in the whole brain along the evolutionary process, as preconized by theories such as the mosaic evolution (Barton and Harvey 2000) and the concerted evolution (Finlay and Darlington 1995) of the brain. Thus, it is necessary to understand the brain not as a unique structure, but as a complex system formed by several regions with particular circuits and specializations. Today, more than look solely to the brain size relative to individual body mass, scientists must try to disentangle the anatomical particularities of specific brain regions related to a giving behavior (Barton and Harvey 2000; Herculano-Houzel et al. 2015; Kabadayi et al. 2018). For instance, by testing association between behavior and the variations in neurons – i.e., the core cells controlling behavior –, and nonneuronal cells that supports neurons. Thus, we tested the association between the number and density of both neuronal and nonneuronal brain cells in cichlid fishes with different mating system and parental care, hypothesizing that more complex social species – i.e., monogamous substrate brooders – would present more brain cells.

Cichlid fish is a group wherein we found several types of parental care, mating systems and a huge social complexity. The way cichlid parents take care of the fry may also influence the way they coordinate their behaviors in this phase. Species that take care of the offspring on the substrate are considered to had evolved in environments with a reduced predation pressure (Dupuis and Keenleyside 1982). Substrate guarding is considered to be the ancestral state of parental care, and mouthbrooding – in which the fry is guarded in a parents (or both) mouth – is derived from this type of parental care (Oppenheimer 1970; Dupuis and Keenleyside 1982; Goodwin et al. 1998). There are also species that take care of the eggs on the substrate until they hatch, and the fry can be taken in a parent's mouth as a temporary refuge if a predator approaches (Hanon 1975; Dupuis and Keenleyside 1982). This is considered the most derived type of parental care (Timms and Keenleyside 1975). According to the Stepping-stone Model, parental care evolved from a "no care" of the offspring to a biparental monogamous care via two stages considered the intermediate ones, one with a male-only care in and the other with a female-only type of care (Gross and Sargent 1985; Balshine and Sloman 2011) in polygamous species. Therefore, we can speculate that different types of parental care and mating systems have been selected differently for brain structures, receptors and circuits related to these behaviors.

Here we tested four different Neotropical cichlid species with different parental care types and mating systems. *Geophagus brasiliensis* is a monogamous species in which both parents take care of the offspring (i.e., biparental care) in the substrate. *Geophagus sveni* is also a monogamous species with biparental care, in which either male or female may eventually take the fry raised on the substrate on their mouth if a predator appears (i.e., facultative mouthbrooders). *Satanoperca pappaterra* is a polygynous species in which female take care of the offspring on its mouth, while males, after reproduction, leave to fertilize new females. *Apistogramma agassizii* is also a polygynous species, were females take care alone of the

offspring in the substrate. In this study, we hypothesized that species considered more derived from a mating system and parental care point of view (e.g., monogamous species with biparental care of the fry) would be the ones with the higher number of brain cells, particularly neurons, as stated by the Social Intelligence hypotheses. We also looked at the relative size of particular brain structures to observe whether there are clear correlations between brain cells and brain volumes, as stated by several studies concerning these social arrangements.

Methods

Fish housing

In this study, we used 6 male and 6 female individuals of each species (Fig. 1), except in *S. pappaterra*, where we have only 2 females. The species used in here were collected in natural rivers in Brazil: *G. brasiliensis* and *S. pappaterra* were collected in the Rio Grande river; *G. sveni* were collected in the Tietê river; and *A. agassizii* were collected in the Amazon river. Rio Grande e Tietê are rivers from the southeast Brazil included in the Paraná river basin, while Amazon river is located in the north of Brazil, and it is part of the Amazon river basin. Fish were transported and kept in the laboratory in polyethylene water tanks (ca. 500 L, 1 fish/10 L) in a regime of 27 ºC and light from 7AM to 7PM. They were fed once a day to apparent satiation with commercial food for tropical fish (28% protein). Water quality was maintained by using biological filters (400 L/h) and constant aeration until subjects' sacrifice for brain collection.

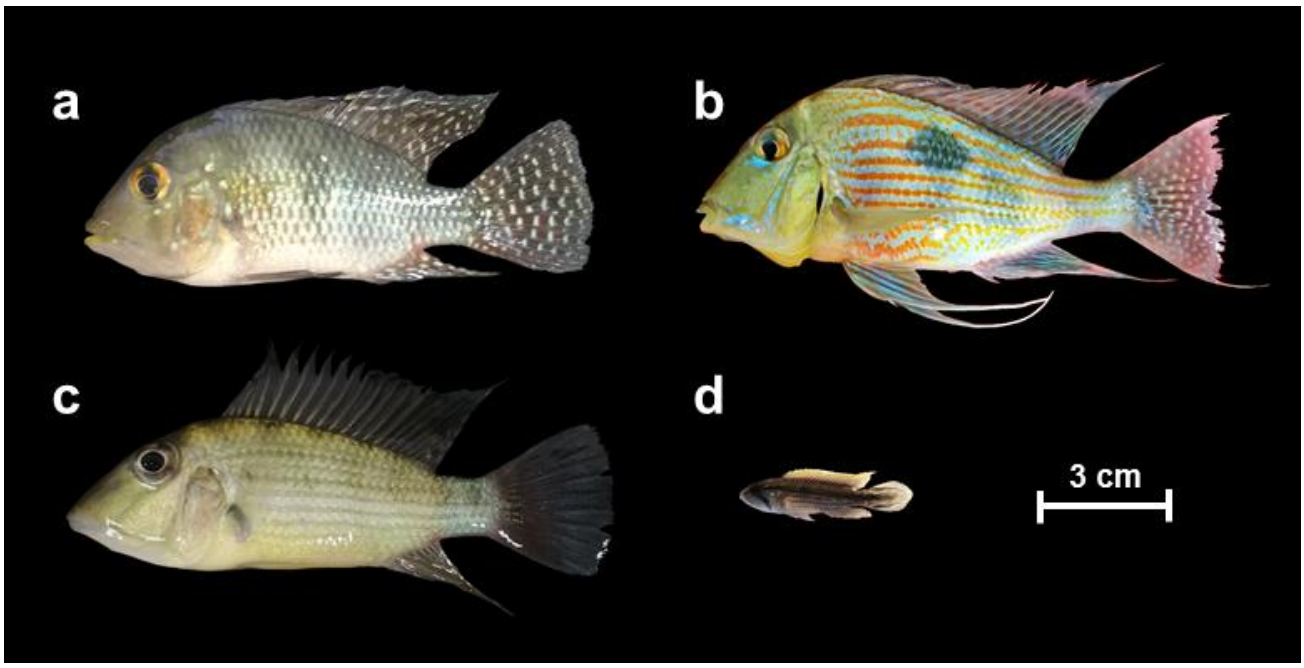

Figure 1. The four Neotropical cichlids used in this study, **a.** *Geophagus brasiliensis,* a monogamous species with biparental care of the offspring in the substrate; **b.** *Geophagus sveni,* a monogamous species with biparental care, and facultative mouthbrooding; **c.** *Satanoperca pappaterra* a polygynous species with female mouthbrooding; **d.** the dwarf cichlid *Apistogramma agassizii*, a polygynous species with female care of the offspring in the substrate. Photos from the author M.L. Brandão.

Brain collection

Fish were killed with benzocaine (0.18 $g.L^{-1}$) fallowed by skull removal and brain collection. The brain was photographed, cleaned to remove blood vessels, and immediately dissected into major structures: the telencephalon, diencephalon, optic tectum and cerebellum. After that, we weighted and fixed individually each structure in 4% paraformaldehyde for 24 hours, followed by transferring to a sucrose solution during overnight (4 °C) and storing in an antifreeze solution (-20 °C) until processing. Olfactory bulb and brainstem were also collected, but were not analyzed in this work.

Isotropic fractionator

This step followed the protocol already used for several vertebrates, which consist in dissociate the brain structure in free nuclei of cells (Herculano-Houzel 2011). The structure was removed from the antifreeze solution and placed in a glass homogenizer containing 1 ml of buffer-detergent solution (1% Triton™ X-100 and 40 mM sodium citrate). After that, it is gently smashed with a pestle, using rotatory and up and down movements until no more cell' clustering is seen in the glass homogenizer with the full pestle inserted. With this, highly anisotropic tissue is transformed in a nuclei solution (Valério-Gomes et al. 2018). In the fish species used in here, the

dissociation took about 15 minutes in the softer structures (i.e., telencephalon, diencephalon and cerebellum) and up to 1 hour in the optic tectum.

After tissue dissociation, we transferred the 1ml solution of free nuclei to 1.5 ml Eppendorf and added 1mg/ml fluorescent nuclear stain DAPI (4', 6-diamidino- 2-phenylindole, a dye with high affiliation to DNA) to afford us to count for the total free nuclei in the sample. After DAPI adding, the Eppendorf tube was inverted a few times to homogenized the sample. Then, four aliquots of 10 µl of the same sample were collected and placed in Neubauer chambers for counting DAPI stained nuclei. We counted 10 central squares of the Neubauer chamber in this study, for all the samples. The formula used to achieve the total number of free nuclei is the mean number of nuclei counted in the four aliquots multiplied by the volume expected in the 10 central squares in the Neubauer chamber multiplied, one more time, by the volume in ml of the initial sample (1 ml of solution with free nuclei).

For the neuronal and nonneuronal cells quantification, we collected a new aliquot of 500 µl from the original sample to realize an immunocytochemical protocol. In this phase, the original protocol suffered a few adjustments, after previous experiments, which are discriminated in here. The spare volume was stored in 4 °C for future use, whether necessary. The aliquots were centrifuged, the supernatants were discarded and the pellets were washed twice in 0.1 M PBS (8000 rpm at 4 °C during 5 minutes, each wash). After that, the aliquots were incubated at 70 °C for one hour in 0.2 M boric acid, being washed again twice with 0.1 M PBS. Then, we used a blocking step (100 µl of 1% Albumin Bovine Fraction V – BSA for each sample in a covered plate under agitation for 30 minutes) in the protocol to reduce fluorescence due to nonspecific antibody binding, which was observed in previous analysis. In the end of the 30 minutes, the samples were centrifuged, the supernatants were discarded and the samples were incubated overnight (350 rpm in a covered plate at 4 °C) with 100 µl of the anti-NeuN primary antibody diluted in 1% BSA at a 1:100 concentration.

Next day, the samples were centrifuged, the supernatants were discarded and the pellets were washed twice in 0.1 M PBS (8000 rpm at 4 °C during 5 minutes, each wash). The samples were suspended in final volumes of 50-400 µl depending on the pellet size (there is a substantial loss of free nuclei during the immunocytochemical step). After that, we placed a new aliquot of this sample in the Neubauer chamber and counted the percentage of DAPI-labeled nuclei that was also labeled with the antibody. We have to count a minimum of 500 DAPI-labeled to achieve statistical power. This count was done by using a macro developed for the free software Fiji. Besides the cells counting, this macro also provided us a feret diameter of the component cells in each brain structure, although without discrimination between neuronal and nonneuronal cells. The formula to determine the total number of neurons (the NeuN-positive nuclei) is the percentage of NeuN-positive nuclei multiplied by the total number of nuclei, with the result divided by 100. The nonneuronal cells number was achieved by the total number of cells minus the total number of neurons.

Volume measurements

As several works had correlate brain volumes with behavioral plasticity and cognitive process, here we wanted to look at this variable also to observe whether there is a pattern that occurs both in the number of brain cells and in the volume of a giving structure. Nevertheless, we had collect the volumes of just the three bigger species, *G. brasiliensis*, *G. sveni* and *S. pappaterra*. This was done after skull dissection, placing the brain in a Petri dish and taking digital images of its dorsal, lateral (left side) and ventral sides (Fig. 2) using a dissection microscope (Opton TIM-2T) and a digital camera (Opton TA-0124-B and TCapture software). The methodology used for the measurements were based on the ellipsoid model already used by Pollen et al. (2007) and Gonzalez-Voyer & Kolm (2010) to measure brain volume in other cichlid species: $S_v = (L \times W \times H)\ \pi/6$. To obtain the structure volume (S_v), each structure's length (L), width (W) and height (H) were measured (Fig. 2) exactly as in Pollen et al. (2007) using ImageJ software (v.1.50i, National Institutes of Health, USA). For two-hemisphere structures, we just doubled the structure's final volume.

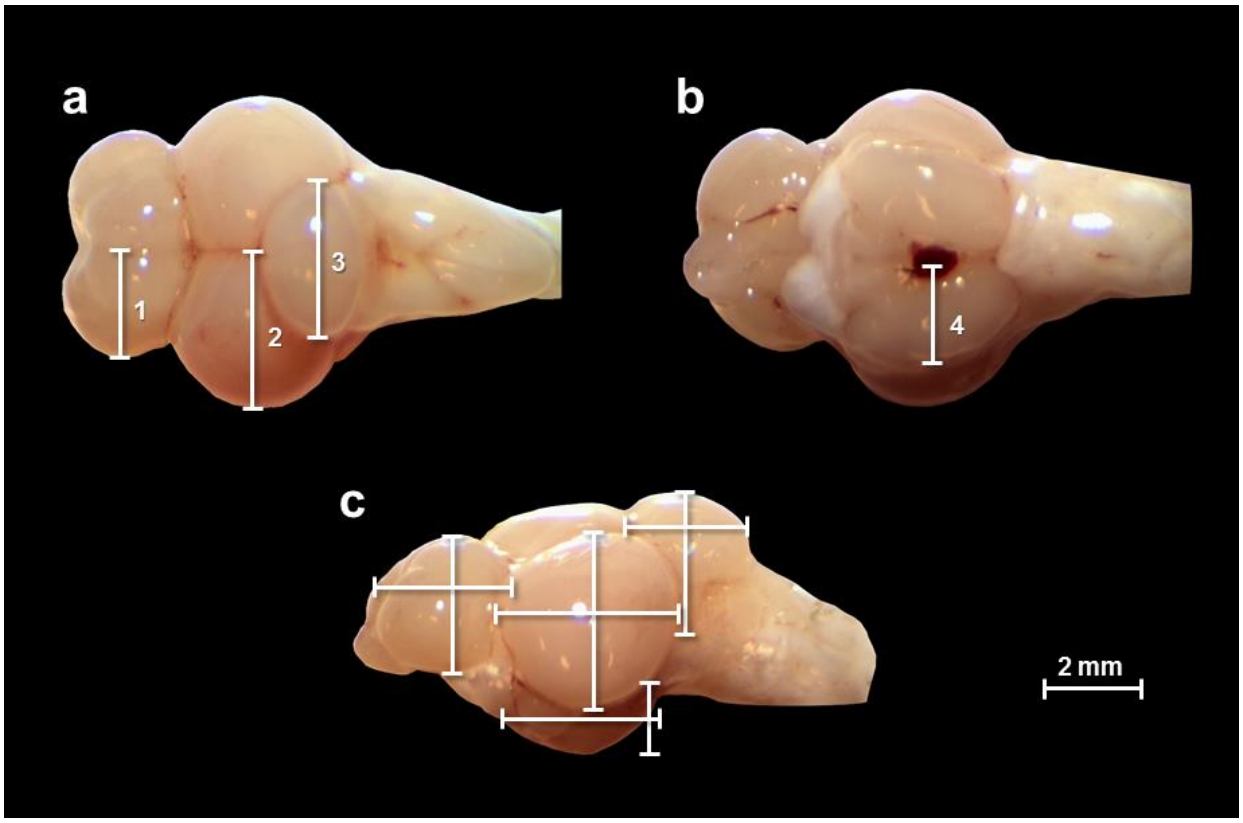

Figure 2. Measurements made from the **a.** dorsal (length), **b.** ventral (length), and **c.** lateral (width and height) sides of the brain to determine the structures' volumes: 1. telencephalon; 2. optic tectum; 3. cerebellum; 4. diencephalon. The brain reference used in here is from *G. brasiliensis*. Photos from the author M.L. Brandão.

Statistical analysis

Data analysis was done by using the free software R, version 3.5.1 (http://www.r-project.org). Normality and homoscedasticity were tested using Kolmogorov-Smirnov and Fmax test (Lehner 1998), respectively. Since data fitted normality, but was heterogeneous, we used a square-root transformation. Number and densities of neurons and nonneuronal cells, as well as feret diameters and structure volumes of the brain cells, were used as response variables in our models. Data were then analyzed with Linear Mixed-Models (LMMs, using sex, species, parental care type and mating system as predictors and brain structures as random effect – "lmer" function of the "lme4" package (Bates et al. 2015) and Linear models completed with Tukey HSD post-hoc test ["glht" function of the "multcomp" package (Hothorn et al. 2008)] for comparing between the four species. Finally, we compared the volume of the brain structures with neurons and nonneuronal cells using Pearson's correlation test adjusted with Bonferroni's ["psych" package (Revelle 2018)]. Statistical significance was set at $p \leq 0.05$. However, we considered a marginal trend towards significance when $p < 0.10$, based on Pollen et al. (2007) for Pearson's correlations.

Ethical statement

We conducted this study according to the ethical principles on animal experimentation adopted by the National Council for the Control of Animal Experimentation (CONCEA – Brazil). It was approved by the Committee on Ethics in Animal Use, UNESP, São José do Rio Preto, permit number 156/2016. This project was also registered in the Authorization and Information System in Biodiversity (SISBIO-ICMBio, Brazil, permit number 54287-1), which allowed the collection of biological material in natural environments for research purposes.

Results

As the four species investigated in our study was represented by individuals with different body sizes (e.g., *Apistogramma agassizii*, a dwarf cichlid), we used two different approaches to analyze our data instead of total number of brain cells. In the first one, number of brain cells in each structure was divided by the weight (mg) of the respective brain structure, affording us to have the density of neuronal and nonneuronal cells in each one of the four brain regions collected. This density was then divided by the individual standard length (SL), measured from the individual snout until the caudal peduncle in centimeters. The second approach consisted of dividing the total number of brain cells by the fish SL. The both approaches are separated in this section with their respective results. As there was no significant difference between the sexes in neither of the different approaches, as mentioned below, we compared each structure between species putting males and females' data together. For last, we compared the brain cells feret diameters (µm) in each brain area between species, without discrimination of neuronal and nonneuronal cells.

Density of brain cells

Here, we compared neurons and nonneuronal cells densities with individual sex, species, parental care type and mating system.

Linear mixed models using species, sexes, parental care and mating system as predictors

Neuronal cells density per fish size

There was no significant difference between males and females concerning the interaction with brain structures ($\chi^2 = 0.48$, $p = 0.48$), but there was a significant difference in the interaction between species and structures ($\chi^2 = 263.72$, $p < 0.0001$). Using Tukey post-hoc test, we observed that the difference in neurons density was between *A. agassizii* and the other three cichlid species, *S. pappaterra* ($p < 0.0001$), *G. brasiliensis* ($p < 0.0001$) and *G. sveni* ($p < 0.0001$), with *A. agassizii* presenting the higher density of neurons in the brain.

Nonneuronal cells density per fish size

There was no significant difference between males and females concerning the interaction with the structures ($\chi^2 = 0.94$, $p = 0.33$), but there was a significant difference in the interaction between species and structures ($\chi^2 = 162.02$, $p < 0.0001$), such as the one say for neuronal density. Using Tukey post-hoc test, here the differences now appeared between the dwarf cichlid, *A. agassizii* and the other three cichlids, *G. brasiliensis* ($p < 0.001$), *G. sveni* ($p < 0.001$) and *S. pappaterra* ($p < 0.001$), with *A. agassizii* again presenting the highest densities among the tested cichlids.

Mating system and parental care

Regarding density of cells, there was a difference between neurons in mouthbrooders and substrate brooders individuals ($\chi^2 = 32.36$, $p < 0.0001$), as well as between monogamous and polygynous species ($\chi^2 = 57.85$, $p < 0.0001$). These differences persisted when looking at the density of nonneuronal cells (mouthbrooders × substrate brooders: $\chi^2 = 28.21$, $p < 0.0001$; monogamous × polygynous: $\chi^2 = 47.99$, $p < 0.0001$).

Analyzing each structure between species

Neuronal cells density per fish size

There was significant differences between species in all the four structures investigated. *Apistogramma agassizii*, the dwarf cichlid, presented the highest densities of neurons in all the four brain structures investigated when compared with the other three species (table 1; Fig. 3). There was no statistical difference between *G. brasiliensis, G. sveni* and *S. pappaterra* concerning density of neurons within the four brain structures.

Table 1: Linear models completed with Tukey's test showing the differences in neurons and nonneuronal cells density between *Apistogramma agassizii* and the other three cichlids used in this study.

Neurons		
Anova by brain structure	**Species compared to *A. agassizi***	**p**
tl: $F_{(3,37)} = 44.03$; $p < 0.0001$	*G. brasiliensis*	$p < 0.001$
	G. sveni	$p < 0.001$
	S. pappaterra	$p < 0.001$
di: $F_{(3,34)} = 29.25$; $p < 0.0001$	*G. brasiliensis*	$p < 0.001$
	G. sveni	$p < 0.001$
	S. pappaterra	$p < 0.001$
ot: $F_{(3,34)} = 69.93$; $p < 0.0001$	*G. brasiliensis*	$p < 0.001$
	G. sveni	$p < 0.001$
	S. pappaterra	$p < 0.001$
cb: $F_{(3,29)} = 7.99$; $p < 0.0005$	*G. brasiliensis*	$p < 0.002$
	G. sveni	$p < 0.001$
	S. pappaterra	$p < 0.03$

Nonneuronal cells		
Anova by brain structure	**Species compared to *A. agassizi***	**p**
tl: $F_{(3,37)} = 30.56$; $p < 0.0001$	*G. brasiliensis*	$p < 0.001$
	G. sveni	$p < 0.001$
	S. pappaterra	$p < 0.001$
di: $F_{(3,34)} = 34.24$; $p < 0.0001$	*G. brasiliensis*	$p < 0.001$
	G. sveni	$p < 0.001$
	S. pappaterra	$p < 0.001$
ot: $F_{(3,34)} = 40.43$; $p < 0.0001$	*G. brasiliensis*	$p < 0.001$
	G. sveni	$p < 0.001$
	S. pappaterra	$p < 0.001$

Nonneuronal cells density

There was significant differences between species in all the four structures investigated also in nonneuronal cells. As for density of neurons, *A. agassizii* presented the highest densities of nonneuronal cells in the telencephalon, diencephalon and optic tectum when compared with *G. brasiliensis, G. sveni* and *S. pappaterra* (table 1; Fig. 4). In the optic tectum, there was also a difference between the mouthbrooder species *G. sveni* and *S. pappaterra,* in which the last one presented higher density of nonneuronal cells ($p = 0.02$; Fig 4f). The cerebellum did not show differences in nonneuronal densities between the four species.

