THAIS SASSO LOPES

SPATIAL AND TEMPORAL OCCURRENCE OF STREAM FROGS IN THE ATLANTIC FOREST AND THEIR DETECTION THROUGH ENVIRONMENTAL DNA

PADRÕES ESPACIAIS E TEMPORAIS DE OCORRÊNCIA DE ANUROS EM RIACHOS DE MATA ATLÂNTICA E SUA DETECÇÃO POR MEIO DE DNA AMBIENTAL

UNIVERSIDADE DE SÃO PAULO

SÃO PAULO

2016

THAIS SASSO LOPES

SPATIAL AND TEMPORAL OCCURRENCE OF STREAM FROGS IN THE ATLANTIC FOREST AND THEIR DETECTION THROUGH ENVIRONMENTAL DNA

PADRÕES ESPACIAIS E TEMPORAIS DE OCORRÊNCIA DE ANUROS EM RIACHOS DE MATA ATLÂNTICA E SUA DETECÇÃO POR MEIO DE DNA AMBIENTAL

Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Mestre em Ciências, na área de Ecologia.

Orientador : Prof. Dr. Marcio Roberto Costa Martins

Coorientadora : Dra. Carla Martins Lopes

UNIVERSIDADE DE SÃO PAULO

SÃO PAULO

2016

Lopes, Thais Sasso

Padrões espaciais e temporais de ocorrência de anuros em riachos de Mata Atlântica e sua detecção por meio de DNA ambiental

93 páginas

Dissertação (Mestrado) – Instituto de Biociências da Universidade de São Paulo. Departamento de Ecologia.

Versão do título em inglês: Spatial and temporal occurrence of stream frogs in the Atlantic forest and their detection through environmental DNA

1. environmental DNA 2. Microhabitat 3. Ecology

I. Universidade de São Paulo. Instituto de Biociências. Departamento de Ecologia.

COMISSÃO JULGADORA:

_____ _____

Prof(a). Dr(a). Prof(a). Dr(a).

_____

Prof. Dr. Marcio Roberto Costa Martins

Orientador

PARA A MINHA FAMÍLIA E A TODOS QUE TIVEREM CURIOSIDADE POR ANFÍBIOS E EDNA.

- Art by Tim Hopgood

Everybody should be quiet
near a little stream and listen

- by Ruth Krauss, 1982.

AGRADECIMENTOS

Este trabalho se tornou possível graças à colaboração e ao incentivo de muitas pessoas. A elas agradeço e com elas divido a alegria de concluí-lo.

Ao Prof. Dr. Marcio Martins, meu orientador, agradeço o irrestrito apoio, entusiasmo e reconhecimento em todos os momentos deste trabalho. Obrigada por participar em mais uma etapa da minha formação como cientista.

Obrigada ao Prof. Dr. Célio Haddad por me aceitar neste grande projeto e estar pronto a ajudar a todo momento. Agradeço também à Prof. Dra. Kelly Zamudio o importante envolvimento e as interessantes discussões sobre o projeto. À Dra. Carla Martins Lopes, por me ajudar em tantos aspectos deste trabalho, me ensinar tantas coisas, me receber em Rio Claro e ser minha co-orientadora.

Agradeço à Prof. Dra. Cristina Miyaki e ao Prof. Dr. Taran Grant por comporem meu Comitê de Acompanhamento e disponibilizarem tempo a esse projeto. Obrigado pelos seus conselhos e por estarem sempre atentos.

À CAPES e à FAPESP (processo 2014/06795-8) agradeço o auxílio financeiro. Ao COTEC e ICMBio agradeço as autorizações.

Agradeço aos professores desta instituição com quem tive o prazer de aprender biologia. Com certeza vocês marcaram minha modesta empreitada até aqui. Aos funcionários da pós-graduação, em especial à Vera, Shirlene, Luis e Lili por sempre atenderem atenciosamente às minhas mil dúvidas.

Um agradecimento à Ananda Brito, Eleonora Domenico e ao Prof. Dr. Marcelo Pompêo por me ajudarem quanto a equipamento de campo e se disponibilizarem atenciosamente. Meu obrigado à Prof. Dra. Renata Moreira por me atender prontamente e emprestar equipamento.

Agradeço aos colegas de laboratório as ajudas ou simples convivência, em especial: Dra. Laura Alencar por permitir que eu aprendesse com ela na minha iniciação científica e estar sempre disposta a ajudar; Marilia Gaiarsa por tirar minhas dúvidas prontamente mesmo estando na Nova Zelândia e por todo seu trabalho de campo junto à Laura Alencar; Erika Santos por me assegurar desde o começo que tudo daria certo; Melina por tirar

minhas dúvidas; Dra. Bianca Berneck por sempre se disponibilizar a me ajudar, até mesmo indo a campo; ao “Azeitona” e “Quase” por carregarem peso riacho acima.