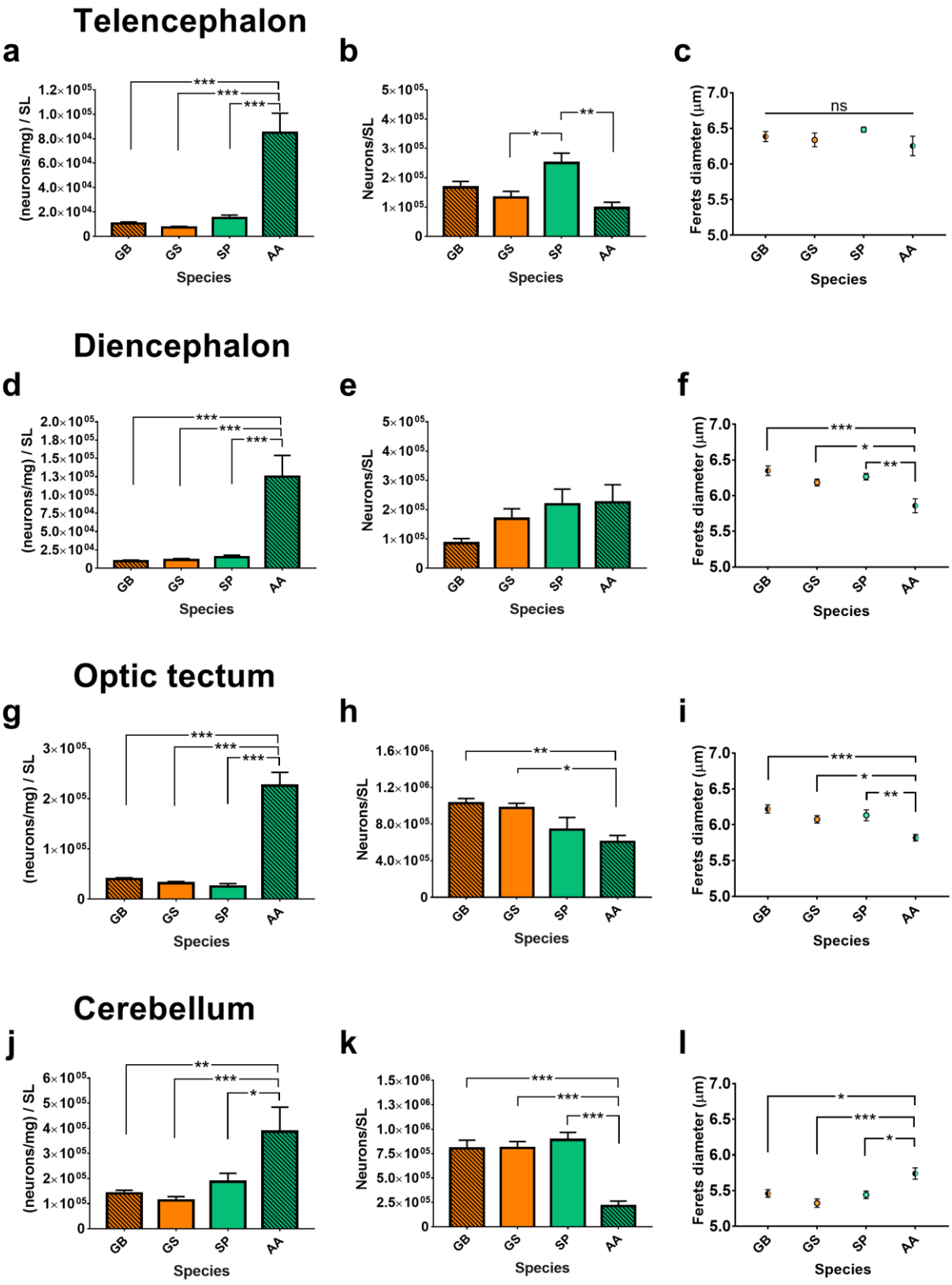

Figure 3. Neurons densities (**a, d, g, h**) and number of neurons standardized by fish standard length (**b, e, h, k**), and ferets diameters of brain cells (**c, f, i, l**) compared between the four species (Linear regression completed by Tukey's test). Orange bars and circles represent monogamous species (*Geophagus brasiliensis* and *Geophagus sveni*); green bars and circles represent polygynous species *(Satanoperca pappaterra* and *Apistogramma agassizii).* Dashed lines show species that are substrate brooders (*G. brasiliensis* and *A. agassizii*) while the absence of dashed lines represent mouthbrooder (*G. sveni* and *S. pappaterra*) type of parental care. Data are represented as mean ± SEM.

Mating system and parental care types

Comparing brain structures grouped in monogamous and polygynous species, we can observe that polygynous individuals presented a significant higher density of neurons in all the four brain structures (all of them with $p < 0.003$, table 2), which is contrary to our predictions. For the nonneuronal cells, these differences were maintained for the telencephalon, diencephalon and optic tectum ($p < 0.001$), except for cerebellum, that showed no differences between the mating systems (table 2).

Table 2. Linear Models completed by Anova test between the four cichlid species concerning density of neurons and nonneuronal cells in the respective brain structures.

	Mating system				Parental care			
	Neurons		Nonneuronal cells		Neurons		Nonneuronal cells	
tl	$F_{(1,39)} = 27.47$	$p < 0.0001$	$F_{(1,39)} = 19.47$	$p < 0.0001$	$F_{(1,39)} = 11.03$	$p = 0.002$	$F_{(1,39)} = 9.88$	$p = 0.003$
di	$F_{(1,36)} = 13.53$	$p = 0.0007$	$F_{(1,36)} = 17.68$	$p = 0.0003$	$F_{(1,36)} = 5.86$	$p = 0.02$	$F_{(1,36)} = 16.3$	$p = 0.0003$
ot	$F_{(1,36)} = 10$	$p = 0.003$	$F_{(1,36)} = 32.01$	$p < 0.0001$	$F_{(1,36)} = 18.02$	$p = 0.0001$	$F_{(1,36)} = 10.12$	$p = 0.003$
cb	$F_{(1,31)} = 11.82$	$p = 0.001$	$F_{(1,31)} = 2.06$	$p = 0.16$	$F_{(1,31)} = 3.39$	$p = 0.07$	$F_{(1,31)} = 0.19$	$p = 0.66$

Concerning type of parental care, substrate brooders presented higher density of neuronal cells in the telencephalon ($p = 0.002$), diencephalon ($p = 0.02$) and optic tectum ($p < 0.001$) when compared to mouthbrooders (table 2). No difference was observed for cerebellar neurons. Nonneuronal cells densities was also different between parental care types in the telencephalon ($p = 0.003$), diencephalon ($p = 0.0003$) and optic tectum ($p = 0.003$), where substrate brooders presented a higher density of cells (table2). As in the neuronal cells, cerebellum did not show differences between substrate brooders and mouthbrooders. Both results regarding mating system and parental care seem to show a strong bias due to *A. agassizii*'s data and will be better discussed in the following section.

Brain cells standardized by fish's size

Linear models using species, sexes and structure identification as predictors

Neuronal cells per fish size

Concerning total number of neurons divided by fish standard length, there was no significant difference between males and females concerning the interaction with brain structures ($\chi^2 = 0.04$, $p = 0.83$), but there was a significant difference in the interaction between species and structures ($\chi^2 = 15.66$, $p = 0.001$). Using Tukey post-hoc test, we observed that the difference in neurons density was between *A. agassizii* and the other three cichlid species, *S. pappaterra* ($p = 0.003$), *G. brasiliensis* ($p = 0.01$) and *G. sveni* ($p = 0.004$), but now with *A. agassizii* presenting the lowest numbers of neurons when compared with the other three species.

Nonneuronal cells per fish size

There was no significant difference between males and females concerning the interaction with the structures ($\chi^2 = 0.24$, $p = 0.62$), but there was a significant difference in the interaction between species and structures ($\chi^2 = 104.16$, $p < 0.0001$). Using Tukey post-hoc test, here the differences now appeared between the dwarf cichlid, *A. agassizii* and the other three cichlids, *G. brasiliensis* ($p < 0.001$), *G. sveni* ($p < 0.001$) and *S. pappaterra* ($p < 0.001$), and also between *S. pappaterra* and *G. sveni* ($p = 0.05$).

Mating system and parental care

Using as dependent variables the total number of neurons and nonneuronal cells, some previous significances observed for density of brain cells persisted. Mouthbrooders were still different from substrate brooders concerning neurons number ($\chi^2 = 6.55$, $p = 0.01$). In monogamous and polygynous individuals this was now just a trend ($\chi^2 = 3.16$, $p = 0.07$). For nonneuronal cells, there was significant differences both in mating system (monogamous × polygynous: $\chi^2 = 10.01$, $p = 0.001$) and parental care (mouthbrooders × substrate brooders: $\chi^2 = 30.28$, $p < 0.0001$).

Analyzing each structure between species

Neuronal cells per fish standard length

There was significant differences between species in all the four structures investigated. Telencephalon presented differences ($F_{(3,37)} = 5.08$, $p = 0.005$) between *S. pappaterra* and *G. sveni* ($p = 0.02$) and *S. pappaterra* and *A. agassizii* ($p = 0.004$; Fig. 3b). In the optic tectum ($F_{(3.34)} = 5.86$, $p = 0.002$; Fig. 3h), *G. brasiliensis* ($p = 0.009$) and *G. sveni* ($p = 0.01$) showed the highest number of neurons when compared to *A. agassizii,* and with *S. pappaterra* concerning *G. brasiliensis* ($p = 0.05$). For last, *A. agassizii* presented the lowest number of cerebellar cells ($F_{(3.29)} = 17.71$, $p < 0.0001$) when compared to *G. brasiliensis, G. sveni* and *S. pappaterra,* all of them with $p < 0.001$ (Fig. 3k). There was no statistical difference concerning the diencephalon.

Nonneuronal cells per fish standard length

In the telencephalon, *A. agassizii* is the species with the lower number of nonneuronal cells ($F_{(3.37)} = 14.34$; $p < 0.0001$; Fig. 4b) when compared to *G. brasiliensis*, *G. sveni* and *S. pappaterra* ($p < 0.0001$ for the three species). The diencephalon presented a difference only between the two polygynous species ($F_{(3.34)} = 2.76$; $p = 0.05$; Fig. 4d), with *S. pappaterra* presenting more cells the *A. agassizii* ($p = 0.03$). *S. pappaterra* has also more nonneuronal cells in the optic tectum than all the other three species ($F_{(3.34)} = 28.82$; $p < 0.0001$), *G. brasiliensis*, *G. sveni* and *A. agassizii,* with a $p < 0.001$ (Fig. 4f). *G. brasiliensis* and *G. sveni* also presented more optic tectum nonneuronal cells than *A. agassizii* ($p < 0.001$ and $p = 0.002$, respectively). In the cerebellum, all the three species, *G. brasiliensis*, *G. sveni* and *S. pappaterra* presented more nonneuronal cells ($F_{(3.29)} = 28.18$; $p < 0.001$) than *A. agassizii* ($p < 0.001$ for all, Fig. 4h).

Mating system and parental care types

Geophagus species presenting monogamy did not show differences with polygynous individuals when looking at the telencephalon. Nevertheless, *G. brasiliensis* and *G. sveni* presented less neurons in the diencephalon ($p = 0.04$), but more of these cells in the optic tectum ($p < 0.001$) and cerebellum ($p = 0.03$) when compared to *S. pappaterra* and *A. agassizii* (table 3). Nonneuronal cells were significantly different between mating system in the telencephalon ($p = 0.03$) and in the cerebellum ($p = 0.0003$), with a higher number of these cells being presented by monogamous species, but with no difference in the diencephalon an optic tectum (table 3).

Table 3: Linear Models completed by Anova test between the four cichlid species concerning number of neurons and nonneuronal cells in the respective brain structures.

	Mating system				**Parental care**			
	Neurons		**Nonneuronal cells**		**Neurons**		**Nonneuronal cells**	
tl	$F_{(1,39)} = 0.21$	$p = 0.65$	$F_{(1,39)} = 4.66$	$p = 0.03$	$F_{(1,39)} = 1.52$	$p = 0.22$	$F_{(1,39)} = 8.05$	$p = 0.007$
di	$F_{(1,36)} = 4.51$	$p = 0.04$	$F_{(1,36)} = 0.13$	$p = 0.72$	$F_{(1,36)} = 1.99$	$p = 0.16$	$F_{(1,36)} = 2.53$	$p = 0.12$
ot	$F_{(1,36)} = 18.08$	$p = 0.0001$	$F_{(1,36)} = 0.0002$	$p = 0.99$	$F_{(1,36)} = 0.07$	$p = 0.79$	$F_{(1,36)} = 10.19$	$p = 0.003$
cb	$F_{(1,31)} = 5.03$	$p = 0.03$	$F_{(1,31)} = 16.47$	$p = 0.0003$	$F_{(1,31)} = 6.97$	$p = 0.01$	$F_{(1,31)} = 8.05$	$p = 0.007$

Comparing parental care's types, mouthbrooders as *G. sveni* and *S. pappaterra* presented more cerebellar cells ($p = 0.02$) than substrate brooders *G.brasiliensis* and *A. agassizii* (table 3). Nonneuronal cells (table 3) was also in a significate higher number for mouthbrooders in the telencephalon ($p = 0.007$), optic tectum ($p = 0.003$) and cerebellum ($p = 0.007$). In spite of all these differences, again we can observe a bias provided by the dwarf cichlid, *A. agassizii*, which will be better explored in our discussion section.

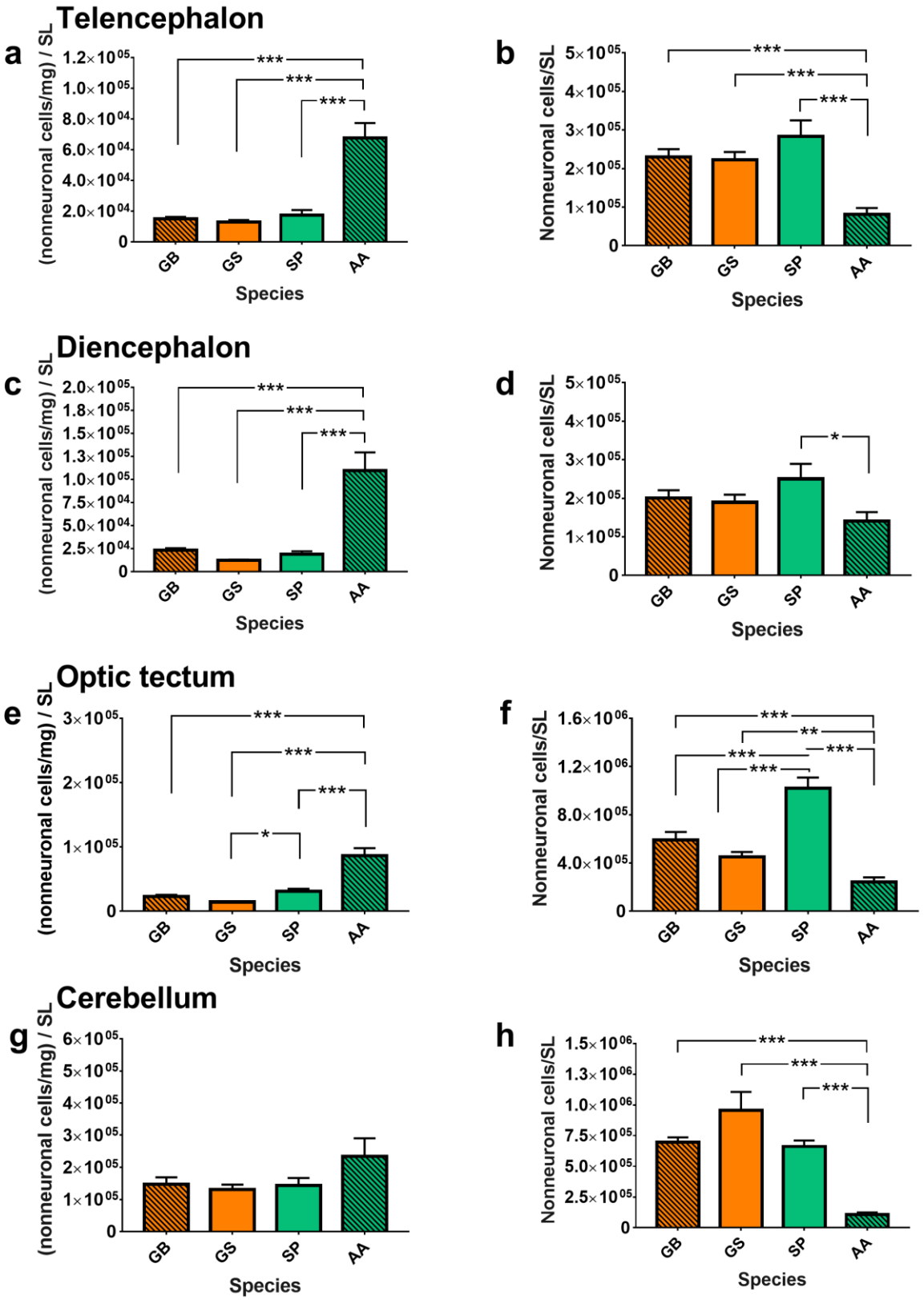

Figure 4. Nonneuronal cells densities (**a, c, e, g**) and number of nonneuronals cells standardized by fish standard length (**b, d, f, h**) compared between the four species (Linear regression completed by Tukey's test). Orange bars and circles represent monogamous species (*Geophagus brasiliensis* and *Geophagus sveni*); green bars and circles represent polygynous species *(Satanoperca pappaterra* and *Apistogramma agassizii).* Dashed lines show species that are substrate brooders (*G. brasiliensis* and *A. agassizii*), while the absence of dashed lines represent mouthbrooder (*G. sveni* and *S. pappaterra*) type of parental care. Data are represented as mean ± SEM.

Ferets diameters of brain cells

Ferets diameters were obtained using a mean of 200 cells measurements for each brain structure and individual. These diameters were not standardized by fish standard length or brain structure weight such as the other brain cells measurements used in this work. Using linear mixed models, we observed that there was no difference between ferets diameters and sex (χ^2 = 0.69, p = 0.4; Fig. 3), but the difference appears when using species as predictors (χ^2 = 14.04, p = 0.003). *A. agassizii* presented feret diameters significantly different from the substrate brooder *G. brasiliensis* (p = 0.003) and the polygynous *S. pappaterra* (p = 0.03), revealing a possible major effect of the miniaturization in this species rather than a difference caused by mating system and type of parental care. There was no statistical significance when using as predictors mating system (F = 2.03, p = 0.21) and type of parental care (F = 0.04, p = 0.84).

Comparing ferets diameters of each brain structure between species, we did not find any difference in the telencephalon, following the tendency of what we observed in our other results concerning this brain structure. Nevertheless, the diencephalon seems to be smaller in *A. agassizii* ($F_{(3.35)}$ = 9.39, p = 0.0001; Fig. 3f) when compared to the other three species, *G. brasiliensis* (p < 0.001), *G. sveni* (p = 0.01) and *S. pappaterra* (p = 0.003). In the optic tectum, feret presented the same pattern, with *A. agassizii* ($F_{(3.36)}$ = 8.19, p = 0.0003; Fig. 3i) presenting cells with small diameters than the monogamous species, *G. brasiliensis* (p < 0.001), *G. sveni* (p = 0.02), and the polygynous *S. pappaterra* (p = 0.008). The cerebellum was the only brain structure where the cells presented a bigger feret diameter in the dwarf cichlid *A. agassizii* ($F_{(3.36)}$ = 7.59, p = 0.0004; Fig. 3l) than in *G. brasiliensis* (p = 0.01), *G. sveni* (p < 0.001) and *S. pappaterra* (p = 0.02). This shows a possible compensation for this area to be the one with the smallest number of neurons when compared with the other cichlids studied here, as showed before.

Volume of brain structures in *G. brasiliensis*, *G. sveni* and *S. pappaterra*

Brain cells density per fish size and volumes

When we used only these three species in the LMs analysis, density of neurons still showed a significant interaction between structure and species ($F_{(6,88)}$ = 3.63, p = 0.003), different from the density of nonneuronal cells, in which the significant difference disappeared when *A. agassizii* ($F_{(6,88)}$ = 0.74, p = 0.62) was withdrawn from the analysis. Using the structure volume as the response variable there is no significant difference also ($F_{(6,88)}$ = 0.72, p = 0.63). The interaction between structure and sex continued non-significant when using neurons ($F_{(3,92)}$ = 0.50, p = 0.68) and nonneuronal cells density ($F_{(3,92)}$ = 0.75, p = 0.52) or even volume ($F_{(3,92)}$ = 0.80, p = 0.49) as response variable.

For parental care type, density of neurons ($F_{(3,92)}$ = 0.81, p = 0.49), nonneuronal cells ($F_{(3,92)}$ = 0.77, p = 0.51) and volumes ($F_{(3,92)}$ = 0.99, p = 0.40) did not show significant

interactions between the three species. The same occurred for mating system interacting with structure for nonneuronal cells ($F_{(3,92)}$ = 0.95, p = 0.42) and volumes ($F_{(3,92)}$ = 0.49, p = 0.67), but not for density of neurons ($F_{(3,92)}$ = 5.56, p = 0.001), that now shows a significant difference.

For last, we made correlation between density of neurons, nonneuronal cells and the brain structure's' volumes. Although not significant, *G. sveni* presented strong correlations between density of cells (table 2), mainly concerning nonneuronal cells. *S. pappaterra* showed a trend between nonneuronal cells density and structure volume in the optic tectum (table 2). There was no significant correlation concerning brain cells densities and structure volume in *G. brasiliensis.*

Table 4. Pearson's correlations between structure volume and brain cells density in the respective region.

Species	Neurons density per SL				Nonneuronal cells density per SL			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
G. brasiliensis	r = -0.34	r = 0.13	r = -0.27	r = -0.31	r = 0.11	r = 0.19	r = 0.10	r = 0.34
	p = 0.37	p = 0.74	p = 0.47	p = 0.41	p = 0.76	p = 0.62	p = 0.79	p = 0.37
G. sveni	r = 0.98	r = 0.44	r = -0.56	r = -0.64	r = -0.97	r = -0.98	r = -0.98	r = -0.98
	p = 0.13	p = 0.71	p = 0.62	p = 0.55	p = 0.14	p = 0.11	p = 0.13	p = 0.13
S. pappaterra	r = 0.46	r = 0.58	r = 0.41	r = -0.34	r = -0.31	r = 0.09	***r = -0.68***	r = -0.41
	p = 0.29	p = 0.16	p = 0.35	p = 0.45	p = 0.49	p = 0.85	***p = 0.09***	p = 0.36

Significance was settled at p ≤ 0.05. Significant correlations are highlighted in bold and gray. Trends (p < 0.10) are highlighted in bold and italic.

Brain cells per fish standard length and volumes – *G. brasiliensis*, *G. sveni* and *S. pappaterra*

When we used only these three species in the LMs analysis, neurons ($F_{(6,88)}$ = 2.18, p = 0.05) and nonneuronal cells number still showed a significant interactions between structure and species ($F_{(6,88)}$ = 5.72, p < 0.0001). The interaction between structure and sex continued non-significant when using number of neurons ($F_{(3.92)}$ = 1.64, p = 0.18) and nonneuronal cells ($F_{(3.92)}$ = 0.12, p = 0.94) as response variable.

For parental care type, number of neurons ($F_{(3.92)}$ = 2.05, p = 0.11) and nonneuronal cells ($F_{(3.92)}$ = 0.51, p = 0.67) did not show significant interactions between the three species. For mating system, there was a significant interaction between structure and mating type in neuronal ($F_{(3.92)}$ = 3.28, p = 0.02) and nonneuronal cells ($F_{(3.92)}$ = 7.29, p = 0.0002). Finally, correlations between neurons and nonneuronal cells standardized by fish SL and the volumes of each corresponding brain structure revealed an strong and positive correlation only between neurons number and structure volume in the telencephalon (r = 0.97, p = 0.03; table 3) for *Geophagus sveni*. Although without significance, *G. brasiliensis* and *S. pappaterra* presented a positive trend of correlation between volume and nonneuronal cells in the cerebellum, and a negative trend between nonneuronal cells in the optic tectum and its volume, respectively.

Table 5. Pearson's correlations between structure volume and brain cells standardized by fish standard length in the respective region.

Species	Neurons per fish SL				Nonneuronal cells per fish SL			
	tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
G. brasiliensis	r = -0.14	r = -0.23	r = -0.19	r = -0.30	r = 0.24	r = -0.38	r = -0.01	***r = 0.61***
	p = 0.68	p = 0.52	p = 0.59	p = 0.39	p = 0.49	p = 0.28	p = 0.97	***p = 0.06***
G. sveni	**r = 0.97**	r = -0.13	r = -0.83	r = -0.55	r = -0.30	r = -0.53	r = -0.77	r = -0.19
	p = 0.03	p = 0.87	p = 0.17	p = 0.44	p = 0.69	p = 0.47	p = 0.22	p = 0.81
S. pappaterra	r = 0.46	r = 0.58	r = 0.41	r = -0.34	r = -0.31	r = 0.09	***r = -0.68***	r = -0.41
	p = 0.29	p = 0.16	p = 0.35	p = 0.45	p = 0.49	p = 0.85	***p = 0.08***	p = 0.36

Significance was settled at $p \leq 0.05$. Significant correlations are highlighted in bold and gray. Trends ($p < 0.10$) are highlighted in bold and italic.

Discussion

Our study compared four Neotropical cichlid species with different mating systems and types of parental care regarding their brain cells' composition. As both reproductive traits are considered powerful forces driven evolution of several behaviors, we expected to find species considered more complexes from a social point of view – i.e., monogamous individuals that coordinate their behavior to care for the youngest – showing a higher number of brain cells. Nevertheless, this was true only for some brain structures, mainly when we used total number of brain cells for comparison between the species. This result seems to agree with the mosaic evolution idea, in which brain regions was selected apart from one another and due to their evolutionary importance for the individual.

The size of a social group may predict its social complexity, as well as the way this social group is organized (Dunbar 1998; Dunbar and Shultz 2007). In such a variable social environment, an individual must to learn how to deal with different conspecifics with different sexes, ages, personalities and social status within the group. In this context, physiological changes in the control center of the behavior, the brain, would be selected because these changes help the individual to cope with the social group as a part of it (Ashton et al. 2018). Nevertheless, our hypothesis that monogamous species would be the most influenced by the social coordination of males and females during brood care in relation to brain complexity was not strongly confirmed, as discussed below. Moreover, some possible biases in our results may have come up, mainly concerning the density of neurons, maybe due to the use of the dwarf cichlid *A. agassizii* – the most derived species used in this study (Arbour and López-Fernández 2016; Ilves et al. 2018). Nevertheless, the four cichlids species we choose are still very close from a phylogenetic point of view. Although we have not used phylogenetic data in our statistical models, we think that our two data approaches, one using density of brain cells, and the other with the total number of cells, both of them standardized by fish's length and analyzed with robust parametric models, have reduced the bias promoted by different fish' lengths.

The polygynous dwarf cichlid, *Apistogramma agassizii*, in general presented the highest density of neurons and nonneuronal cells (per fish SL) when compared with the other three cichlids. Nevertheless, this scenario was not maintained for the total number of cells (per fish SL), where there was an inversion in the results previously observed, with the dwarf cichlid, now presenting the lowest numbers of total cells in several structures. These differences in brain cells' numbers in *A. agassizii* seem to be due to the miniaturization faced by this species along evolution rather than to its mating system and parental roles. This idea is in accordance with what we observed for cells' ferets diameters. The miniaturization process does not concerns a simple reduction in body size, but has several impacts – and *vice-versa* – in a species' physiology, ecology, life history and behavior by reducing the costs as well as the benefits of having a larger body (Hanken and Wake 1993). *Apistogramma agassizii* presented a reduction in cell size in the diencephalon and optic tectum, while in the telencephalon there was no difference of cell size when compared with the other species. Moreover, this lack of difference can be a result of the higher variance presented by cells diameters of this species in the telencephalon. Our results are in accordance with a recent study of Marhounová et al. (2019) with guppies, the *Poecilia reticulata*, and discuss that indeed small fish species present high neuronal densities, which could be a compensation for the small brain size in these individuals with the same ecological and social complexity faced by non-miniaturized fishes. The most interesting difference in ferets diameters occurred in the cerebellum, in which the cells of the dwarf cichlid were much bigger than *Geophagus* species and *S. pappaterra.* This shows that not only the body of *A. agassizii* suffered a miniaturization, but also its brain, both in cell size – i.e., reduced diameters in cells of the diencephalon and optic tectum – and in cell quantity – i.e. cerebellum with fewer number of total cells

Our results are not in accordance with results commonly observed for mammals. In this group, cells densities inversely correlate to cell size (see Mota and Herculano-Houzel 2014). Nevertheless, here we had the cerebellum showing us exactly the opposite, a structure with bigger cells and higher density of them. In general, the cerebellum was the structure that presented the lowest number of neuronal and nonneuronal cells when compared with the other brain regions. This augmentation in size in the cerebellar cells may be a "compensation" due to the reduced number of the cells in this area, as bigger nervous cells are faster in synaptic transmissions. The opposite was already observed for mice, in which an increased number of brain cells is compensated by a reduction in cells' size (Herculano-Houzel et al. 2015), showing that more cells not necessarily results in a bigger and heavier brain. Nevertheless, the body miniaturization suffered by *A. agassizii* was not reflected in an increased number of brain cells, but in an increased density of brain cells. A study of Kverková et al. (2018) with social and solitary species of mole-rats suggests that when a reduction in body size is not followed by an increase of relative brain size it may be due to a weak evolutionary pressure of sociality in this brain size that not overcomes metabolic constraints – not strong as previously defended in

theories as the SBH, for example –, which not outweigh the metabolic demands of having a big brain in a small body. This view may somewhat limits the reach of the Social Brain Hypothesis (Kverková et al. 2018), showing an alternative way to interpret the results we found here.

Looking to the other polygynous species of this study, *Satanoperca pappaterra,* this fish also presented differences in brain cells' composition when compared to the monogamous cichlids. Concerning the telencephalon, *S. pappaterra* presented more neurons than *G. sveni*, this last one also a mouthbrooder species. This tendency was followed by nonneuronal cells, but only in the optic tectum structure, in which *S. pappaterra* surpasses the both *Geophagus* species in number of cells. On the other hand, other brain measurements, as ferets diameters, did not presented differences between the *Geophagus* species and *S. pappaterra.* Variations between monogamous and polygynous species were made clearer when we grouped individuals of both groups for analysis. In the telencephalon and diencephalon – regions considered highly involved in social behavior control (see Shumway 2010; O'Connell and Hofmann 2011; Teles et al. 2016) –, polygynous species presented a higher density of neurons and nonneuronal cells when compared to monogamous ones, although this seems to be biased by the huge density of cells presented by *A. agassizii*.

Using our second approach of looking at the total number of brain cells, we did not observe differences between the neurons in the telencephalon by comparing mating systems. This was not expected, as theoretical basis predict that monogamous individuals would present more cells in this brain region. Interestingly, nonneuronal cells were higher in monogamous species when compared to the polygynous. Although neurons are considered the cells' units behind brain complexity (Fang and Yuste 2017), the glial cells – i.e., the greatest part of what we called "nonneuronal cells" – have not only the task to maintain the neuronal circuits working properly, but are known to be involved in several behavioral and cognitive processes (see Bracchi-Ricard et al. 2008; Kyrargyri et al. 2015; Sardinha et al. 2017; Mu et al. 2019), which may be a confirmation of our hypothesis that monogamy is more complex from a social point of view. For last, the diencephalon showed the opposite of what we observed for the telencephalon: *Geophagus* species presenting less neurons than polygynous individuals do, and this difference disappearing for diencephalic nonneuronal cells. This reduced number of neurons presented by monogamous species is being influenced by *G. brasiliensis*, the monogamous species that is exclusively a substrate brooder. Our results about total number of brain cells in the telencephalon and diencephalon are somewhat in accordance with the study of Pollen et al. (2007), in which they observed that monogamous species of African cichlids present larger telencephalons and smaller hypothalamus when compared to polygynous species. Why this was not a scenario also observed for *G. sveni* is speculative. In fact, Pollen et al. (2007) highlighted that all the monogamous species used in their study were substrate brooders, while all the polygynous individuals used were mouthbrooders, what made it difficult to state whether the differences in brain regions are a result of the mating system, of the type of

parental care, or both (Pollen et al. 2007). In our study, we can speculate that the answer for our results would be the mating system. For last, in polygynous species, males defend their territories more frequently and intensively when compared to monogamous males (Pollen et al. 2007). The hypothalamus controls these aggressive behaviors by neuroendocrine pathways, which may have favored morphological changes that results in different sizes of hypothalamus in the different mating systems (Pollen et al. 2007; Shumway 2010).

Thus, as several works already compared brain volumes and reproductive behaviours, we also looked for the link between mating system and parental care related to brain cells composition. Different from the above mentioned studies, volumes were not correlate to any of our predictor variables, such as sex, species, mating system or parental care type. Regarding correlations, the only positive correlation observed was found between volume and number of neurons in the telencephalon of *G. sveni,* showing that more neurons are related to a bigger telencephalon. This species was the only one that also showed several strong r-values in practically all the brain structures concerning density of cells, although not significant ones. These correlations where interesting, since in the number of cells or in their densities, the strong r-values were about negative correlations. Thus, if the volume of a giving structure is increasing, while number and density of cells are decreasing, it means that, in this scenario, the average cells size are also increasing. This was the case in all the brain regions of *G. sveni,* except in the forebrain, where densities of brain cells did not show strong r-values in relation to structure volumes. Moreover, although sharing with *G. brasiliensis* the monogamy, and with *S. pappaterra,* the mouthbrooding behaviour, these two species did not show such strong tendencies of correlations between brain cells and structure volumes. These results reveal that the social aspects of reproductive behaviours found for *G. sveni* do not drive the relations we observed between structure volume and brain cells' composition, at least not clearly.

Concerning type of parental care, substrate brooders presented a higher density of cells, both neuronal and nonneuronal, in the telencephalon, diencephalon and optic tectum than mouthbrooders, the first ones considered the ancestral care state and highly represented by New World cichlids (Kuwamura 1986; Goodwin et al. 1998; Barlow 2002). Nevertheless, this seems to be a bias provided by the huge density of brain cells presented by *A. agassizii.* Thus, we could observe that the total number of cells was not as disparate as densities among types of parental care, and we will look closer to this approach. Firstly, there was an interesting difference appearing in the cerebellum, in which the number of neurons as well as nonneuronal cells were higher in mouthbrooders. This make sense, as mouthbrooders must take care of the brood inside their mouths without swallowing it, a refined motor control that may have selected a more complex cellular architecture in the cerebellum.

Nonneuronal cells was also higher in mouthbrooders in the telencephalon and optic tectum. In fact, mouthbrooding is considered a more effective type of care when occurring in situations where the predation pressure is higher (Dupuis and Keenleyside 1982), selecting

more cautious and appraiser individuals that knows better when to liberate or take the brood in their mouth during brood development. This seems to be true for the occasionally moutbrooder *G. sveni.* Although it is a monogamous species, both males and females of *G. sveni* can take the brood in their mouth if a threat is perceived. This is considered a rare state among New World cichlids, and may have been selected due to an increase in predation pressure towards the fry (Dupuis and Keenleyside 1982). For *S. pappaterra*, a polygynous species with a long period of parental care (Lopes et al. 2015), the dynamic of caretaking can be even more challenger. In this species, the mother has to take care of the brood, while chases away predators and evaluate the surroundings for sudden environmental changes common in the tropics, for example. This constant state of alert may had selected more tuned brains to deal with such threats. On the other hand, *S. pappaterra* polygynous males have to display aggressive behaviors towards possible opponents, while mating with receptive females, all this without leaving their territory, which demands elaborated mental states of decision-making (Brandão et al. 2019). Therefore, it makes sense to evidence a higher number of nonneuronal cells in the telencephalon and optic tectum of mouthbrooders. More cells in the telencephalon confers a better cognitive processing for individuals inserted in a more unstable environment, as this brain area is highly related to spatial learning and memory (see Kotrschal et al. 1998; Broglio et al. 2011). More cells in the optic tectum confer a better visual acuity during parental care than those demanded by monogamous and substrate brooders species as *G. brasiliensis,* for example, in which both parents share the tasks during parental care in a more stable environment.

In the optic tectum and cerebellum, the density of cells in general was again higher for *A. agassizii*, probably due to the already discuss situation of the miniaturization faced by this species. Interestingly, in nonneuronal cells of the cerebellum there was no difference between the dwarf cichlid and the other three species, which was already discussed due to the fact the ferets diameter somewhat seems to be compensating for this reduced density of cells. The total number of cells, nevertheless, shows a reduced number of cells in these areas regarding the dwarf cichlid in comparison to the both *Geophagus* and *S. pappaterra* species*.* There are no differences between these last three cichlids, which will only appear when they are grouped in their respective mating system and type of parental care. Therefore, comparing total number of neurons in the optic tectum and the cerebellum, monogamous species as *G. brasiliensis* and *G. sveni* have more of these cells than polygynous *S. pappaterra* and *A. agassizii.* This might be due to a reduced number of neurons in the cerebellum in *A. agassizii*, which also was repeated in nonneuronal cells, where monogamous individuals also showed more cells than polygynous. Although a higher number of cells in monogamous species composes the optic tectum, this does not seems to be due to a strong reduction factor provided by *A. agassizii*, as in the cerebellum, at least in neuronal cells. For example, there was no differences between polygynous and monogamous individuals concerning the nonneuronal cells in the optic tectum,

but there are differences for each species alone, with *S. pappaterra* showing the highest quantities of optic tectum nonneuronal cells.

Overall, our study seems to be in accordance to the idea of the mosaic evolution of the brain, which points out that the brain of cichlids, as in mammals, did not evolved as a single unit, but have selected differently the brain regions that compose it according to their specific importance (Barton and Harvey 2000). On the other hand, our initial predictions, related to the SBH – i.e., monogamous individuals would presented more brain cells than polygynous species – was not confirmed for all brain areas and types of brain cells. Even when each species was compared alone, *S. pappaterra* and *A. agassizii* presented more cells in some cases than the monogamous *Geophagus.* A recent study of Kverková et al. (2018) done with African mole-rats showed that solitary individuals presented more neurons in general than social species, bouncing the SBH, so well stablished for mammals. In fact, this study discuss that some practices so common in some mammals, as formation of coalitions and tactical deception may be the key for the selection of larger brains. Therefore, even though some species present associations considered high cognitively demanding, these social interactions would not be so complex from a cognition point of view but, instead, could be solved by the individuals with a simple rule-of-thumb that would not require refined calculations of goals and social consequences (Barrett et al. 2007; Kverková et al. 2018). For last, we need to point that the four species of Neotropical cichlids we used in our study are very few known for scientific purposes, mainly in which concerns their physiology and behavior. Therefore, several factors we do not know about all aspects of the life history of these species, for example, could cause some disarray in our results. For example, our goal was to look at social evolutionary power, although several hypothesis defends the power of ecological factors acting on brain evolution (Clutton-Brock and Harvey 1980; Rosati 2017; González-Forero and Gardner 2018). However, and as mentioned above, Neotropical cichlid fish are represented a huge diversity of species with unique social behaviors that worth investigating when we aim comparative studies such this; moreover, our results hypothesis was in part confirmed by looking of number of brain cells. Moreover, and for the next step, some studies defends that today, even more important than the number of neurons that composes the brain is the cells' connections occurring between brain structures, as well as dendrites density and conduction velocity (Dicke and Roth 2016; Genç et al. 2018).

In summary, this work brings, for the first time, interesting insights about the brain cells' composition in cichlids species with different social organizations, as mating system and the type of parental care, both considered such two strong evolutionary forces driving brain evolution. These findings seems to agree with the mosaic evolution of the brain, as we did not observe larger brains for one species or another, but an increasing in different regions with different roles in fish behavior and ecology. The SBH, on the other hand, does not seems to explain all the results we have observed here, revealing that the sociability enforced by

monogamy is not such a powerful evolutionary force in order to increase number of brain cells, as we expected. In which regards the brain, all information must be interpreted together. Besides volume and number of cells, now the next step may be to observe how these cells are connected between brain regions, and how fast and effectively behavioral information is passed along.

Acknowledgements

We thank Ana Carolina dos Santos Gauy, Marcela Cesar Bolognesi and Magda Teles for technical assistance during data collection, and Nuno Pimpão Martins for the development of the macro used in brain cells' counting. This research was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), research grant #2016/26160-2, to EGF. MLB was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES #001) – DS for PhD Student and by FAPESP (scholarship #2019/10808-1 – TT-3).