Um obrigado cheio de carinho às companheiras de mestrado Gabriela Marin e Luísa Novara que entraram nessa jornada comigo e a tornaram mais prazerosa. Agradeço a todos os momentos que vocês me socorreram, me confortaram e me fortaleceram, do início ao fim. Agradeço aos amigos da escalada que me ensinaram a ser persistente e o lado ruim da auto-sabotagem. Aos amigos e professores da *Organization for Tropical Studies* por tudo que me ensinaram e pela melhor companhia.

Um agradecimento sem fim ao meu namorado Fábio Henrique Kuriki Mendes por ter sido meu tutor, meu amor, meu revisor, meu psicólogo e minha motivação. Nunca me cansarei de agradecer o seu apoio, carinho, risadas e conforto que jamais me faltaram, mesmo a 8.000 km de distância. Obrigada por ser meu modelo, ir na frente nesta empreitada na ciência e sempre responder minhas mil dúvidas de biologia e programação quando eu corria até você.

Finalmente, agradeço à minha família, principalmente meus preciosos pais por me amarem e por sempre confiarem mais do que qualquer pessoa no meu sucesso. Obrigada por lutarem desde sempre para que nada me faltasse e me darem todo tipo de suporte que eu precisei para chegar até aqui. Obrigada!

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 2

LIST OF TABLES

Chapter 1

Chapter. 2

OVERVIEW

Brazil ranks as the country with one of the highest amphibian species diversity. Streams in the Atlantic forest of southeastern Brazil have an important availability of microenvironments and harbors a particular richness in amphibian species. Monitoring herpetofauna and knowledge on their spatial and temporal dynamics provide primary information for ecological studies, and are essential to the development of other areas such as conservation biology. In this work we gather information on the occurrence and abundance of three torrent frogs, *Cycloramphus boraceiensis, Hylodes asper* and *Hylodes phyllodes* and examine the reliability of eDNA analysis to detect anuran communities. Samplings occurred within a 95 to 115 m transect in four streams in Núcleo Picinguaba, at the Parque Estadual da Serra do Mar, São Paulo, Brazil. Number of frogs observed and their habitat were monthly recorded from January 2007 to December 2010 and every two months in 2011. We searched for post-metamorphic individuals while walking upstream for 30-60 min, checking all visually accessible spots in the streambed. We mapped the location of each active and inactive frog and characterized its microhabitat use in relation to five parameters. We also collected eDNA samples at 16 sites on April, 2015. We used eDNA metabarcoding approach with a universal primer of a mitochondrial marker (12S) to detect amphibian communities. Throughout the 5-year study, we recorded a total of 6335 visual observations. The abundance of the three species varied along and between streams, and only *Hylodes phyllodes* was found in the stream 2. Abundance of *C. boraceiensis* and *H. asper* was significantly higher in the wet seasons. The three species were found active mainly in wet rocks, without moss and without cover. Inactive individuals of *H. asper* and *H. phyllodes* were found mainly in dry leaves, without moss or cover. Through eDNA metabarcoding, we detected nine species, which were consistent with traditional survey results. DNA of riparian species and species with higher constancy in traditional surveys were detected in higher proportions. Our study showed that traditional survey and DNA metabarcoding results can be complementary and both methodologies can be combined in future ecological studies.