References

Arbour JH, López-Fernández H (2016) Continental cichlid radiations: functional diversity reveals the role of changing ecological opportunity in the Neotropics. Proc R Soc B Biol Sci 283:20160556. doi: 10.1098/rspb.2016.0556

Ashton BJ, Thornton A, Ridley AR (2018) An intraspecific appraisal of the social intelligence hypothesis. Philos Trans R Soc B. doi: http://dx.doi.org/10.1098/rstb.2017.0288

Balshine S, Sloman K (2011) Parental Care in Fishes. In: Farrel AP (ed) Encyclopedia of Fish Physiology: From Genome to Environment. Academic Press, San Diego, pp 670–677

Barlow G (2002) The Cichlid Fishes: Nature's Grand Experiment In Evolution, 1st edn. Basic Books, New York

Barrett L, Henzi P, Rendall D (2007) Social brains, simple minds: does social complexity really require cognitive complexity? Philos Trans R Soc B Biol Sci 362:561–575. doi: 10.1098/rstb.2006.1995

Barton RA, Harvey PH (2000) Mosaic evolution of brain structure in mammals. Nature 405:1055–1058. doi: 10.1038/35016580

Bates D, Mächler M, Bolker B, Walker S (2015) Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw. doi: 10.18637/jss.v067.i01

Bludau A, Royer M, Meister G, et al (2019) Epigenetic Regulation of the Social Brain. Trends Neurosci 42:471–484. doi: 10.1016/j.tins.2019.04.001

Bracchi-Ricard V, Brambilla R, Levenson J, et al (2008) Astroglial nuclear factor-κB regulates learning and memory and synaptic plasticity in female mice. J Neurochem 104:611–623. doi: 10.1111/j.1471-4159.2007.04993.x

Brandão ML, Fernandes AMT de A, Gonçalves-de-Freitas E (2019) Male and female cichlid fish show cognitive inhibitory control ability. Sci Rep 9:15795. doi: 10.1038/s41598-019-52384-2

Broglio C, Gómez A, Durán E, et al (2011) Brain and Cognition in Teleost Fish. In: Brown C, Laland K, Krause J (eds) Fish Cognition and Behavior, 2nd edn. Wiley-Blackwell, Oxford, UK, pp 325–358
Clutton- Brock TH, Harvey PH (1980) Primates, brains and ecology. J Zool 190:309–323. doi: 10.1111/j.1469-7998.1980.tb01430.x
Dicke U, Roth G (2016) Neuronal factors determining high intelligence. Philos Trans R Soc B Biol Sci 371:20150180. doi: 10.1098/rstb.2015.0180
Dunbar RIM (2009) Social Brain. Soc Brain Evol 58–81.
Dunbar RIM (1998) The social brain hypothesis. Evol Anthropol Issues, News, Rev 6:178–190. doi: 10.1002/(SICI)1520-6505(1998)6:5<178::AID-EVAN5>3.3.CO;2-P
Dunbar RIM, Shultz S (2007) Evolution in the Social Brain. Science (80-) 317:1344–1347. doi: 10.1126/science.1145463
Dupuis HMC, Keenleyside MHA (1982) Egg-Care Behavior of *Aequidens paraguayensis* (Pisces, Cichlidae) in Relation to Predation Pressure and Spawning Substrate. Can J Zool Can Zool 60:1794–1799.
Fang W-Q, Yuste R (2017) Overproduction of Neurons Is Correlated with Enhanced Cortical Ensembles and Increased Perceptual Discrimination. Cell Rep 21:381–392. doi: 10.1016/j.celrep.2017.09.040
Fernald RD (2015) Social behaviour: can it change the brain? Anim Behav 103:259–265. doi: 10.1016/j.anbehav.2015.01.019
Fernald RD (2017) Cognitive skills and the evolution of social systems. J Exp Biol 220:103–113. doi: 10.1242/jeb.142430
Finlay B, Darlington R (1995) Linked regularities in the development and evolution of mammalian brains. Science (80-) 268:1578–1584. doi: 10.1126/science.7777856
Garcia AN, Bezner K, Depena C, et al (2017) The effects of long-term estradiol treatment on social behavior and gene expression in adult female rats. Horm Behav 87:145–154. doi: 10.1016/j.yhbeh.2016.11.011
Genç E, Fraenz C, Schlüter C, et al (2018) Diffusion markers of dendritic density and arborization in gray matter predict differences in intelligence. Nat Commun 9:1905. doi: 10.1038/s41467-018-04268-8
González-Forero M, Gardner A (2018) Inference of ecological and social drivers of human brain-size evolution. Nature 557:554–557. doi: 10.1038/s41586-018-0127-x
Gonzalez-Voyer A, Kolm N (2010) Sex, Ecology and the Brain: Evolutionary Correlates of Brain Structure Volumes in Tanganyikan Cichlids. PLoS One 5:e14355. doi: 10.1371/journal.pone.0014355
Goodwin NB, Balshine-Earn S, Reynolds JD (1998) Evolutionary transitions in parental care in cichlid fish. Proc R Soc London Ser B Biol Sci 265:2265–2272. doi: 10.1098/rspb.1998.0569
Gross TR, Sargent RC (1985) The Evolution of Male and Female Parental Care in Fishes.

Hanken J, Wake DB (1993) Miniaturization of Body Size: Organismal Consequences and Evolutionary Significance. Annu Rev Ecol Syst 24:501–519. doi: 10.1146/annurev.es.24.110193.002441
Hanon L (1975) Adaptations morphologiques et comportementales a l'incubation buccale chez les poissons cichlides: oeufs et alevins. Ann la Société R Zool Belgique 105:169–192.
Herculano-Houzel S (2011) The Isotropic Fractionator: A Fast, Reliable Method to Determine Numbers of Cells in the Brain or Other Tissues. 391–403. doi: 10.1007/7657_2011_13
Herculano-Houzel S, Messeder DJ, Fonseca-Azevedo K, Pantoja NA (2015) When larger brains do not have more neurons: increased numbers of cells are compensated by decreased average cell size across mouse individuals. Front Neuroanat 9:64. doi: 10.3389/fnana.2015.00064
Holekamp KE, Dantzer B, Stricker G, et al (2015) Brains, brawn and sociality: A hyaena's tale. Anim Behav 103:237–248. doi: 10.1016/j.anbehav.2015.01.023
Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models. Biometrical J 50:346–363. doi: 10.1002/bimj.200810425
Huffman LS, Mitchell MM, O'Connell LA, et al (2012) Rising StARs: Behavioral, hormonal, and molecular responses to social challenge and opportunity. Horm Behav 61:631–641. doi: 10.1016/j.yhbeh.2012.02.016
Ilves KL, Torti D, López-Fernández H (2018) Exon-based phylogenomics strengthens the phylogeny of Neotropical cichlids and identifies remaining conflicting clades (Cichliformes: Cichlidae: Cichlinae). Mol Phylogenet Evol 118:232–243. doi: 10.1016/j.ympev.2017.10.008
Kabadayi C, Bobrowicz K, Osvath M (2018) The detour paradigm in animal cognition. Anim Cogn 21:21–35. doi: 10.1007/s10071-017-1152-0
Kotrschal K, Van Staaden MJ, Huber R (1998) Fish brains: evolution and environmental relationships.
Kuwamura T (1986) Parental care and mating systems of cichlid fishes in Lake Tanganyika: a preliminary field survey. J Ethol 4:129–146. doi: 10.1007/BF02348115
Kverková K, Bělíková T, Olkowicz S, et al (2018) Sociality does not drive the evolution of large brains in eusocial African mole-rats. Sci Rep 8:9203. doi: 10.1038/s41598-018-26062-8
Kyrargyri V, Vega-Flores G, Gruart A, et al (2015) Differential contributions of microglial and neuronal IKKβ to synaptic plasticity and associative learning in alert behaving mice. Glia 63:549–566. doi: 10.1002/glia.22756
Lehner PN (1998) Handbook of Ethological Methods, 2nd edn. Cambridge University Press
Lihoreau M, Latty T, Chittka L (2012) An exploration of the social brain hypothesis in insects. Front Physiol 3:1–7. doi: 10.3389/fphys.2012.00439
Lopes TM, Garcia De Oliveira F, Bialetzki A, Agostinho AA (2015) Early development in the mouth-brooding cichlid fish *Satanoperca pappaterra* (Perciformes: Cichlidae). Rev Biol Trop 63:139–153.
Lucon-Xiccato T, Gatto E, Bisazza A (2017) Fish perform like mammals and birds in inhibitory

motor control tasks. Sci Rep 7:13144. doi: 10.1038/s41598-017-13447-4
MacLean EL, Hare B, Nunn CL, et al (2014) The evolution of self-control. Proc Natl Acad Sci U S A 111:E2140-8. doi: 10.1073/pnas.1323533111
MacLean EL, Matthews LJ, Hare B a., et al (2012) How does cognition evolve? Phylogenetic comparative psychology. Anim Cogn 15:223–238. doi: 10.1007/s10071-011-0448-8
Marhounová L, Kotrschal A, Kverková K, et al (2019) Artificial selection on brain size leads to matching changes in overall number of neurons. Evolution (N Y) 73:2003–2012. doi: 10.1111/evo.13805
Maruska KP, Zhang A, Neboori A, Fernald RD (2013) Social Opportunity Causes Rapid Transcriptional Changes in the Social Behaviour Network of the Brain in an African Cichlid Fish. J Neuroendocrinol 25:145–157. doi: 10.1111/j.1365-2826.2012.02382.x
Mota B, Herculano-Houzel S (2014) All brains are made of this: a fundamental building block of brain matter with matching neuronal and glial masses. Front Neuroanat 8:1–21. doi: 10.3389/fnana.2014.00127
Mu Y, Bennett D V., Rubinov M, et al (2019) Glia Accumulate Evidence that Actions Are Futile and Suppress Unsuccessful Behavior. Cell 178:27–43. doi: 10.1016/j.cell.2019.05.050
Munley KM, Rendon NM, Demas GE (2018) Neural Androgen Synthesis and Aggression: Insights From a Seasonally Breeding Rodent. Front Endocrinol (Lausanne). doi: 10.3389/fendo.2018.00136
O'Connell LA, Hofmann HA (2011) The Vertebrate mesolimbic reward system and social behavior network: A comparative synthesis. J Comp Neurol 519:3599–3639. doi: 10.1002/cne.22735
Oliveira RF (2009) Social behavior in context: Hormonal modulation of behavioral plasticity and social competence. Integr Comp Biol 49:423–440. doi: 10.1093/icb/icp055
Oppenheimer JR (1970) Mouthbreeding in fishes. Anim Behav 18:493–503. doi: 10.1016/0003-3472(70)90045-X
Pitnick S, Jones KE, Wilkinson GS (2006) Mating system and brain size in bats. Proc R Soc B Biol Sci 273:719–724. doi: 10.1098/rspb.2005.3367
Pollen AA, Dobberfuhl AP, Scace J, et al (2007) Environmental complexity and social organization sculpt the brain in Lake Tanganyikan cichlid fish. Brain Behav Evol 70:21–39. doi: 10.1159/000101067
Revelle W (2018) psych: Procedures for Psychological, Psychometric, and Personality Research. Northwestern University, Evanston, Illinois
Rosati AG (2017) Foraging Cognition: Reviving the Ecological Intelligence Hypothesis. Trends Cogn Sci 21:691–702. doi: 10.1016/j.tics.2017.05.011
Sardinha VM, Guerra-Gomes S, Caetano I, et al (2017) Astrocytic signaling supports hippocampal-prefrontal theta synchronization and cognitive function. Glia 65:1944–1960. doi: 10.1002/glia.23205

Schillaci MA (2006) Sexual Selection and the Evolution of Brain Size in Primates. PLoS One 1:e62. doi: 10.1371/journal.pone.0000062
Shumway C a. (2010) The evolution of complex brains and behaviors in African cichlid fishes. Curr Zool 56:144–156.
Teles MC, Gozdowska M, Kalamarz-Kubiak H, et al (2016) Agonistic interactions elicit rapid changes in brain nonapeptide levels in zebrafish. Horm Behav 84:57–63. doi: 10.1016/j.yhbeh.2016.05.020
Timms AM, Keenleyside MHA (1975) The Reproductive Behaviour of *Aequidens paraguayensis* (Pisces, Cichlidae). Z Tierpsychol 39:8–23. doi: 10.1111/j.1439-0310.1975.tb00896.x
Valério-Gomes B, Guimarães DM, Szczupak D, Lent R (2018) The Absolute Number of Oligodendrocytes in the Adult Mouse Brain. Front Neuroanat 12:90. doi: 10.3389/fnana.2018.00090
Zeng Y, Lou SL, Liao WB, et al (2016) Sexual selection impacts brain anatomy in frogs and toads. Ecol Evol 6:7070–7079. doi: 10.1002/ece3.2459

4 PAPER 3. Neurons and nonneuronal cells are associate to sociality and cognition in fish

Manuela Lombardi Brandão[1], Eliane Gonçalves-de-Freitas[1,2] and Rui F. Oliveira[3,4,5].

[1]Departamento de Zoologia e Botânica, Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista (UNESP), Cristóvão Colombo, 2265, 15054-000, São José do Rio Preto, SP, Brazil.
[2]Centro de Aquicultura da UNESP, SP, Brazil.
[3]Instituto Gulbenkian de Ciência, Oeiras, Portugal.
[4]ISPA - Instituto Universitário, Lisbon, Portugal.
[3]Champalimaud Neuroscience Programme, Champalimaud Centre for the Unknown, Lisbon, Portugal.

ABSTRACT

A social group encompass individuals interacting with others from different sexes, ages, personalities and hierarchical ranks, which demands individual refined cognitive processing to deal with such an unpredicted social environment. As social and cognitive behaviors are associated to brain complexity, we hypothesized that individual brain architecture explains the individual cognitive flexibility and sociality. Therefore, we compared the behavior and brain structures of a monogamous Neotropical fish, the pearl cichlid *Geophagus brasiliensis*, in which both sexes finely coordinate their behavior during parental care. We tested males and females under aggressiveness, sociability and inhibitory control tests. Those behaviors then were tested for correlation with neuronal and nonneuronal brain cells in the telencephalon, diencephalon, optic tectum and cerebellum. There was no significant correlations regarding males and females. Therefore, both sexes were analyzed together. The telencephalon was the only brain structure in which there was no association between brain cells and the tested behaviors. Diencephalon, cerebellum and optic tectum, however, were correlated to the brain cells, which seems to be linked to a better individual evaluation of the social environment. For example, higher number of cells in the diencephalon and in the cerebellum were positively correlated to individuals that faster succeeded in a detour-reaching task. There are more cells in the cerebellum of individuals that presented less aggressive behaviors. A higher number of cells in the optic tectum was associated to individuals that take more time to engage in a fight. In general, pearl cichlids that presents a higher number of brain cells are less aggressive, spent more time near conspecifics and are less persistent in irrelevant situations, revealing that the higher the number of brain cells, the more cautious is the social animal.

Key words: sociability; aggressiveness; inhibitory control; *Geophagus brasiliensis*; monogamy; substrate brooding; isotropic fractionator.

Introduction

Social animals live in groups whose adaptive value surpass the individual's gain of living alone (Dunbar and Shultz 2007; Favati et al. 2017). Nevertheless, living with conspecifics in large groups is considered highly challenging from a behavioral point of view. The Social Brain Hypothesis postulates that animals living in a bigger social group will have a higher cognitive demand, resulting in a more complex brain (Dunbar 1998; Dunbar and Shultz 2007). In spite of this theory has been already evidenced in several animal species, there are a growing questioning about the importance of other factors concerning cognition and intelligence besides brain size. Nowadays, the complexity of neural structures and the way this structures are connected between each other are considered to be equally or more important for cognitively-demanding tasks than only the size of the brain (Ashton et al. 2018). Moreover, having bigger brains not even means having more neurons, the cells considered to be the principal responsible for mental processing and individual cognitive ability (Herculano-Houzel 2017; Kverková et al. 2018). Thus, more than looking at the brain size, it is important to know the relationships between the complex social behaviors, cognitive demands, and the complexity of brain architecture coordinating these behaviors.

Reproduction, for instance, is a particular behavior that is intrinsically related to brain evolution due to its social context (see Boucherie, Loretto, Massen, & Bugnyar, 2019; Cummings, 2015; Schillaci, 2006). The sociality behind mating systems can be simpler, as polygamous systems, or more complex, as the monogamous interrelationships. Moreover, the mating system is directly related to the type of parental care, as it interferes with further opportunities of mating (Kuwamura 1986). Thus, we consider the polygamy to be less complex because only the male or only the female take care of the fry (Emlen and Oring 1977), and, therefore, the individual do not have to coordinate its behavior with the other parent during brood care. On the other hand, monogamous species form pairs and have to coordinate the brood care between them: while a parent is chasing away predators, the other parent must to look to youngsters and keep them close (Emlen and Oring 1977).

Besides the interactions among different sexes, a social group is also made up of individuals interacting with others from different ages, personalities and hierarchical ranks, which demands individual refined cognitive processing to deal with such interactions, and to alters its behavior according to such an unpredictable social environment (Emery et al. 2007; Wascher et al. 2018). Therefore, an individual's cognitive ability may be modulated by its social environment, as well as the social environment may be driving to a remodeling in the coordination center of these social behaviors – the brain – with a highly intricate bi-directional

relationship (Wascher et al. 2018). Thus, we hypothesized that individual brain architecture explains the individual cognitive flexibility and sociality.

Several works have been correlating brain volumes with behavior and cognitive processes (see Shumway 2010; Broglio et al. 2011; Holekamp et al. 2015; Marhounová et al. 2019). However, brain volume includes all brain cell types, such as neurons and nonneuronal cells. Here, we used the isotropic fractionator method to evaluate whether these two sets of brain cells are associated to individual social and cognitive performance in a Neotropical fish, the pearl cichlid *Geophagus brasiliensis*, a monogamous substrate-brooder species with biparental care of the fry. Pearl cichlids are a wide spread species in South America, occupying several water body types, such as streams, ponds, lagoons and even estuaries (Bastos et al. 2011). Such as other cichlids, *G. brasiliensis* defends its territory using aggressive interactions (Kadry and Barreto 2010; Sanches et al. 2012) and have males and females tuned during offspring care. Males and females of pear cichlid display asymmetrical parental care activities, as males seem to invest more energy in guarding the nest and the fry (Bastos et al. 2011). In this context, we aimed to test whether a species considered more derived in a mating system and parental care behavior point of view would present individual complex social and cognitive behaviors associated to the composition of brain cells. Moreover, we aimed to test whether males and females would differ in these characteristics due to the different roles played by each sex during reproduction and brood care. We showed, for the first time, that neurons and nonneuronal cells from specific brain areas, such as diencephalon, cerebellum, and optic tectum are associated to individuals that take more time to engage in a fight, are less aggressive, more sociable and are less persistent in irrelevant situations, revealing that the higher the number of brain cells the more cautious is the social animal.

Methods

Fish housing

We used 6 males and 6 females pearl cichlids *Geophagus brasiliensis* (Quoy & Gaimard, 1824) collected in the Rio Grande river, MG, southern Brazil (20°16'58.0"S and 49°09'22.1"W). Then, fish were transported and kept in the laboratory in polyethylene tanks (ca. 500 L, 1 fish/10 L) where they were acclimated for at least 15 days. The water temperature was controlled at 27 °C with heaters and thermostats, and light regime was settled from 7AM to 7PM. Water quality was maintained with biological filters (400 L/h, constant aeration) and also with 25% water renewal every week to clean accumulated organic residuals without disturbing social group stability (Gauy et al. 2018). Fish were fed once a day to apparent satiation with commercial food for tropical fish (28% CP).

Experimental design

After acclimation, fish were anesthetized (tricaine methanosulphonate, MS222, 150 mg.L^{-1}) measured, weight and allocated individually in a home aquarium (40 × 30 × 40 cm, ca. 432 L) until the next day, when the behavioral tests started (Fig. 1). Although we allocated fish individually, aquaria were always paired along experiments in a way that two fish could always see each other through a lateral transparent wall of the tank. These transparent walls were, however, covered with an opaque partition during behavioral tests. We used this design aiming to avoid detrimental effects from social isolation on fish learning and welfare (Brandão et al. 2015).

The subjects were submitted to three behavioral tests in a sequence (Fig. 1). The first one was an aggressiveness test, which measures individual's aggressive behavior towards conspecifics using a mirror-elicited fight paradigm. The second one was designed to test individual's sociability, by observing whether subjects would rather to stay close to the social group or in an isolation situation. The third behavioral test was based on the detour-reaching paradigm, to test inhibitory control abilities and cognitive flexibility. After the tests, individuals were killed with anesthetic overdose (i.e., benzocaine 180 mg.L^{-1}) for sex confirmation and brain collection.

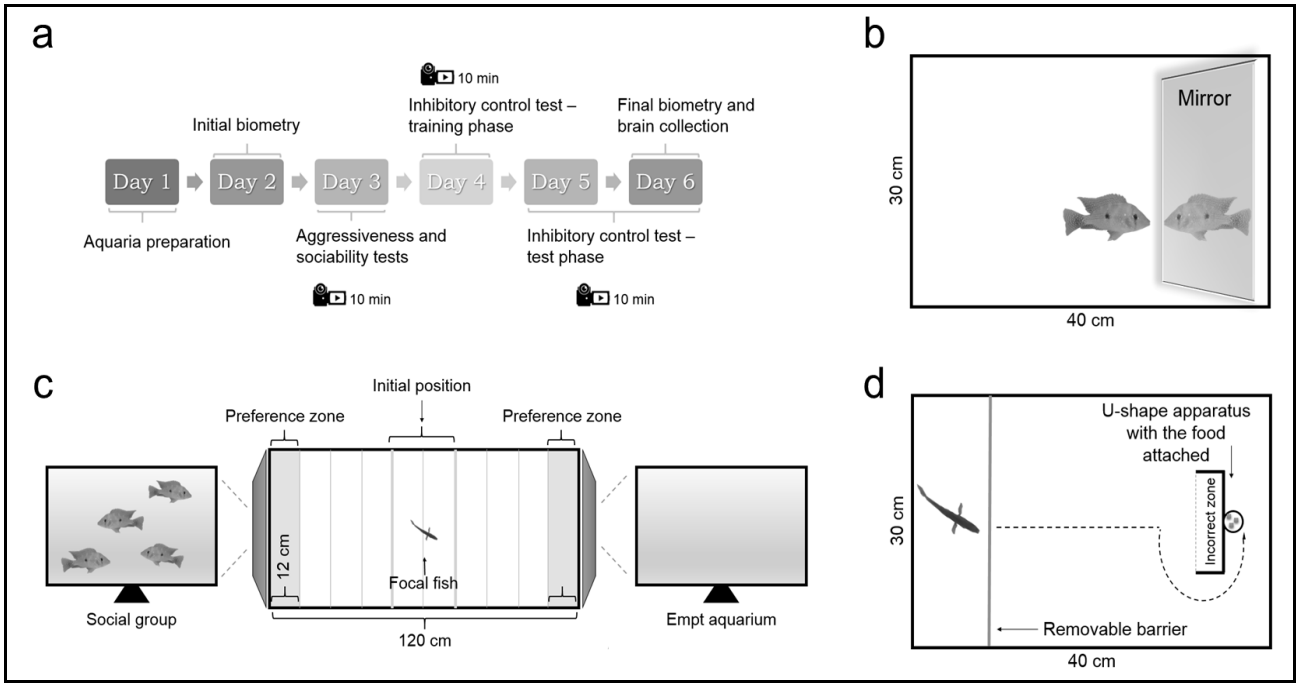

Figure 1. Experimental design with the **a.** tests' chronogram and schematic views of the **b.** aggressiveness test, **c.** the sociability test and the **d.** inhibitory control test. The aquarium showed in **b.** was only used during the sociability test. Both **c.** and **d.** schemes show a view from above of the individual's home aquarium. Fish images and schemes are from M.L. Brandão authorship.

Aggressiveness test

We quantified the frequency of aggressive behaviors and the latency for the first aggressive interaction to be displayed by the focal fish towards its mirror image. The use of mirrors to elicit fights in fish is a well-established methodology applied to measure aggressiveness, as cichlid fishes interact with their images as if they were real opponents (see Oliveira et al. 2005; Desjardins and Fernald 2010; Reddon et al. 2012; Teles et al. 2016; Scherer et al. 2016; Scherer et al. 2017). The mirror test reduces physical injuries avoiding fights between two real opponents. Moreover, this methodology allows preventing sexual bias that could happen, as *G. brasiliensis* do not present a clearly sexual dimorphism. Thus, we introduced a mirror of 40 × 30 cm in the home aquarium (Fig. 1b) and the aggressive behaviors showed by the focal fish towards its image were video recorded for 30 minutes in the morning of the first day of experiments. The behaviors were quantified using an adaptation of the ethogram already developed for pearl cichlid in real opponent interactions (e.g., Kadry and Barreto 2010). Nevertheless, as we used mirror-elicited fights, some aggressive behaviors were adjusted for this context (e.g., Brandão et al. 2018). Moreover, aggressive behaviors were grouped in displays – interactions without physical contact – and attacks – physical encounters that may result in body injuries. In our study, we grouped undulation and butting as attacks, and frontal and lateral exhibitions, as displays.

Sociability test

This test measured the preference of the focal fish to stay close to the social group, formed by conspecifics, or to stay on the opposite side of the test aquarium, isolated from social group. In this protocol, the subject preference to stay closer to conspecific indicates higher sociability (Magurran 1990). Two computer screens (18 inches each, identical video configurations) were placed in each side of the glass aquarium (120 × 60 × 40 cm, ca. 432 L);,with one screen exhibiting a video record of an aquarium housing a group of 4 conspecifics – two males and two females –, and the other exhibiting an aquarium filled only with water (Fig. 1c). The use of images and video records simulating conspecifics was already validated and is currently a well-used methodology to test fish sociability (see Kohda et al. 2015; Müller et al. 2017; Scherer et al. 2017). The aquarium's floor was divided into 10 zones (12 cm each), according to its length. The preference zone was delimited in the length of one zone, meaning that 12 cm was the maximum distance that the focal fish could be from the screens in order to consider that there was a preference by one out of the two social conditions (Fig. 1c). The focal fish was transferred from its home tank to the test aquarium where behavioral quantification took place (10 min video recording). After that, fish was returned to its home tank, handled by a small net.

We confined the focal fish in the middle of the test aquarium before start recordings. Fish displacement was filmed three times (10 min each3 hours) by a camera placed above the aquarium. We quantified the position of the focal fish in each zone as well as the time spent by

the individual in these preference zones (fish was considered to be inside a preference zone when its eyes had crossed the preference zone line). The sides exhibiting the social group *vs*. the empty aquarium was counterbalanced between the replicates. For analysis, we made a ratio of preference, which was calculated by dividing the time spent by the subject in the social group zone by the sum of time spent in both preference zones.

Inhibitory control test

The inhibitory control test is considered a robust method to measure cognitive flexibility in problems' resolution (Amici et al. 2008; MacLean et al. 2014; Lucon-Xiccato et al. 2017), and it demands high self-control from as individual to resist to a stimulus when the animal is aiming a higher or more advantageous goal (Hauser 1999). This executive motor function measures the individual capability to understand that there is a barrier between itself and its goal, and the animal must, therefore, realize an alternative route, the detour, to reach the goal (Diamond 1990; Lucon-Xiccato et al. 2017; Kabadayi et al. 2018).

This test was carried out in the home tank wherein the focal fish lived individually since the day 1. Here, the fish should learn to realize a detour around a U-shaped apparatus to reach a food reward (i.e., small slices of processed meat, Fig. 1d). The test had two phases: the first one called a training phase, and the second with a test phase. In the training phase, the U-shaped apparatus was opaque, covered with a black adhesive, while in the test phase the apparatus was transparent. During the training, the focal fish was restrained by a transparent barrier in one of the aquarium's ends, while the opaque U-shaped apparatus was allocated in the opposite side. After that, the barrier was removed and the focal fish had 10 minutes to detour the apparatus and eat. The trainings consisted of 20 trials subdivided into 10 trials in the morning and 10 trials in the afternoon, filmed by upper cameras during 10 minutes each session. The training phase aimed to teach the animal that there was a barrier (12 cm width by 27 cm height, with two lateral wings of 3 cm each) to be detoured in order to achieve the food reward. In this phase, the focal fish must achieve a learning criterion to be promoted to the test phase, started in the next day. To account for the learning criterion, the 20 training sessions were separated into four blocks of 5 trials and the learning criterion used was that the animal detoured the opaque apparatus correctly (i.e., without entering the incorrect zone formed by the apparatus' wings; Fig. 1d) in 4 out of 5 trials (80% of correct responses). This protocol was based in the one used for Nile tilapia in our laboratory (see Brandão et al. 2019), although some methodological adjustments have been made.

In the test phase, we changed the opaque U-shaped apparatus by a transparent one, composed by the same dimensions of the previously used. As the apparatus was now transparent, subjects would initially try to reach reward directly through the glass, without perform detour-reaching task learnt in the previous training phase. The test phase had also 20 trials, but was realized into two consecutive days (10 trials/day of 10 minutes each, with 5 trials

in the morning and 5 trials in the afternoon), differently from what we done in the training phase. We considered this phase to be more exhaustive for the fish, as the individual should recall the learned task, avoid trying cross a transparent barrier and detour it. We quantified the number of trials fish took to learn the task (i.e., fish had to detour the opaque apparatus and eat, without entering the incorrect zone formed by the wings of the U-shaped apparatus) in the training phase. In the test phase, we quantified the time fish spent trying to achieve the food through the glass in the, the latency for fish to detour the transparent apparatus and the percentage of correct responses (i.e., fish had now to detour an transparent U-shaped apparatus, without entering the incorrect zone formed by the apparatus wings) in the 20 trials.

Brain collection

At the end of day 6, immediately after the inhibitory control test was concluded, fish were euthanized with benzocaine (180 $mg.L^{-1}$) fallowed by skull removal and brain collection. The brain was photographed (dorsal, lateral and ventral views, Fig. 2) and immediately dissected into four brain structures – telencephalon, diencephalon, optic tectum and cerebellum – that were finally weighted, cleaned to remove any blood vessel that might have remained in the tissue, and fixed individually in 4% paraformaldehyde for 24 hours. After that, each structure was transferred to a sucrose solution during overnight (4 °C) and stored in an antifreeze solution (-20 °C) until processing. Olfactory bulb and brainstem were also collected, even though not analyzed in this work.

Isotropic fractionator and brain cells quantification

This step followed the protocol already used for several vertebrates, which consist in dissociate a giving structure in a free nuclei solution of neuronal and nonneuronal cells (Herculano-Houzel 2011). The structures were removed from the antifreeze solution and gently homogenized into an isotropic solution. After tissue dissociation, we stained the free nuclei with DAPI (4', 6-diamidino- 2-phenylindole, a dye with high affiliation to the DNA in all the cells) to afford us to count for the total free nuclei in the sample. This was done placing four aliquots of 10 µl of the same sample were collected and placed in Neubauer chambers and counting 10 central squares of the Neubauer chamber, for all the samples. The total number of free nuclei was achieved multiplying the mean number of nuclei counted in the four aliquots by the volume of the 10 central squares in the Neubauer chamber multiplied, one more time, by the volume in ml of the initial sample (1 ml of solution with free nuclei).

Neurons' number was determined by immunocytochemistry. New aliquots of 500µl from the original sample were collected and anti-NeuN primary antibody (Merck Millipore, Germany) was used for stain the neuronal nuclei. After that, a new aliquot of 10 µl of this sample was again placed in the Neubauer chamber and we counted the percentage of DAPI-labeled nuclei that was also NeuN-positive. A minimum of 500 DAPI-labeled was counted in order to achieve

statistical power and aliquots were replaced when they were necessary. Total neurons were calculated using the percentage of NeuN-positive nuclei multiplied by the total number of DAPI-labeled nuclei and divided by 100. The nonneuronal cells was inferred using the total number of cells minus the total number of neurons.

After achieving the number of neuronal and nonneuronal cells in each brain structure, we used three different approaches to compare these cells with behavioral tests. In the first one, we used brain cells densities, that was the number of cells divided by the weight of the structure (mg). In the second, we used the total number of cells without any normalization and, for last, the number of brain cells divided by the fish standard length (SL, cm), measured from the fish snout to the end of its caudal peduncle.

Measurements of brain area's volume

After brain had being removed from the skull, it was placed in a Petri dish and photographed by the dorsal, lateral (left side) and ventral sides (Fig. 2) using a dissection microscope (Opton TIM-2T) and a digital camera (Opton TA-0124-B and TCapture software). The methodology used for the measurements were based on the ellipsoid model already used by Pollen et al. (2007) and Gonzalez-Voyer & Kolm (2010) to measure brain volume in other cichlid species: $S_v = (L \times W \times H)\ \pi/6$. To obtain the structure volume (S_v), each structure's length (L), width (W) and height (H) were measured (Fig. 2) exactly as in Pollen et al. (2007) using ImageJ software (v.1.50i, National Institutes of Health, USA). For two-hemisphere structures, we just doubled the structure's final volume.

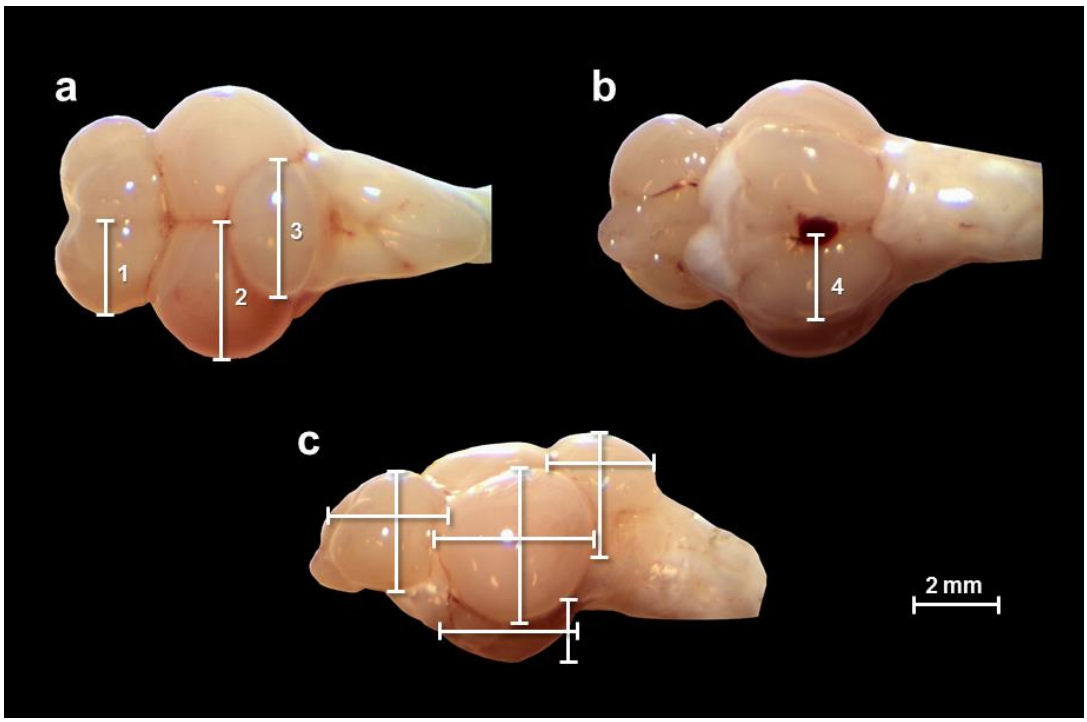

Figure 2. Measurements made from the **a.** dorsal (length), **b.** ventral (length), and **c.** lateral (width and height) sides of the brain to determine the structures' volumes of the **1.** telencephalon, **2.** optic tectum, **3.** cerebellum and **4.** diencephalon. Photos from the author M.L. Brandão.

Statistical analysis

Data analysis was done by using the free software R, version 3.5.1 (http://www.r-project.org). We checked our data for normality using Shapiro-Wilks test, and homoscedasticity by Fmax test (Quinn and Keough 2002). After that, we checked for males and females' differences in behavioral tests using Linear Models (LM), with quantified behaviors as the response variables, and sex as predictor variables. Although LM data fitted parametrical assumption, in the correlation tests some variables presented non-normal distribution. Therefore, we used the non-parametric Spearman's correlation test corrected by Bonferroni ["corr.test" function of the "psych" package (Revelle 2018)], to look for interactions between quantified behaviors and neuroanatomical measures (i.e., brain cells' densities, total number of brain cells and total number of brain cells divided by fish SL). Statistical significance was set at $p = 0.05$. However, in correlations with $R > 0.6$ we considered a marginal trend towards significance when $p \leq 0.08$, based on Pollen et al. (2007).

Ethical statement

This project was registered in the Authorization and Information System in Biodiversity (SISBIO-ICMBio, Brazil, permit number 54287-1), which allows the collection of biological material in natural environments for research purposes. It was also conducted according to the ethical principles on animal experimentation adopted by the National Council for the Control of Animal

Experimentation (CONCEA – Brazil) and approved by the Committee on Ethics in Animal Use, UNESP, São José do Rio Preto, SP, permit number 156/2016.

Results

Behavioral tests

Here, we checked whether the performance between behavioral tests was correlated (table 1). Indeed, we could observe interesting correlations showing that the data we quantified were in fact good predictors for social and cognitive factors. In the aggressiveness test, fish that emitted more attacks were also those that showed more displays towards the mirror, revealing animals we a higher predisposition to engage in an aggressive encounter. In the inhibitory control test, there was also positive correlation between time that fish spent trying to cross the transparent barrier to achieve the food and the latency to realize the detour (table 1), as expected. The percentage of correct responses along the test phase was also negatively correlated with the time fish took trying to cross the glass and the latency to realize the detour, revealing worse individual performance on the task in general.

Table 1: Spearman's correlations between quantified behaviors.

Behavioural tests	Quantified behaviours	Attacks	Displays	Latency for the first aggression	Ratio close to the group	Number of trials to learn	Time on the glass	Latency to detour the barrier
Aggressiveness (mirror- elicited fights)	**Attacks**							
	Displays	**r = 0.80** **p = 0.01**						
	Latency for the first aggression	r = -0.43 p = 0.29	r = -0.56 p = 0.14					
Sociability (social group preference)	**Ratio close to the group**	r = -0.05 p = 0.91	r = -0.02 p = 0.96	r = 0.41 p = 0.32				
Inhibitory control (detour-reaching task)	**Number of trials to learn**	r = 0.53 p = 0.17	r = 0.58 p = 0.13	r = -0.21 p = 0.61	r = 0.31 p = 0.44			
	Time on the glass	r = 0.09 p = 0.82	r = 0.08 p = 0.84	r = -0.14 p = 0.73	r = -0.15 p = 0.71	r = -0.34 p = 0.40		
	Latency to detour the barrier	r = 0.05 p = 0.91	r = 0.29 p = 0.49	r = -0.33 p = 0.42	r = -0.23 p = 0.58	r = -0.02 p = 0.95	**r = 0.78** **p = 0.02**	
	% correct responses	r = 0.33 p = 0.42	r = 0.34 p = 0.41	r = -0.28 p = 0.50	r = 0.19 p = 0.65	r = 0.44 p = 0.27	**r = -0.81** **p = 0.01**	**r = -0.70** **p = 0.05**

Significance was settled at $p \leq 0.05$. Significant correlations are in bold and gray.

By looking at the variation of our behavioral data in a standardized form (Fig. 3), we could see that our number of samples were sufficient to provide a variation in behavioral performances. For this, we used an individual score of each subject standardized by subtracting the mean and dividing by the standard deviation of each quantified behavior. In each behavior, we could see a variation by more than one standard deviation around the mean. This showed us that the lack of correlation was not an artifact of low variance in our sample.

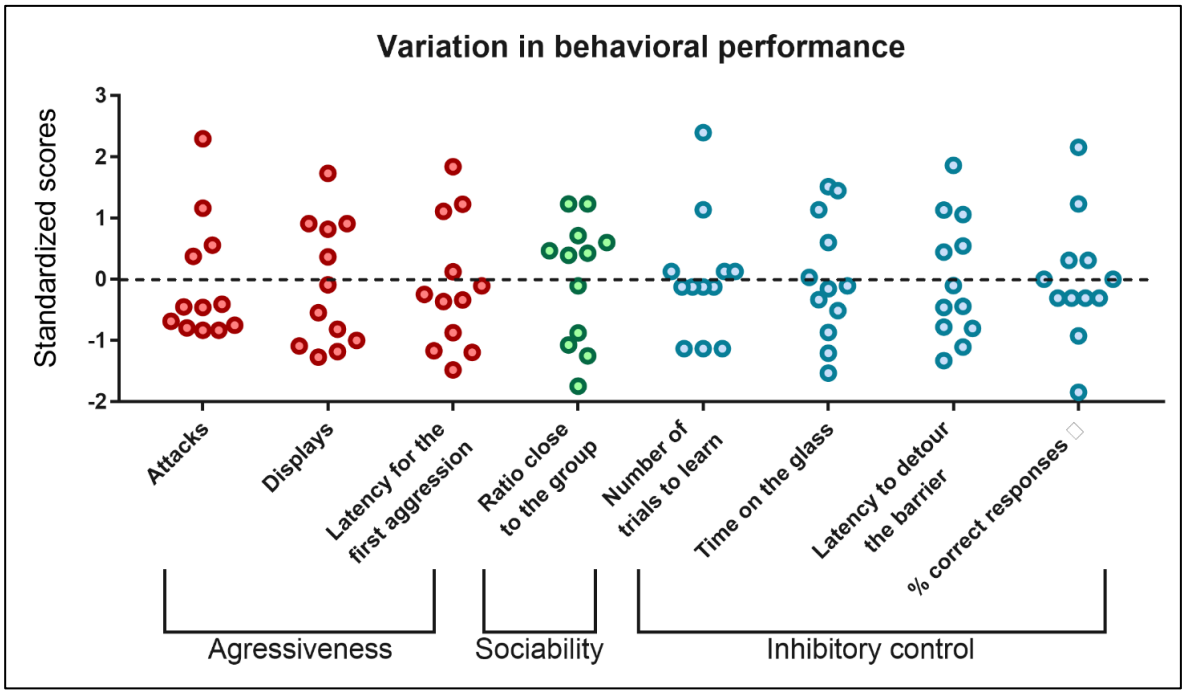

Figure 3. Variation in behavioral performance for each quantified behavior (red: aggressiveness test; green: sociability test; and blue: inhibitory control test).

Males and females did not show differences concerning behavioral tests

There was no significant differences between males and females concerning aggressiveness, sociability and inhibitory control tests (table 2). Thus, we analyzed the data of both sexes together.

Table 2: Linear regressions using individual sex as predictor variable.

Behavioural tests	Quantified behaviours	F	P
Aggressiveness (mirror- elicited fights)	**Attacks**	0.46	0.51
	Displays	3.08	0.11
	Latency for the first aggression	0.03	0.85
Sociability (social group preference)	**Ratio close to the group**	2.16	0.17
Inhibitory control (detour-reaching task)	**Number of trials to learn**	0.32	0.58
	Time on the glass	0.28	0.6
	Latency to detour the barrier	0.34	0.57
	% correct responses	0.11	0.74

Significance assumed with $p \leq 0.05$.

To check for correlations between behavioral test and brain, we used neuronal and nonneuronal cells densities, total number of neurons and nonneuronal cells and total number of neurons and nonneuronal cells divided by the fish standard length. We correlated brain volumes with behavioral and cognitive complexity, and also compared structure volumes with our quantified behaviors.

Table 3. Spearman's correlations between behavioral tests and densities of neurons and nonneuronal cells. Values were correct by Bonferroni test.

Behavioral tests	Quantified behaviors	Density of neurons				Density of nonneuronal cells			
		tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Aggressiveness (mirror- elicited fights)	**Attacks**	r = -0.21	r = 0.47	r = -0.50	r = -0.28	r = -0.45	r = 0.07	r = 0.14	**r = -0.74**
		p = 0.61	p = 0.23	p = 0.21	p = 0.49	p = 0.26	p = 0.86	p = 0.73	**p = 0.03**
	Displays	r = -0.25	r = 0.42	r = -0.42	r = -0.22	r = -0.07	r = 0.01	r = 0.09	**r = -0.73**
		p = 0.55	p = 0.30	p = 0.31	p = 0.59	p = 0.86	p = 0.98	p = 0.82	**p = 0.04**
	Latency for the first aggression	r = 0.59	r = 0.24	**r = 0.78**	r = 0.52	r = 0.31	r = -0.16	r = 0.33	r = 0.55
		p = 0.12	p = 0.57	**p = 0.02**	p = 0.18	p = 0.45	p = 0.69	p = 0.42	p = 0.16
Sociability (social group preference)	**Ratio close to the group**	r = 0.15	r = 0.24	r = -0.01	***r = 0.63***	r < 0.01	r = 0.20	***r = 0.66***	r = -0.31
		p = 0.71	p = 0.57	p = 0.98	***p = 0.09***	p = 1	p = 0.63	***p = 0.07***	p = 0.45
Inhibitory control (detour-reaching task)	**Number of trials to learn**	***r = -0.68***	***r = 0.65***	r = -0.13	r = 0.42	r = -0.49	r = 0.13	r = -0.02	**r = -0.80**
		p = 0.06	***p = 0.07***	p = 0.74	p = 0.30	p = 0.21	p = 0.75	p = 0.95	**p = 0.01**
	Time on the glass	r = 0.31	r = -0.54	r = -0.40	**r = -0.71**	r =0.02	r = -0.40	r = -0.16	r = 0.14
		p = 0.45	p = 0.16	p = 0.32	**p = 0.04**	p = 0.95	p = 0.32	p = 0.69	p = 0.73
	Latency to detour the barrier	r < 0.01	r = -0.52	r = -0.31	*r = -0.64*	r = -0.05	***r = -0.66***	r = -0.55	r = 0.05
		p = 1	p = 0.18	p = 0.45	*p = 0.08*	p = 0.91	***p = 0.07***	p = 0.16	p = 0.91
	% correct responses	r = -0.40	r = 0.57	r = -0.11	r = 0.47	r = -0.09	***r = 0.64***	r = 0.39	r = -0.57
		p = 0.32	p = 0.14	p = 0.80	p = 0.23	p = 0.82	***p = 0.08***	p = 0.34	p = 0.14

Significance was settled at $p \leq 0.05$. Significant correlations are highlighted in bold and gray. Trends ($p < 0.10$) are highlighted in bold and italic.