RESUMO GERAL

O Brasil apresenta uma das maiores diversidade de espécies de anfíbios, sendo reconhecidas em torno de 500 espécies endêmicas no país, as quais são encontradas predominantemente em área de Mata Atlântica. O monitoramento da herpetofauna e o conhecimento da dinâmica espacial e temporal destas espécies são informações básicas, porém, fundamentais ao desenvolvimento de diversas áreas de pesquisa e conservação. Neste trabalho reunimos informações sobre ocorrência e abundância de três espécies típicas de riacho, *Cycloramphus boraceiensis, Hylodes asper* e *Hylodes phyllodes* e testamos o uso de DNA ambiental para detecção de comunidades de anuros. As amostragens ocorreram em um transecto de 95 a 115 m em quatro riachos no Núcleo de Picinguaba, localizado no Parque Estadual da Serra do Mar, São Paulo, Brasil. A abundância e o microhabitat destas espécies foram amostrados mensalmente de janeiro de 2007 a dezembro de 2010 e em meses alternados em 2011. Indivíduos pós-metamórficos foram amostrados por procura visual a montante de cada riacho, verificando-se todos os locais ao longo do leito. A localidade de cada indivíduo ativo e inativo foi mapeada e o uso do ambiente foi caracterizado em relação a cinco parâmetros. As amostras de DNA ambiental foram coletadas em 16 pontos em Abril de 2015. eDNA *metabarcoding* foi realizado com primer universal de anfíbios para uma região do gene mitocondrial (12S). Ao longo de cinco anos, registramos um total de 6335 observações visuais. A abundância das três espécies variou entre e ao longo dos riachos, sendo que apenas a espécie *Hylodes phyllodes* foi observada no riacho 2. Houve uma sazonalidade na abundância de *C. boraceiensis* e *H. asper*, sendo ambas espécies encontradas em maior número na estação chuvosa. As três espécies foram encontradas ativas majoritariamente em rochas úmidas ou molhadas, sem musgo e sem cobertura. Indivíduos inativos de *H. asper* e *H. Phyllodes* foram encontrados majoritariamente em folhas secas sem musgo e sem cobertura. Por meio da técnica de eDNA metabarcoding, foram detectadas nove espécies, todas compatíveis com a amostragem tradicional anterior. O DNA de espécies com fases do ciclo de vida atreladas aos riachos e com maior constância na amostragem tradicional foi detectado em maior proporção. Nossos estudos demonstraram que os resultados da amostragem tradicional e de eDNA *metabarcoding* fornecem informações fundamentais e complementares, sendo uma combinação de ambas metodologias potencialmente útil a futuros estudos de ecologia.

GENERAL INTRODUCTION

Understanding the structure and dynamics of communities is a central goal in ecology. Achieving this goal entails describing local diversity patterns – such as species composition and abundance – and also temporal and spatial patterns of species distribution. The more detailed and thorough these descriptions are, the more diverse their applications can be, ranging from systematics and biogeography to conservation biology.

Brazil is a world leader in amphibian diversity, with 1026 recognized species (Silvano and Segalla, 2005; SBH, 2014). Almost 500 of these species are endemic to Brazil (True *et al.*, 2010) and predominantly recorded in the Atlantic Forest domain, even after the heavily destruction of its vegetation coverage (Ribeiro *et al*., 2009). However, amphibian surveys are sorely lacking for large areas in Brazil and a greater resolution is required at some of the already investigated locations (Silvano and Segalla, 2005). Therefore, the ecology of many Brazilian amphibians is still poorly known and few species are well characterized with respect to their temporal variation, spatial distribution, and natural history (Silvano and Segalla, 2005). Insufficient knowledge of amphibian ecology proves to be even more critical when we realize that freshwater environments, on which the majority of amphibians rely, are among the most threatened habitats in the world (Dudgeon *et al*., 2006). More reliable monitoring of amphibian assemblages and information of temporal and spatial patterns of species associated with aquatic environments are critical, particularly to provide conservation guidelines (Rees *et al*., 2014).

Field surveys enable researchers to assess species occurrence and are traditionally conducted through a myriad of methods (e.g., active surveys or traps placement). However, various difficulties might arise when fauna inventories are carried out with traditional methods. One difficulty is, for example, the detection of small, rare, cryptic or less

conspicuous species (MacKenzie *et al*., 2002; Silveira *et al*., 2010). Furthermore, some methods may be not effective for some species, habits or environments, such as acoustic survey for amphibian species that do not vocalize or pitfall traps for arboreal species (Rödel and Ernst, 2004). Moreover, species identification based on morphological characters is subject to researcher biases such as its taxonomic experience (Hopkins and Freckleton, 2002; Silveira *et al.*, 2010). Other difficulties may lie in the fact that the vast majority of methods are seasonally constrained and fail to survey species in certain seasons when individuals are inactive, or identify species in certain life stages such as eggs, larval or juveniles (Ficetola *et al*., 2008). The combination of these factors affects wildlife surveys by allowing for false-positive (i.e., the species is detected where is not actually present) and especially false-negative results (i.e., the species is not detected when is in fact present), which blurs the true occurrence and distribution of species.

In the last 20 years, sequencing technologies progressed rapidly and the emergence of next-generation sequencing (NGS) allowed researchers to overcome costly and time-consuming steps. These advances in sequencing procedures have prompted the growth of molecular databases (e.g. NCBI: http://www.ncbi.nlm.nih.gov; EMBL, http://www.ebi.ac.uk/embl, BOLD, http://www.barcodinglife.org) containing genic and genomic sequences from several taxa, making molecular data readily accessible to answer new questions (Valentini *et al*., 2009). The availability of molecular data allowed the exploration of innovative tools in fields such as molecular ecology, particularly with regard to the development DNA barcode (short stretch of a species-specific DNA sequence, capable to distinguish species) for species identification (Valentini *et al*., 2009).