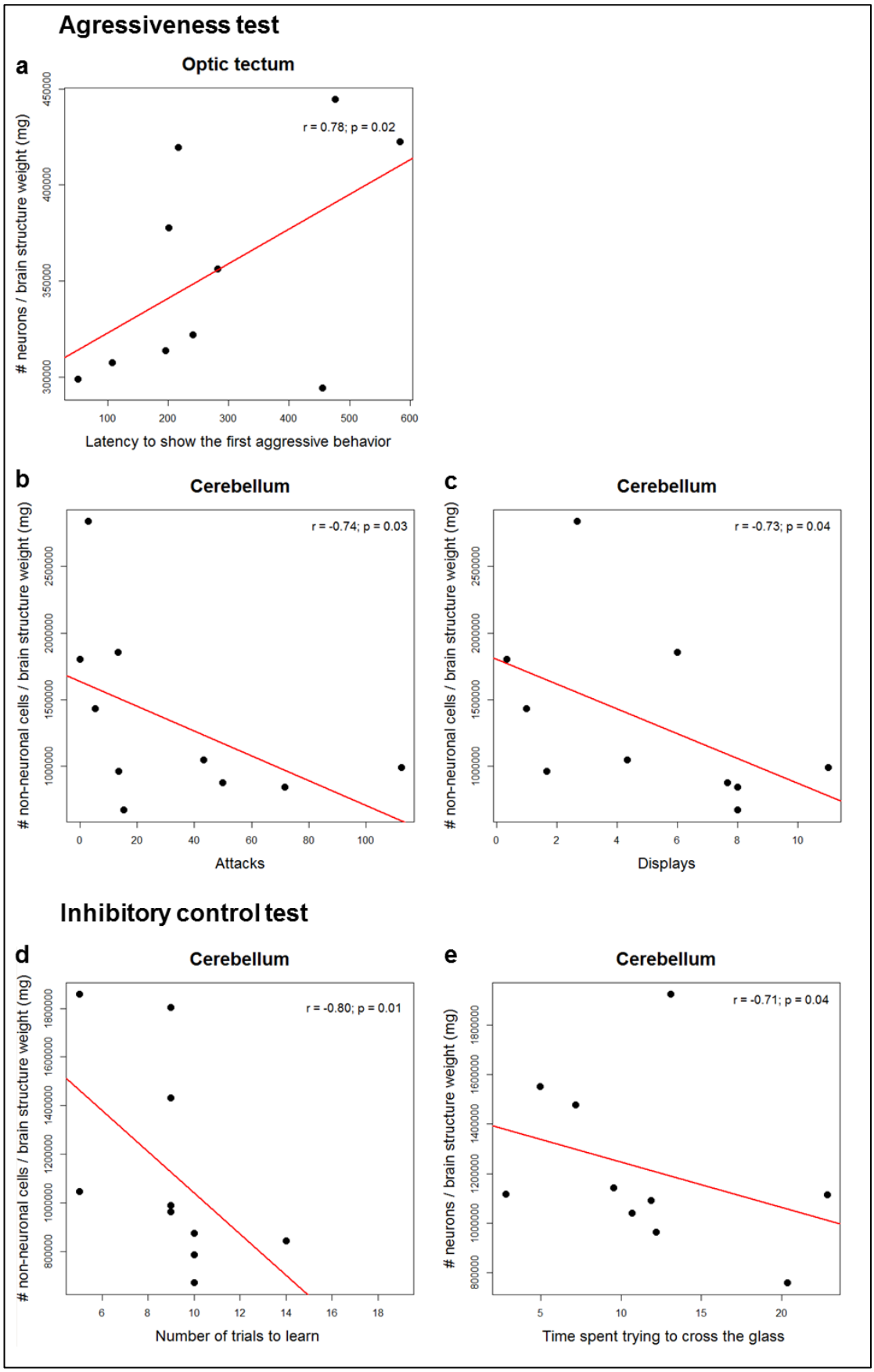

Figure 4. Spearman's correlations between behavioral tests (Aggressiveness test: **a.** optic tectum**,** and **b.**, **c.** cerebellum; Inhibitory control test: **d., e.** cerebellum) and brain cells densities. Only graphics with statistical differences are shown.

Aggressiveness and brain cells

Putting females and males' data together, attacks and displays showed a negative correlation with density of nonneuronal cells in the cerebellum (table 3; Fig. 4b, c). Latency to emit the first aggressive behavior was positively correlated to the density of neurons in the optic tectum (Fig. 4a), showing a relation to the brain visual center and time individuals took to engage in a fight.

Sociability and brain cells

For the sociality test, we used a ratio of individual preference to stay close to the social group. Different from the other two behavioral tests realized in this work (i.e., the aggressiveness and the inhibitory control tests), the Spearman's correlation did not show any significant correlation between the sociability test and the other variables tested in this study (tables 3, 4 and 5). There was just a statistical and positive trend regarding density of nonneuronal cells in the optic tectum and the preference to stay close to conspecifics, and although the correlation was somewhat strong ($r = 0.66$, table 3), it was marginally significant ($p = 0.07$).

Table 4: Spearman's correlations between behavioral tests and total number of neurons and nonneuronal cells.

Behavioral tests	Quantified behaviors	Number of neurons				Number of nonneuronal cells			
		tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Aggressiveness (mirror-elicited fights)	**Attacks**	r = 0.14 p = 0.73	r = 0.24 p = 0.57	r = 0.05 p = 0.91	r = 0.52 p = 0.18	r = -0.05 p = 0.91	r = -0.19 p = 0.65	r = 0.36 p = 0.38	r = -0.19 p = 0.65
	Displays	r = 0.07 p = 0.86	r = 0.41 p = 0.31	r = 0.32 p = 0.43	r = 0.48 p = 0.23	r = 0.33 p = 0.41	r = 0.01 p = 0.98	r = 0.38 p = 0.35	r = 0.01 p = 0.98
	Latency for the first aggression	r = 0.43 p = 0.29	r = 0.19 p = 0.65	r = 0.28 p = 0.49	r = -0.09 p = 0.82	r = -0.24 p = 0.57	r = -0.38 p = 0.35	r = 0.12 p = 0.78	r = 0.28 p = 0.49
Sociability (social group preference)	**Ratio close to the group**	r = 0.15 p = 0.71	r = 0.24 p = 0.57	r = -0.20 p = 0.63	r = 0.57 p = 0.13	r = -0.06 p = 0.89	r = -0.37 p = 0.36	r = 0.39 p = 0.33	r = -0.07 p = 0.86
Inhibitory control (detour-reaching task)	**Number of trials to learn**	r = -0.43 p = 0.28	**r = 0.71** **p = 0.04**	r = 0.11 p = 0.79	r = 0.44 p = 0.27	r = -0.25 p = 0.55	r = 0.03 p = 0.93	r = -0.08 p = 0.83	r = -0.62 p = 0.10
	Time on the glass	r = 0.45 p = 0.26	***r = -0.64*** ***p = 0.08***	r = 0.19 p = 0.65	r = 0.02 p = 0.95	r = 0.26 p = 0.53	r = 0.07 p = 0.86	r = 0.16 p = 0.69	r = 0.24 p = 0.57
	Latency to detour the barrier	r < 0.01 p = 1	r = -0.38 p = 0.35	r = 0.33 p = 0.42	r = -0.24 p = 0.57	r = 0.14 p = 0.73	r = 0.07 p = 0.86	r = -0.21 p = 0.61	r = -0.02 p = 0.95
	% correct responses	r = -0.31 p = 0.44	r = 0.56 p = 0.15	r = -0.33 p = 0.43	r = 0.39 p = 0.34	r = 0.02 p = 0.95	r = 0.01 p = 0.98	r = 0.19 p = 0.64	r = -0.18 p = 0.66

Significance was settled at $p \leq 0.05$. Significant correlations are highlighted in bold and gray. Trends ($p < 0.10$) are highlighted in bold and italic.

Inhibitory control and brain cells

The response variables used in here are part of the training phase and test phase of the inhibitory control test. Spearman's correlation showed a significant and negative correlation between the number of trials to learn the detour-reaching task in the training phase and the cerebellar density of nonneuronal cells (table 3; Fig. 4d). A negative correlation was also seen between time fish spent trying to cross the glass and the density of neurons in the cerebellum (Fig. 4e). For last, the total number of neurons in the diencephalon was positive correlated to the number of trials to learn (table 4, Fig.5). Using total number of brain cells adjusted with fish standard length, two other correlations appeared (table 5): a positive correlation between diencephalic neurons and the number of trials to learn the task in the training phase (Fig. 6a) and a negative correlation between nonneuronal cells and this same behavior (Fig. 6b).

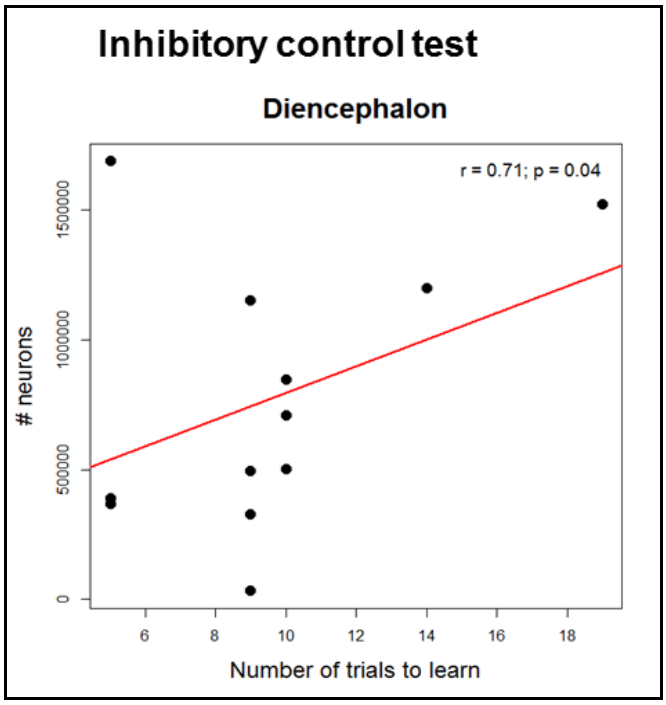

Figure 5. Spearman's correlations between number of trials to learn the detour-reaching task (inhibitory control test – training phase) and the total number of neurons in the diencephalon. Only the graphic with statistical difference is shown.

Table 5: Spearman's correlations between behavioral tests and total number of neurons and nonneuronal cells divided by the fish standard length.

Behavioral tests	Quantified behaviors	Number of neurons / fish SL (cm)				Number of nonneuronal cells / fish SL (cm)			
		tl	**di**	**ot**	**cb**	**tl**	**di**	**ot**	**cb**
Aggressiveness (mirror-elicited fights)	**Attacks**	r = -0.05 p = 0.91	r = 0.24 p = 0.57	r = -0.21 p = 0.61	r = 0.28 p = 0.49	r = -0.09 p = 0.82	r = -0.12 p = 0.78	r = 0.35 p = 0.38	r = -0.28 p = 0.49
	Displays	r = -0.11 p = 0.80	r = 0.41 p = 0.31	r = -0.14 p = 0.73	r = 0.29 p = 0.49	r = 0.27 p = 0.51	r = 0.07 p = 0.86	r = 0.38 p = 0.35	r = -0.09 p = 0.82
	Latency for the first aggression	r = 0.52 p = 0.18	r = 0.19 p = 0.65	r = 0.40 p = 0.32	r = -0.05 p = 0.91	r = -0.19 p = 0.65	r = -0.59 p = 0.12	r = 0.12 p = 0.78	r = 0.24 p = 0.57
Sociability (social group choice)	**Ratio close to the group**	r = 0.03 p = 0.93	r = 0.24 p = 0.57	r = -0.51 p = 0.19	r = 0.48 p = 0.23	r = -0.21 p = 0.61	r = -0.49 p = 0.21	r = 0.39 p = 0.33	r = -0.09 p = 0.82
Inhibitory control (detour-reaching task)	**Number of trials to learn**	r = -0.62 p = 0.10	**r = 0.71** **p = 0.04**	r = -0.13 p = 0.75	r = 0.32 p = 0.44	r = -0.37 p = 0.36	r = 0.07 p = 0.86	r = -0.08 p = 0.84	**r = -0.71** **p = 0.04**
	Time on the glass	r = 0.40 p = 0.32	***r = -0.64*** ***p = 0.08***	r = 0.12 p = 0.78	r = -0.26 p = 0.53	r =0.33 p = 0.42	r < 0.01 p = 1	r = 0.16 p = 0.69	r = 0.36 p = 0.38
	Latency to detour the barrier	r < 0.01 p = 1	r = -0.38 p = 0.35	r = 0.21 p = 0.61	r = -0.52 p = 0.18	r = 0.21 p = 0.61	r = 0.09 p = 0.82	r = -0.21 p = 0.61	r = 0.05 p = 0.91
	% correct responses	r = -0.40 p = 0.32	r = 0.56 p = 0.15	r = -0.48 p = 0.22	r = 0.58 p = 0.13	r = -0.11 p = 0.80	r = 0.12 p = 0.77	r = 0.19 p = 0.64	r = -0.29 p = 0.48

Significance was settled at $p \leq 0.05$. Significant correlations are highlighted in bold and gray. Trends ($p < 0.10$) are highlighted in bold and italic.

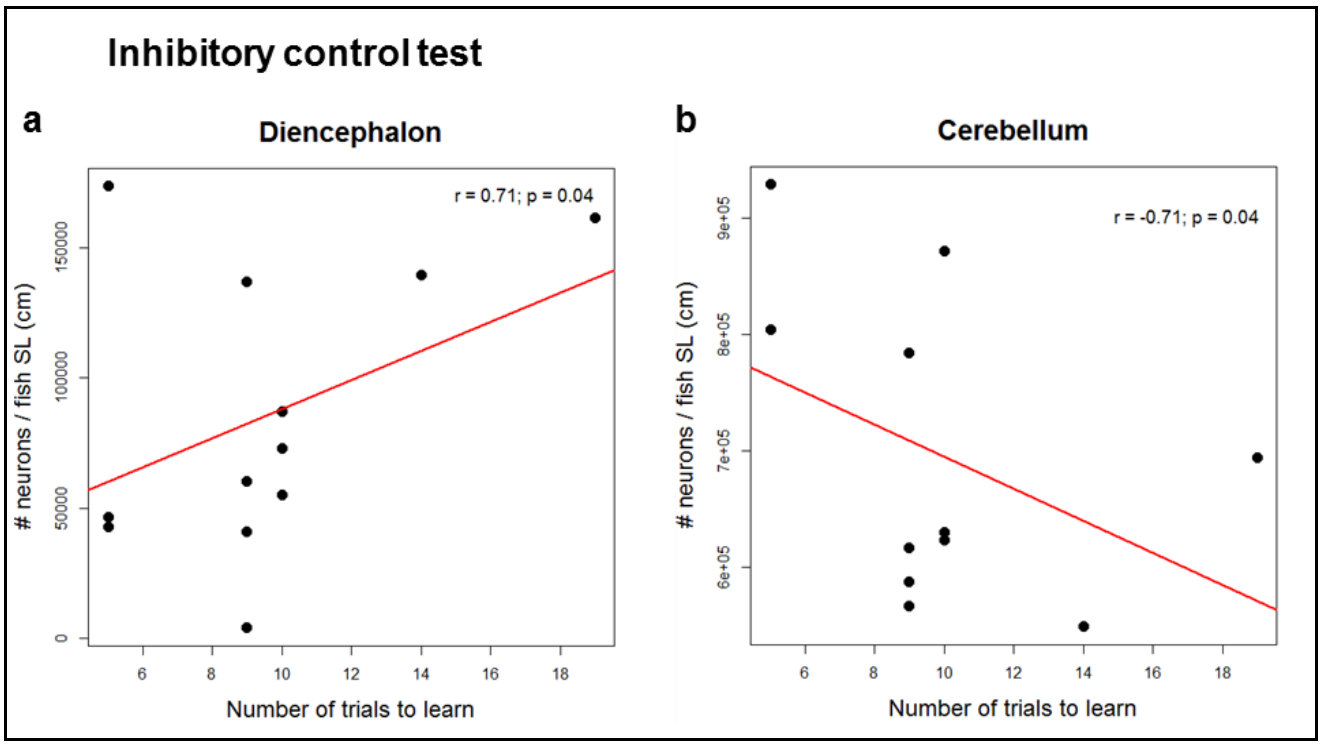

Figure 6. Spearman's correlations between number of trials to learn de detour-reaching task in the inhibitory control test and the ratio of neurons by standard length in the **a.** diencephalon and **b.** cerebellum. Only graphics with statistical differences are shown.

Brain structure volumes

As several works have correlated brain volumes with behavior and cognitive processes, here we wanted to look at this variable to see whether this was also true for our quantified behaviors. There was a positive correlation between diencephalon volume and displays towards the mirror image (table 7; Fig. 7a). There was also two positive correlations between optic tectum volume and the time fish spent trying to cross the glass and the latency to realize the detour in the inhibitory control test (table 6; Fig. 7b, c).

Table 6. Spearman's correlations between quantified behaviors and structure volumes.

Behavioral tests	Quantified behaviors	Brain structure volumes (mm^3)			
		tl	**di**	**ot**	**cb**
Aggressiveness (mirror-elicited fights)	**Attacks**	r = 0.09	r = 0.57	r = 0.21	r = -0.09
		p = 0.82	p = 0.14	p = 0.61	p = 0.82
	Displays	r = -0.04	**r = 0.80**	r = 0.36	r = 0.27
		p = 0.91	**p = 0.01**	p = 0.38	p = 0.51
	Latency for the first aggression	r = -0.19	r = -0.05	r = -0.59	r = -0.24
		p = 0.65	p = 0.91	p = 0.12	p = 0.57
Sociability (social group preference)	**Ratio close to the group**	r = -0.09	r = 0.23	r = -0.44	r = 0.18
		p = 0.82	p = 0.59	p = 0.27	p = 0.67
Inhibitory control (detour-reaching task)	**Number of trials to learn**	r = 0.16	r = 0.58	r = -0.21	r = 0.08
		p = 0.70	p = 0.13	p = 0.62	p = 0.84
	Time on the glass	r = 0.09	r = 0.14	**r = 0.83**	r = 0.62
		p = 0.82	p = 0.73	**p = 0.01**	p = 0.10
	Latency to detour the barrier	r = -0.19	r = 0.26	**r = 0.88**	***r = 0.66***
		p = 0.65	p = 0.53	**p = 0.004**	***p = 0.07***
	% correct responses	r = 0.07	r = 0.08	r = -0.56	r = -0.42
		p = 0.86	p = 0.84	p = 0.15	p = 0.29

Significance was settled at $p \leq 0.05$. Significant correlations are highlighted in bold and gray. Trends ($p < 0.10$) are highlighted in bold and italic.

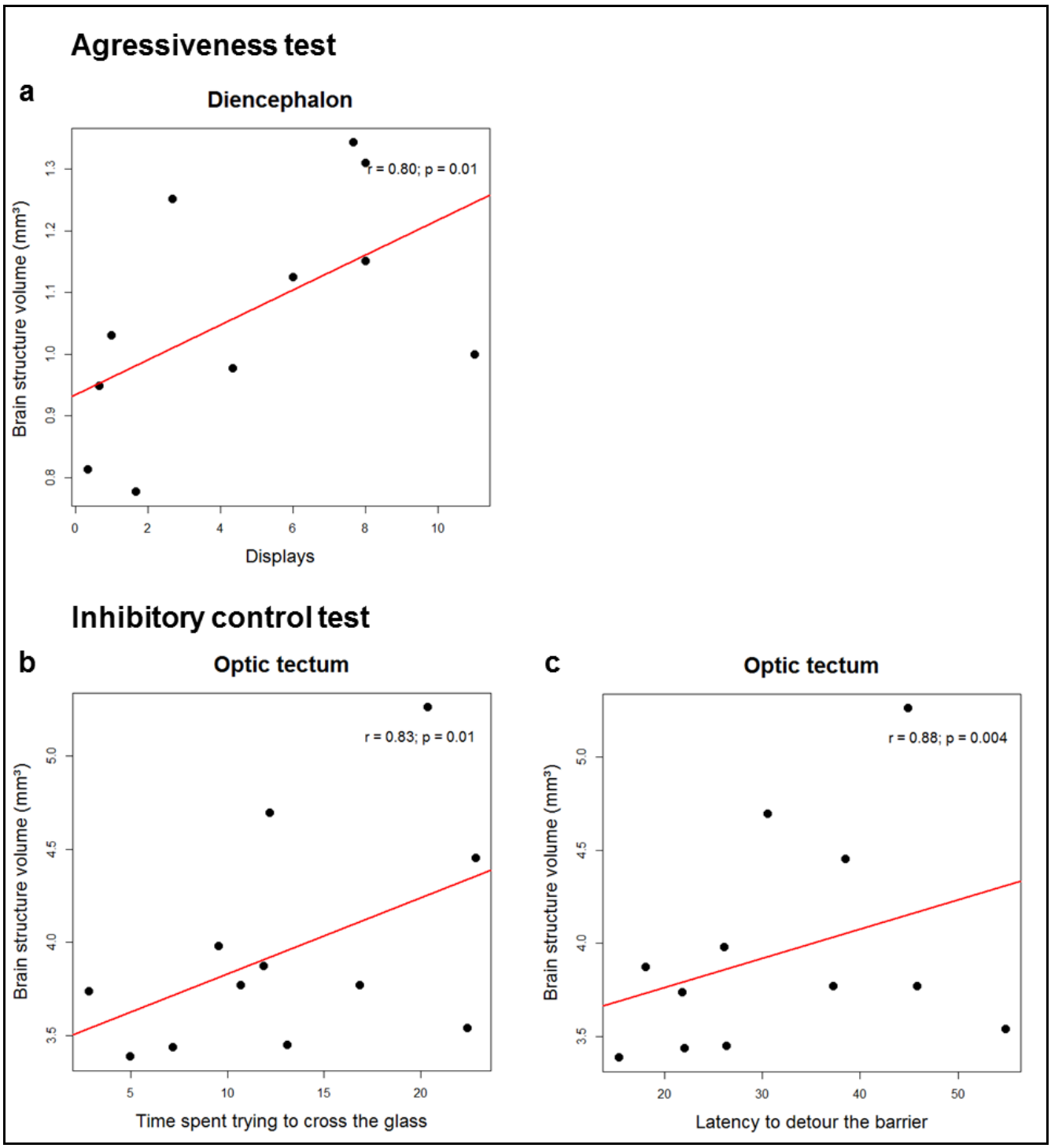

Figure 7. Spearman's correlations between behavioral tests (Aggressiveness test: **a.** diencephalon; Inhibitory control test: **d., e.** optic tectum) and brain structure volumes. Only graphics with statistical differences are shown.

Discussion

In this study, we used three behavioral tasks to infer social behaviors and cognitive flexibility in a monogamous cichlid species with biparental care of the fry, the *Geophagus brasiliensis*. We hypothesized that a social species would present correlations between sociality indicators and brain structures known to be linked to social behavior, as the

telencephalon, for example. Nevertheless, significant correlations appeared mainly in the cerebellum, revealing an important role of this structure in the social indicator tests used here. Monogamous cichlid fish, such as *G. brasiliensis*, usually present biparental brood care. Although males and females can change their roles during parental care, generally females will be in charge of ventilate the eggs and pay attention to the newborn fry, while males will spend most of their time defending the nest and chasing away predators (Wimberger 1992; Bastos et al. 2011). These differences, however, were not translated in significant differences between behaviors and brain structures among sexes, as we expected. Therefore, males and females pearl cichlids were grouped for analysis in the behavioral tests, and data are discussed accordingly.

The tests we choose to measure sociality and cognition (i.e., aggressiveness, sociability and inhibitory control tests) showed interesting correlations between them. In the aggressiveness test, fish that gave more attacks were also those that showed more displays towards the mirror, revealing animals with a higher predisposition to engage in fights. In the inhibitory control test, we found a positive correlation between the time that fish spent trying to cross through the transparent barrier and the latency to realize the detour. Percentage of correct responses along the test phase was negatively correlated with the time fish took trying to cross the barrier and the latency to realize the detour. These correlations show that data variation occurred as expected for such behavioral indicators and therefore it is indeed a representative of *G. brasiliensis* behavioral performance.

Among brain structures, the telencephalon is considered to be more associated to the organization of social behaviors. Nevertheless, our analysis only showed a tendency to significance with a strong correlation value between neurons density in the telencephalon and number of trials to learn in the inhibitory control task. Individuals with fewer cells in this area took more trials to learn the detour-reaching task, evidencing the well-known role of this region in learning skills (see Shumway 2010; Broglio et al. 2011). Nevertheless, this trend was the only association observed for the telencephalic region and behavioral repertoire in this study. On the other hand, we observed significant associations for the other three brain structures analyzed in this work – the diencephalon, optic tectum and cerebellum, which indicates the role of brain cells quantity controlling some aspect of fish social behavior.

Diencephalic volume was related to a higher frequency of displays exhibited towards a virtual opponent, but not to attacks. Indeed, the diencephalon is a key structure related to social behavior in cichlid fishes (Pollen et al. 2007), mainly for its role in the production and expression of two important neuropeptides in the hypothalamic preoptic area, the vasotocin and the isotocin (Hoyle 1999; Reddon et al. 2017). Vasotocin regulates

dominant and subordinate behaviors in several cichlid species (see Greenwood et al. 2008; Almeida et al. 2012; Almeida and Oliveira 2015; Maruska and Fernald 2018), while isotocin, although not well known as vasotocin, seems to be related to increased responsiveness in front of social information (Reddon et al. 2012), such as affiliative behavior (Reddon et al. 2015; Reddon et al. 2017). Isotocin and vasotocin are produced in three neuronal groups located in the preoptic area: the parvocellular, containing the smaller population of cells; and the magnocellular and gigantocellular areas, that present the bigger cells (Bradford and Northcutt 1983). Studies with different fish species seems to show a tendency of the bigger cells to be more related to dominant status and territorial behavior than the smaller cell' population (Greenwood et al. 2008). Therefore, we can speculate that these cells' size might be reflected in the volume of the diencephalon. Thus, the positive correlation between diencephalic volume and displays seen in our study may be related to a more cautious aggressive behavior when a possible opponent appears. Individuals with a higher number of cells in the diencephalon may have a more refined way of assessing the social environment and making decisions about the contests with which they will be involved.

Besides nonapeptides hormones, the hypothalamus also regulates the hormonal cascade of androgens in the hypothalamus-pituitary-gonad axis, which are associated to social challenge (Almeida et al. 2014). In Neotropical cichlids, for instance, estradiol also is associated to social interactions (see Ramallo et al. 2017; Scaia et al. 2018). The hypothalamus also has a well-conserved role in fight-or-flight responses across vertebrates (Wingfield 2013), which can interfere in the behaviors we tested here. The hypothalamus-pituitary-interrenal axis modulates the way individuals interact with the social environment and with the risks of predation, fights, injuries and detrimental consequences from this environment. The final patch of this axis, the interrenal cells in fish, will therefore produce and liberate glucocorticoids that works catabolizing energy for the individuals to deal with a giving stressful situation. On the other hand, extreme situations may lead to a detrimental effect of glucocorticoids in several cognitive processes in fish, as learning and memory (see Barreto et al. 2006; Gaikwad et al. 2011). In here, we observed that the total neuronal cells in the diencephalon was positively correlated to a longer time to learn the detour-reaching task, a trend also showed regarding density of neurons in this area, which may indicate a possible influence of HPA axis on this learning process. Nevertheless, we did not measure cortisol hormones in this study. Besides the role of glucocorticoids, a recent study with zebrafish pointed a behavioral homology of fear response generated by hypothalamic regions, also seen in mammals (Lal et al. 2018). Thus, besides the effects originated from stress, the fear response initiated by hypothalamic neurons may be another way affecting the inhibitory

control task. A possible neophobia could make animals to take more trials to learn the detour-reaching task, not by a lack of cognitive capability, but because of an initial state of fear (e.g., Regolin et al. 1995; Brandão et al. 2019). In spite of that, after achieve the learn criterion, individuals with more cells in the diencephalon presented a tendency to show a higher percentage of correct responses in the test phase, with a reduced time spent trying to cross the glass and detour the transparent apparatus. This shows us that, even if the test itself could produce a stressful experience to individuals at the beginning, it did not seem to affect the final steps of it.

As seen for the diencephalon, the interactions we found between cerebellum and behavioral responses would also be related to emotional states in the pearl cichlid. In our study, subjects with a higher density of cerebellar cells seems to be slower to attack, spent more time near conspecifics and persist less in irrelevant situations – i.e., time trying to cross the transparent glass – revealing a more cautious animal. In mammals, several studies have shown that the cerebellum is very important not only for motor control, but also to coordinates reason, emotion and aggression (Demirtas-Tatlidede and Schmahmann 2013). Electrical stimulations at cerebellum was critical to ameliorate behaviors related to aggression and violence (Cooper et al. 1976; Heath 1977) in humans. In fish, cerebellum is also linked to emotional conditioning, spatial cognition, associative learning and memory processes (Rodríguez et al. 2005; Brown et al. 2011; Ebbesson and Braithwaite 2012; Warren and Sawtell 2016). A recent study with African cichlids of the genus *Ophthalmotilapia* showed that the cerebellum was the structure with the highest gene expression of arginine vasotocin in the brain (Derycke et al. 2018), the nonapeptide above mentioned regulating social interaction in fish. In another study using four labrid cleaner fish species, authors found a significant higher concentration of arginine vasotocin in the cerebellum of species with obligatory cleaners than in facultative ones, which seems to be strongly related to the mutualistic behavior (Kulczykowska et al. 2015). In addition to these results, we found that a higher number of cells in the cerebellum is also related to a decreased aggressiveness in a monogamic cichlid. Individuals with higher densities of cerebellar cells seems to be more cautious to engage in a fight, as they took more time to display the first aggressive behavior towards the mirror and also are less aggressive when the fight actually begins. Thus, it is plausible to assume that the cerebellum has an important role in the control of social aggressive behavior in cichlids, modulated by its cell's density.

Concerning the inhibitory control test, the cerebellar region is known to contribute to the understanding of the errors performance and to the consequent behavioral adjustments in both cognitive and motor domains in humans (Blakemore et al. 2001; Molinari et al. 2008). Cerebellum also regulates the cancellation of a motor response

before the action even starts (Brunamonti et al. 2014) and, when damaged, it causes individuals to perform worse than those with intact cerebellum (Olivito et al. 2017). Giving the brain homologies among vertebrates (Broglio et al. 2005; O'Connell and Hofmann 2011), the cerebellum seems to have a similar role in fish. For example, cerebellar lesions in the goldfish, *Carassius auratus*, led to a dramatic decrease in correct responses when compared with sham animals in a spatial-learning task (Rodríguez et al. 2005). Looking at the anatomical level, a recent study with the guppy, *Poecilia reticulata*, showed that the number of cerebellar neurons outweighs in almost four times the expected number of these cells in this region, when compared with another brain regions, which indicates the importance of the cerebellum in the computational tasks of the brain (Marhounová et al. 2019). Here we observed that individuals with less neuronal and nonneuronal cells in the cerebellum spent more time trying to cross through the transparent barrier, taking more trials to actually learn the task. There was also a trend showing that a slower density of cerebellar neurons was related to a higher latency to realize the detour, which could be somehow related to the previously mentioned emotional evaluation of the situation. Taking together, our results and the previous ones mentioned here indicate that the cerebellum is not a brain structure only controlling fine tune motor skills, but it is also involved in learning and cognitive abilities. Probably, tasks related to spatial learning and motor performance, such as the detour-reaching task, demand more complexity from the cerebellum.

The optic tectum was another structure to present statistic interaction between number of cells and behaviors. A positive correlation between aggressive interactions and a higher density of neurons in the optic tectum seems to be related to an individual that takes more time to engage in a fight. The optic tectum is a brain area well-conserved across vertebrates, and responsible for controlling visual evaluations, individual orientation, and providing egocentric frames of reference for perception and action (Salas et al. 2003; Brown et al. 2011). Here we have used mirror-elicited fights, and fish have fought with their own image, although not so intensively as when the opponent is a real one (personal observation). Studies with other fish species already shown that, in spite of the well-stablished use of the mirror to quantify aggressive behaviors, mirror images may cause divergent physiological and behavioral responses from those seen when a fish fights with a real opponent (Oliveira et al. 2005; Dijkstra et al. 2012; Teles et al. 2016; Li et al. 2018). An even more intriguing study was recent published by Kohda and collaborators (2019), in which they show that the cleaner wrasse, *Labroides dimidiatus,* passes all the phases proposed by the mirror self-recognition test (Kohda et al. 2019), which could be a bias in studies using mirror-elicited fights. Nevertheless, differently from cleaner wrasses, the African cichlid *Neolamprologus pulcher* failed to pass the same mark test, evidencing that this highly social cichlid does not recognize itself in the mirror. However, even though

the pearl cichlid also fights with virtual opponents we cannot discard the possibility that fish somehow lack some important visual information regarding the decision to engage or not in a fight. The number of neurons in the optic tectum could be the responsible for this alternative judgment regarding visual discrimination in aggressive behavior.

We did not find any significant correlation between the time individuals spent close to conspecifics in sociability test and the brain cells in general, but there was statistical trend to significance with strong correlation R-value (i.e., $r > 0.6$). We can interpret this result as the distance provided by our test aquarium was not far enough to provide a visual discrimination between isolation and social group experience for tested subjects. Thus, even when in the opposite side of the group, fish might have perceived itself close to conspecifics. Nevertheless, there was a trend showing that a higher density of neurons in the cerebellum and a higher density of nonneuronal cells in the optic tectum are both associated to individuals that spent more time near the social group. In fact, cichlids have a well-developed visual system, which is indeed important in the social communication (e.g., Castro et al. 2009; Chen and Fernald 2011; Field et al. 2018). White and Brown (2015) demonstrated that species that live in low complexity habitats has a larger optic tectum due to the importance of this area for animals relying strongly on visual cues in these environments. This is the case of *G. brasiliensis*, a species found in low-complex sandy environment (Castro et al. 2004), where a larger optic tectum would provide a greater visual acuity to these species.

The inhibitory control task showed interesting correlations with optic tectum volume and a trend concerning the cerebellum volume. Individuals that took more time trying to cross the glass and, consequently, were slower to detour the transparent apparatus, were those with the higher optic tectum and cerebellum volumes. In a recent study, Fong *et al.* (2019) observed that individuals of the fish *Poecilia reticulata* that were exposed to a spatial-learning task had a larger relative optic tectum size when compared to guppies that faced a reversal-learning task (Fong et al. 2019). We consider that the inhibitory control test we used in this study may represent a spatial-learning task, as the animals have to perform a detour to achieve the food; and also a high flexible and cognitively demanding behavioral task, as those seen in the reversal-learning, as individuals have to inhibit a prepotent response to achieve a higher goal. It is interesting that it was exactly the test parts regarding spatial cognition that were related to optic tectum size in our study, and not the behaviors considered more cognitively demanding, such as the percentage of correct responses, or the number of trials to learn the detour-reaching task, thus showing this could be a rule for brain controlling cognition in fish.

The brain volumes are commonly used in behavioral comparative studies. Several researchers had already found associations between brain structure volumes and mating

systems or parental care. Nevertheless, brain regions are formed by cells with very different roles concerning social behavior and cognition. Neurons are considered to be the cells behind real behavioral complexity and are related to highly demanding cognitive activities (Herculano-Houzel et al. 2015; Fang and Yuste 2017; Kabadayi et al. 2018). Nonneuronal cells of fish are composed in their majority by glial cells (i.e., microglia, radial astrocytes, oligodendrocytes and ependymal cells). However, the traditional view of glial cells only supporting neurons are nowadays considered as a misleading idea (Kjaerby et al. 2017; Mu et al. 2019). Microglial cells, for example, are very important in LTP processing in the mammal hippocampal, and are as important as neurons to learning processes and cognition (Rogers et al. 2011; Kyrargyri et al. 2015). Astrocytes are also related to spatial learning and fear memory (Bracchi-Ricard et al. 2008; Sardinha et al. 2017) and are more important than neurons to stop non-relevant behaviors, driving rapid behavioral changes previously attributed to neurons solely (Mu et al. 2019). Oligodendrocytes present a fundamental role in regulation of cognitive behaviors involving social interactions (Makinodan et al. 2012), in which individuals reared in isolation do not recover normal myelination even after reintroduction to the social group. Thus, besides the importance of neurons in cognitive processes, these studies show that glial cells also present crucial roles for brain and behavior complexity. This might reflect the significant correlations showed in our study concerning nonneuronal cells, or even correlations between some behaviors and particular volume structures.

In this study, we have shown, for the first time, that the number of brain cells is interconnected to social and cognitive behaviors in a cichlid fish. Although the well-documented role of the telencephalon in sociality and cognition regarding its volume and relative size, our study did not reveal any interaction between this brain structure cells and the behaviors investigated. Nevertheless, the other three brain areas – i.e., the diencephalon, cerebellum and optic tectum – presented interesting associations mediating aggressiveness, sociability and executive function. Diencephalon and cerebellum seem to be related to a better evaluation of unrecognized environments and stimuli, revealing individuals that are more cautious in new situations. The optic tectum is in agreement with this idea, showing its importance in visual-information processing, and was the structure that better matches the previously known relationships between structure size and cognitive capacities in fish. In here, we also could differentiate neurons from nonneuronal cells, showing that glial cells seem to be very important in coordinating social interaction and cognitive functions, and not merely structural components of the brain.

Acknowledgements

We thank Ana Carolina dos Santos Gauy, Marcela Cesar Bolognesi and Magda Teles for technical assistance during data collection, and Nuno Pimpão Martins for the development of the macro used in brain cells' counting. This research was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), grant #2016/26160-2 to EGF. MLB was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES #001) – DS for PhD Student and by FAPESP (scholarship #2019/10808-1 – TT-3).

References

Almeida O, Gonçalves-de-Freitas E, Lopes JS, Oliveira RF (2014) Social instability promotes hormone–behavior associated patterns in a cichlid fish. Horm Behav 66:369–382. doi: 10.1016/j.yhbeh.2014.05.007

Almeida O, Gozdowska M, Kulczykowska E, Oliveira RF (2012) Brain levels of arginine–vasotocin and isotocin in dominant and subordinate males of a cichlid fish. Horm Behav 61:212–217. doi: 10.1016/j.yhbeh.2011.12.008

Almeida O, Oliveira RF (2015) Social status and arginine vasotocin neuronal phenotypes in a cichlid fish. Brain Behav Evol 85:203–213. doi: 10.1159/000381251

Amici F, Aureli F, Call J (2008) Suplementar Material - Fission-Fusion Dynamics, Behavioral Flexibility, and Inhibitory Control in Primates. Curr Biol 18:1415–1419. doi: 10.1016/j.cub.2008.08.020

Ashton BJ, Thornton A, Ridley AR (2018) An intraspecific appraisal of the social intelligence hypothesis. Philos Trans R Soc B. doi: http://dx.doi.org/10.1098/rstb.2017.0288

Barreto RE, Volpato GL, Pottinger TG (2006) The effect of elevated blood cortisol levels on the extinction of a conditioned stress response in rainbow trout. Horm Behav 50:484–488. doi: 10.1016/j.yhbeh.2006.06.017

Bastos RF, Condini M V., Varela Junior AS, Garcia AM (2011) Diet and food consumption of the pearl cichlid Geophagus brasiliensis (Teleostei: Cichlidae): relationships with gender and sexual maturity. Neotrop Ichthyol 9:825–830. doi: 10.1590/S1679-62252011005000049

Blakemore S-J, Frith CD, Wolpert DM (2001) The cerebellum is involved in predicting the sensory consequences of action. Neuroreport 12:1879–1884. doi: 10.1097/00001756-200107030-00023

Boucherie PH, Loretto M-C, Massen JJM, Bugnyar T (2019) What constitutes "social complexity" and "social intelligence" in birds? Lessons from ravens. Behav Ecol Sociobiol 73:12. doi: 10.1007/s00265-018-2607-2

Bracchi-Ricard V, Brambilla R, Levenson J, et al (2008) Astroglial nuclear factor-κB

regulates learning and memory and synaptic plasticity in female mice. J Neurochem 104:611–623. doi: 10.1111/j.1471-4159.2007.04993.x
Bradford M, Northcutt R (1983) Organization of the diencephalon and pretectum of the ray-finned fishes. In: Davis R, Northcutt R (eds) Fish neurobiology. University of Michigan Press, Ann Arbor, MI, pp 117–164
Brandão ML, Braithwaite VA, Gonçalves-de-Freitas E (2015) Isolation impairs cognition in a social fish. Appl Anim Behav Sci 171:204–210. doi: 10.1016/j.applanim.2015.08.026
Brandão ML, Colognesi G, Bolognesi MC, et al (2018) Water temperature affects aggressive interactions in a Neotropical cichlid fish. Neotrop Ichthyol 16:1–8. doi: 10.1590/1982-0224-20170081
Brandão ML, Fernandes AMT de A, Gonçalves-de-Freitas E (2019) Male and female cichlid fish show cognitive inhibitory control ability. Sci Rep 9:15795. doi: 10.1038/s41598-019-52384-2
Broglio C, Gómez A, Durán E, et al (2005) Hallmarks of a common forebrain vertebrate plan: Specialized pallial areas for spatial, temporal and emotional memory in actinopterygian fish. Brain Res Bull 66:277–281. doi: 10.1016/j.brainresbull.2005.03.021
Broglio C, Gómez A, Durán E, et al (2011) Brain and Cognition in Teleost Fish. In: Brown C, Laland K, Krause J (eds) Fish Cognition and Behavior, 2nd edn. Wiley-Blackwell, Oxford, UK, pp 325–358
Brown C, Laland K, Krause J (2011) Fish Cognition and Behavior, 2nd edn. Wiley-Blackwell, Oxford, UK
Brunamonti E, Chiricozzi FR, Clausi S, et al (2014) Cerebellar Damage Impairs Executive Control and Monitoring of Movement Generation. PLoS One 9:e85997. doi: 10.1371/journal.pone.0085997
Castro ALS, Gonçalves-de-freitas E, Volpato GL, Oliveira C (2009) Visual communication stimulates reproduction in Nile tilapia, Oreochromis niloticus (L.). Brazilian J Med Biol Res 42:368–374.
Castro RMC, Casatti L, Santos HF, et al (2004) Estrutura e composição da ictiofauna de riachos da bacia do Rio Grande no estado de São Paulo, sudeste do Brasil. Biota Neotrop 4:01–39. doi: 10.1590/S1676-06032004000100006
Chen CC, Fernald RD (2011) Visual information alone changes behavior and physiology during social interactions in a cichlid fish (astatotilapia burtoni). PLoS One 6:1–12. doi: 10.1371/journal.pone.0020313
Cooper IS, Amin I, Riklan M, et al (1976) Chronic Cerebellar Stimulation in Epilepsy: Clinical and Anatomical Studies. Arch Neurol 33:559–570. doi: 10.1001/archneur.1976.00500080037006
Cummings ME (2015) The mate choice mind: studying mate preference, aversion and

social cognition in the female poeciliid brain. Anim Behav 103:249–258. doi: 10.1016/j.anbehav.2015.02.021
Demirtas-Tatlidede A, Schmahmann JD (2013) Morality: incomplete without the cerebellum? Brain 136:e244–e244. doi: 10.1093/brain/awt070
Derycke S, Kéver L, Herten K, et al (2018) Neurogenomic Profiling Reveals Distinct Gene Expression Profiles Between Brain Parts That Are Consistent in Ophthalmotilapia Cichlids. Front Neurosci 12:1–12. doi: 10.3389/fnins.2018.00136
Desjardins JK, Fernald RD (2010) What do fish make of mirror images? Biol Lett 6:744–747. doi: 10.1098/rsbl.2010.0247
Diamond A (1990) Developmental Time Course in Human Infants and Infant Monkeys, and the Neural Bases of, Inhibitory Control in Reaching. Ann N Y Acad Sci 608:637–676. doi: 10.1111/j.1749-6632.1990.tb48913.x
Dijkstra PD, Schaafsma SM, Hofmann HA, Groothuis TGG (2012) 'Winner effect' without winning: Unresolved social conflicts increase the probability of winning a subsequent contest in a cichlid fish. Physiol Behav 105:489–492. doi: 10.1016/j.physbeh.2011.08.029
Dunbar RIM (1998) The social brain hypothesis. Evol Anthropol Issues, News, Rev 6:178–190. doi: 10.1002/(SICI)1520-6505(1998)6:5<178::AID-EVAN5>3.3.CO;2-P
Dunbar RIM, Shultz S (2007) Evolution in the Social Brain. Science (80-) 317:1344–1347. doi: 10.1126/science.1145463
Ebbesson LOE, Braithwaite VA (2012) Environmental effects on fish neural plasticity and cognition. J Fish Biol 81:2151–2174. doi: 10.1111/j.1095-8649.2012.03486.x
Emery NJ, Seed AM, von Bayern AM., Clayton NS (2007) Cognitive adaptations of social bonding in birds. Philos Trans R Soc B Biol Sci 362:489–505. doi: 10.1098/rstb.2006.1991
Emlen ST, Oring LW (1977) Ecology, Sexual Selection, and the Evolution of Mating Systems. Science (80-) 197:215–223.
Fang W-Q, Yuste R (2017) Overproduction of Neurons Is Correlated with Enhanced Cortical Ensembles and Increased Perceptual Discrimination. Cell Rep 21:381–392. doi: 10.1016/j.celrep.2017.09.040
Favati A, Løvlie H, Leimar O (2017) Individual aggression, but not winner–loser effects, predicts social rank in male domestic fowl. Behav Ecol 28:874–882. doi: 10.1093/beheco/arx053
Field KE, McVicker CT, Maruska KP (2018) Sexually-Relevant Visual and Chemosensory Signals Induce Distinct Behaviors and Neural Activation Patterns in the Social African Cichlid, Astatotilapia burtoni. Front Behav Neurosci 12:1–19. doi: 10.3389/fnbeh.2018.00267
Fong S, Buechel SD, Boussard A, et al (2019) Plastic changes in brain morphology in relation to learning and environmental enrichment in the guppy (Poecilia reticulata). J

Exp Biol 222:jeb200402. doi: 10.1242/jeb.200402
Gaikwad S, Stewart A, Hart P, et al (2011) Acute stress disrupts performance of zebrafish in the cued and spatial memory tests: The utility of fish models to study stress–memory interplay. Behav Processes 87:224–230. doi: 10.1016/j.beproc.2011.04.004
Gauy AC dos S, Boscolo CNP, Gonçalves-de-Freitas E (2018) Less water renewal reduces effects on social aggression of the cichlid Pterophyllum scalare. Appl Anim Behav Sci 198:121–126. doi: 10.1016/j.applanim.2017.10.003
Gonzalez-Voyer A, Kolm N (2010) Sex, Ecology and the Brain: Evolutionary Correlates of Brain Structure Volumes in Tanganyikan Cichlids. PLoS One 5:e14355. doi: 10.1371/journal.pone.0014355
Greenwood AK, Wark AR, Fernald RD, Hofmann HA (2008) Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish. Proc R Soc B Biol Sci 275:2393–2402. doi: 10.1098/rspb.2008.0622
Hauser MD (1999) Perseveration, inhibition and the prefrontal cortex: A new look. Curr Opin Neurobiol 9:214–222. doi: 10.1016/S0959-4388(99)80030-0
Heath RG (1977) Modulation of emotion with a brain pacemamer. Treatment for intractable psychiatric illness. J Nerv Ment Dis 165:300–317.
Herculano-Houzel S (2017) Numbers of neurons as biological correlates of cognitive capability. Curr Opin Behav Sci 16:1–7. doi: 10.1016/j.cobeha.2017.02.004
Herculano-Houzel S (2011) The Isotropic Fractionator: A Fast, Reliable Method to Determine Numbers of Cells in the Brain or Other Tissues. 391–403. doi: 10.1007/7657_2011_13
Herculano-Houzel S, Messeder DJ, Fonseca-Azevedo K, Pantoja NA (2015) When larger brains do not have more neurons: increased numbers of cells are compensated by decreased average cell size across mouse individuals. Front Neuroanat 9:64. doi: 10.3389/fnana.2015.00064
Holekamp KE, Dantzer B, Stricker G, et al (2015) Brains, brawn and sociality: A hyaena's tale. Anim Behav 103:237–248. doi: 10.1016/j.anbehav.2015.01.023
Hoyle CHV (1999) Neuropeptide families and their receptors: evolutionary perspectives. Brain Res 848:1–25. doi: 10.1016/S0006-8993(99)01975-7
Kabadayi C, Bobrowicz K, Osvath M (2018) The detour paradigm in animal cognition. Anim Cogn 21:21–35. doi: 10.1007/s10071-017-1152-0
Kadry VO, Barreto RE (2010) Environmental enrichment reduces aggression of pearl cichlid, Geophagus brasiliensis, during resident-intruder interactions. Neotrop Ichthyol 8:329–332. doi: 10.1590/S1679-62252010000200011
Kjaerby C, Rasmussen R, Andersen M, Nedergaard M (2017) Does Global Astrocytic

Calcium Signaling Participate in Awake Brain State Transitions and Neuronal Circuit Function? Neurochem Res 42:1810–1822. doi: 10.1007/s11064-017-2195-y
Kohda M, Hotta T, Takeyama T, et al (2019) If a fish can pass the mark test, what are the implications for consciousness and self-awareness testing in animals? PLOS Biol 17:e3000021. doi: 10.1371/journal.pbio.3000021
Kohda M, Jordan LA, Hotta T, et al (2015) Facial recognition in a group-living cichlid fish. PLoS One 10:1–12. doi: 10.1371/journal.pone.0142552
Kulczykowska E, Cardoso SC, Gozdowska M, et al (2015) Brain levels of nonapeptides in four labrid fish species with different levels of mutualistic behavior. Gen Comp Endocrinol 222:99–105. doi: 10.1016/j.ygcen.2015.06.005
Kuwamura T (1986) Parental care and mating systems of cichlid fishes in Lake Tanganyika: a preliminary field survey. J Ethol 4:129–146. doi: 10.1007/BF02348115
Kverková K, Bělíková T, Olkowicz S, et al (2018) Sociality does not drive the evolution of large brains in eusocial African mole-rats. Sci Rep 8:9203. doi: 10.1038/s41598-018-26062-8
Kyrargyri V, Vega-Flores G, Gruart A, et al (2015) Differential contributions of microglial and neuronal IKKβ to synaptic plasticity and associative learning in alert behaving mice. Glia 63:549–566. doi: 10.1002/glia.22756
Lal P, Tanabe H, Suster ML, et al (2018) Identification of a neuronal population in the telencephalon essential for fear conditioning in zebrafish. BMC Biol 16:45. doi: 10.1186/s12915-018-0502-y
Li C-Y, Hofmann HA, Harris ML, Earley RL (2018) Real or fake? Natural and artificial social stimuli elicit divergent behavioural and neural responses in mangrove rivulus, Kryptolebias marmoratus. Proc R Soc B Biol Sci 285:20181610. doi: 10.1098/rspb.2018.1610
Lucon-Xiccato T, Gatto E, Bisazza A (2017) Fish perform like mammals and birds in inhibitory motor control tasks. Sci Rep 7:13144. doi: 10.1038/s41598-017-13447-4
MacLean EL, Hare B, Nunn CL, et al (2014) The evolution of self-control. Proc Natl Acad Sci U S A 111:E2140-8. doi: 10.1073/pnas.1323533111
Magurran AE (1990) The adaptive significance of schooling as an anti-predator defence in fish. AnnZoolFennici 27:51–66. doi: 10.2307/23736019
Makinodan M, Rosen KM, Ito S, Corfas G (2012) A Critical Period for Social Experience-Dependent Oligodendrocyte Maturation and Myelination. Science (80-) 337:1357–1360. doi: 10.1126/science.1220845
Marhounová L, Kotrschal A, Kverková K, et al (2019) Artificial selection on brain size leads to matching changes in overall number of neurons. Evolution (N Y) evo.13805. doi: 10.1111/evo.13805

Maruska KP, Fernald RD (2018) *Astatotilapia burtoni*: A Model System for Analyzing the Neurobiology of Behavior. ACS Chem Neurosci 9:1951–1962. doi: 10.1021/acschemneuro.7b00496
Molinari M, Chiricozzi FR, Clausi S, et al (2008) Cerebellum and Detection of Sequences, from Perception to Cognition. The Cerebellum 7:611–615. doi: 10.1007/s12311-008-0060-x
Mu Y, Bennett D V., Rubinov M, et al (2019) Glia Accumulate Evidence that Actions Are Futile and Suppress Unsuccessful Behavior. Cell 178:27–43. doi: 10.1016/j.cell.2019.05.050
Müller K, Smielik I, Hütwohl J-M, et al (2017) The virtual lover – Variable and easy guided 3D-fish animations as innovative tool in mate-choice experiments with sailfin mollies. I. Design and implementation. Curr Zool 63:1–19. doi: 10.1093/cz/zow106
O'Connell LA, Hofmann HA (2011) The Vertebrate mesolimbic reward system and social behavior network: A comparative synthesis. J Comp Neurol 519:3599–3639. doi: 10.1002/cne.22735
Oliveira RF, Carneiro L a, Canário AVM (2005) No hormonal response in tied fights. Nature 437:207–208. doi: 10.1038/437207a
Olivito G, Brunamonti E, Clausi S, et al (2017) Atrophic degeneration of cerebellum impairs both the reactive and the proactive control of movement in the stop signal paradigm. Exp Brain Res 235:2971–2981. doi: 10.1007/s00221-017-5027-z
Pollen AA, Dobberfuhl AP, Scace J, et al (2007) Environmental complexity and social organization sculpt the brain in Lake Tanganyikan cichlid fish. Brain Behav Evol 70:21–39. doi: 10.1159/000101067
Quinn GP, Keough MJ (2002) Experimental Design and Data Analysis for Biologists, 1st edn. Cambridge University Press, Cambridge
Ramallo MR, Honji RM, Birba A, et al (2017) A game of two? Gene expression analysis of brain (cyp19a1b) and gonadal (cyp19a1a) aromatase in females of a Neotropical cichlid fish through the parental care period and removal of the offspring. Gen Comp Endocrinol 252:119–129. doi: 10.1016/j.ygcen.2017.08.009
Reddon AR, O'Connor CM, Marsh-Rollo SE, et al (2015) Brain nonapeptide levels are related to social status and affiliative behaviour in a cooperatively breeding cichlid fish. R Soc Open Sci 2:140072. doi: 10.1098/rsos.140072
Reddon AR, O'Connor CM, Marsh-Rollo SE, Balshine S (2012) Effects of isotocin on social responses in a cooperatively breeding fish. Anim Behav 84:753–760. doi: 10.1016/j.anbehav.2012.07.021
Reddon AR, O'Connor CM, Nesjan E, et al (2017) Isotocin neuronal phenotypes differ among social systems in cichlid fishes. R Soc Open Sci 4:170350. doi:

10.1098/rsos.170350
Regolin L, Vallortigara G, Zanforlin M (1995) Object and spatial representations in detour problems by chicks. Anim Behav 49:195–199. doi: 10.1016/0003-3472(95)80167-7
Revelle W (2018) psych: Procedures for Psychological, Psychometric, and Personality Research. Northwestern University, Evanston, Illinois
Rodríguez F, Durán E, Gómez A, et al (2005) Cognitive and emotional functions of the teleost fish cerebellum. Brain Res Bull 66:365–370. doi: 10.1016/j.brainresbull.2004.11.026
Rogers JT, Morganti JM, Bachstetter AD, et al (2011) CX3CR1 Deficiency Leads to Impairment of Hippocampal Cognitive Function and Synaptic Plasticity. J Neurosci 31:16241–16250. doi: 10.1523/JNEUROSCI.3667-11.2011
Salas C, Broglio C, Rodríguez F (2003) Evolution of forebrain and spatial cognition in vertebrates: Conservation across diversity. Brain Behav Evol 62:72–82. doi: 10.1159/000072438
Sanches FHC, Miyai CA, Costa TM, et al (2012) Aggressiveness overcomes body-size effects in fights staged between invasive and native fish species with overlapping niches. PLoS One. doi: 10.1371/journal.pone.0029746
Sardinha VM, Guerra-Gomes S, Caetano I, et al (2017) Astrocytic signaling supports hippocampal-prefrontal theta synchronization and cognitive function. Glia 65:1944–1960. doi: 10.1002/glia.23205
Scaia MF, Morandini L, Noguera C, et al (2018) Can estrogens be considered as key elements of the challenge hypothesis? The case of intrasexual aggression in a cichlid fish. Physiol Behav 194:481–490. doi: 10.1016/j.physbeh.2018.06.028
Scherer U, Buck M, Schuett W (2016) Lateralisation in agonistic encounters: do mirror tests reflect aggressive behaviour? A study on a West African cichlid. J Fish Biol 89:1866–1872. doi: 10.1111/jfb.13069
Scherer U, Godin J-GJ, Schuett W (2017) Validation of 2D-animated pictures as an investigative tool in the behavioural sciences: A case study with a West African cichlid fish, Pelvicachromis pulcher. Ethology 123:560–570. doi: 10.1111/eth.12630
Schillaci MA (2006) Sexual Selection and the Evolution of Brain Size in Primates. PLoS One 1:e62. doi: 10.1371/journal.pone.0000062
Shumway C a. (2010) The evolution of complex brains and behaviors in African cichlid fishes. Curr Zool 56:144–156.
Teles MC, Gozdowska M, Kalamarz-Kubiak H, et al (2016) Agonistic interactions elicit rapid changes in brain nonapeptide levels in zebrafish. Horm Behav 84:57–63. doi: 10.1016/j.yhbeh.2016.05.020
Warren R, Sawtell NB (2016) A comparative approach to cerebellar function: insights from

electrosensory systems. Curr Opin Neurobiol 41:31–37. doi: 10.1016/j.conb.2016.07.012
Wascher CAF, Kulahci IG, Langley EJG, Shaw RC (2018) How does cognition shape social relationships? Philos Trans R Soc B Biol Sci 373:20170293. doi: 10.1098/rstb.2017.0293
White GE, Brown C (2015) Microhabitat Use Affects Brain Size and Structure in Intertidal Gobies. Brain Behav Evol 85:107–116. doi: 10.1159/000380875
Wimberger PH (1992) Plasticity of fish body shape. The effects of diet, development, family and age in two species of Geophagus (Pisces: Cichlidae). Biol J Linn Soc 45:197–218. doi: 10.1111/j.1095-8312.1992.tb00640.x
Wingfield JC (2013) Ecological processes and the ecology of stress: The impacts of abiotic environmental factors. Funct Ecol 27:37–44. doi: 10.1111/1365-2435.12039

5 GENERAL CONCLUSIONS

The evolution of vertebrates‘ brains follows two models that are not excludent between them: the mosaic evolution and the concerted evolution theory. Both theories were demonstrated in cichclid fishes in this study. The social brain hypothesis, on the other hand, do not explains completely our results. Instead of our expectations, other ecological factors seem to contribute to different patterns observed in cichlids‘ brain anatomy – i.e., miniaturization. For last, more complex cognitive and social responses are, indeed, related to a higher number of brain cells, revealing more socially competent individuals.

REFERENCES

Ashton BJ, Thornton A, Ridley AR (2018) An intraspecific appraisal of the social intelligence hypothesis. Philos Trans R Soc B. doi: http://dx.doi.org/10.1098/rstb.2017.0288

Barlow G (2002) The Cichlid Fishes: Nature’s Grand Experiment In Evolution, 1st edn. Basic Books, New York

Barton RA, Harvey PH (2000) Mosaic evolution of brain structure in mammals. Nature 405:1055–1058. doi: 10.1038/35016580

Braithwaite VA (2006) Cognitive Ability in Fish. In: Sloman K, Balshine S, Wilson R (eds) Behaviour and Physiology of Fish, 1st edn. Elsevier Academic Press, London, UK, pp 1–37

Brandão, IL (1981) Não verás país nenhum. Global Editora, 27ed. São Paulo: Liberdade, 384p.

Bshary R (2011) Machiavellian Intelligence in Fishes. In: Brown C, Laland K, Krause J (eds) Fish Cognition and Behavior, 2nd edn. Blackwell Publishing Ltd, Oxford, UK, pp 223–242

Bshary R, Wickler W, Fricke H (2002) Fish cognition: a primate’s eye view. Anim Cogn 5:1–13. doi: 10.1007/s10071-001-0116-5

Dunbar RIM (1998) The social brain hypothesis. Evol Anthropol Issues, News, Rev 6:178–190. doi: 10.1002/(SICI)1520-6505(1998)6:5<178::AID-EVAN5>3.3.CO;2-P

Dunbar RIM, Shultz S (2007) Evolution in the Social Brain. Science (80-) 317:1344–1347. doi: 10.1126/science.1145463

Finlay B, Darlington R (1995) Linked regularities in the development and evolution of mammalian brains. Science (80-) 268:1578–1584. doi: 10.1126/science.7777856

Gonzalez-Voyer A, Kolm N (2010) Sex, Ecology and the Brain: Evolutionary Correlates of Brain Structure Volumes in Tanganyikan Cichlids. PLoS One 5:e14355. doi: 10.1371/journal.pone.0014355

Gonzalez-Voyer A, Winberg S, Kolm N (2009) Brain structure evolution in a basal vertebrate clade: evidence from phylogenetic comparative analysis of cichlid fishes. BMC Evol Biol 9:238. doi: 10.1186/1471-2148-9-238

Herculano-Houzel S, Lent R (2005) Isotropic Fractionator: A Simple, Rapid Method for the

Quantification of Total Cell and Neuron Numbers in the Brain. J Neurosci 25:2518–2521. doi: 10.1523/JNEUROSCI.4526-04.2005
Herculano-Houzel S, Manger PR, Kaas JH (2014) Brain scaling in mammalian evolution as a consequence of concerted and mosaic changes in numbers of neurons and average neuronal cell size. Front Neuroanat 8:77. doi: 10.3389/fnana.2014.00077
Herculano-Houzel S, Messeder DJ, Fonseca-Azevedo K, Pantoja NA (2015) When larger brains do not have more neurons: increased numbers of cells are compensated by decreased average cell size across mouse individuals. Front Neuroanat 9:64. doi: 10.3389/fnana.2015.00064
Kandel ER (2001) The Molecular Biology of Memory Storage: A Dialogue Between Genes and Synapses. Science (80-) 294:1030–1038. doi: 10.1126/science.1067020
Kverková K, Bělíková T, Olkowicz S, et al (2018) Sociality does not drive the evolution of large brains in eusocial African mole-rats. Sci Rep 8:9203. doi: 10.1038/s41598-018-26062-8
Lihoreau M, Latty T, Chittka L (2012) An exploration of the social brain hypothesis in insects. Front Physiol 3:1–7. doi: 10.3389/fphys.2012.00439
Moberg O, Braithwaite V a., Jensen KH, Salvanes AGV (2011) Effects of habitat enrichment and food availability on the foraging behaviour of juvenile Atlantic Cod (*Gadus morhua* L). Environ Biol Fishes 91:449–457. doi: 10.1007/s10641-011-9803-5
O'Connell LA, Hofmann HA (2012) Evolution of a Vertebrate Social Decision-Making Network. Science (80-) 336:1154–1157. doi: 10.1126/science.1218889
Oliveira RF (2009) Social behavior in context: Hormonal modulation of behavioral plasticity and social competence. Integr Comp Biol 49:423–440. doi: 10.1093/icb/icp055
Pollen AA, Dobberfuhl AP, Scace J, et al (2007) Environmental complexity and social organization sculpt the brain in Lake Tanganyikan cichlid fish. Brain Behav Evol 70:21–39. doi: 10.1159/000101067
Ribeiro, D. Jornal Estado de Minas, Belo Horizonte, 17 de fevereiro de 2007.
Salvanes AGVA, Moberg O, Ebbesson LOE, et al (2013) Environmental enrichment promotes neural plasticity and cognitive ability in fish. Proc R Soc B Biol Sci 280:20131331–20131331. doi: 10.1098/rspb.2013.1331
Taborsky B, Oliveira RF (2012) Social competence: an evolutionary approach. Trends Ecol Evol 27:679–688. doi: 10.1016/j.tree.2012.09.003
Wingfield JC (2013) Ecological processes and the ecology of stress: The impacts of abiotic environmental factors. Funct Ecol 27:37–44. doi: 10.1111/1365-2435.12039

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José do Rio Preto

TERMO DE REPRODUÇÃO XEROGRÁFICA

Autorizo a reprodução xerográfica do presente Trabalho de Conclusão, na íntegra ou em partes, para fins de pesquisa.

São José do Rio Preto, 10/03/2020

Assinatura do autor