A recent and promising technique for species identification makes use of what is known as environmental DNA (eDNA). Environmental DNA is the DNA recovered from an environmental sample such as air, soil or water, with no evident sign of biological

material (Ficetola *et al*., 2008). This DNA can then be sampled for sequencing by NGS and used in subsequent identification of the species that released it. Environmental DNA detection provides us an excellent way to determine species occurrences with the advantages of not depending on visual detection or removal of organisms, and not relying on taxonomic knowledge (Darling and Mahon, 2011; Deiner and Altermatt, 2014). On the other hand, eDNA analyses demand molecular biology and bioinformatics skills. Recovery of DNA from environmental samples is therefore a new approach for species survey with the potential to overcome many of the previously mentioned limitations of traditional methods and improve our knowledge of species distribution.

The use of eDNA does not mean replacing traditional methodologies, however. Even though sequencing of environmental samples has the potential to enhance our ability to detect species that are otherwise difficult to spot or collect, it is important to recognize that only by traditional field observations one can gather information on the species natural history, such as habitat use, or the population abundance. Overall, eDNA analysis and traditional methodologies provide baseline data, paramount for our understanding of the studied taxa and for taking the best informed conservation decisions.

STRUCTURE

In this study, we used data from a five-year survey obtained through traditional methods to describe the temporal and spatial occurrence and abundance of amphibians at the Atlantic forest, Ubatuba, Brazil. Also based on the five-year survey, we investigate whether a single visit for eDNA sampling can be effective in detecting anuran communities in tropical streams. The dissertation is divided in two chapters. Specifically, the first chapter describes

the occurrence of three torrent frogs (*Cycloramphus boraceiensis, Hylodes asper* and *H. phyllodes*) in four streams, investigate the abundance of these species over time and characterize their microhabitat use. The second chapter explores the applicability of eDNA metabarcoding to characterize the amphibian community associated with streams and investigate whether eDNA detection coincides with the data obtained by traditional sampling.

eDNA OVERVIEW

In the second chapter we apply a recent and unfamiliar methodology to assess species presence in aquatic environments through eDNA collection. Therefore, we will briefly describe the advances in this methodology to review the state of the art on aquatic eDNA analysis:

Previous sampling of DNA from the environment

The first studies to extract genomic material from environmental samples explored the microbial diversity in soil and water (Schmidt *et al*., 1991; Stein *et al*., 1996; Handelsman *et al*., 1998). Schmidt *et al* (1991) were the first to amplify ribosomal RNA of picoplankton communities extracted from sea water samples. Their approach enabled microbiologists to access the genome of uncultivable microorganisms in the laboratory and showed that microbial diversity can be more complex than previously imagined. Molecular sequences of microbial populations taken directly from the environment were then designated as the "metagenome", and the study of genomes obtained thereby as "metagenomics" (Handelsman *et al*., 1998).

Species detection based on DNA obtained directly from the environment was then extended to macro organisms in paleoecology. By using soil samples, researchers revealed

the pattern of distribution of extinct mammals, birds and plants (Willerslev *et al*., 2003; Willerslev and Cooper, 2005). Subsequently, DNA extractions were also performed from cave sediment (Hofreiter *et al*., 2003) and ice-core (Willerslev *et al*., 2007). The current availability of high-throughput sequencing technology and the design of barcodes boosted the use of eDNA in ecological studies. While the goal of metagenomic and paleoecology has often been to characterize all genomes in the sample and the functional diversity of microbial communities, eDNA analyses in ecology has, at least initially, focused on one or a few species previously known. For instance, the first study to collect DNA from freshwater environments attempted to comprehend the distribution of the invasive bullfrog in France (Ficetola *et al*., 2008).

Early detection of invasive and endangered species

Nowadays, eDNA detection has been used extensively and successfully to monitor several exotic and endangered species (Darling and Mahon, 2011). In freshwater environments, examples include the detection of Asian carp, Chinook salmon, American bullfrogs, snakes (*Python bivittatus*), Bluegill fish, crustaceans (*Procambarus clarkii*) and gastropods (*Potamopyrgus antipodarum*) (Chapter 2 - Supplementary List I). Studies on dispersion of invasive aquatic species confirmed the effectiveness of eDNA analysis as a survey tool for early detection of populations at low densities - as low as 1 individual per 100 m^2 (Secondi *et al.* 2016) - and at any life stage (e.g. Dejean *et al*., 2012; Goldberg *et al*., 2013; Piaggio *et al*., 2013; Takahara *et al*., 2013). These studies support the eDNA detection as a good alternative for the survey of cryptic, elusive and at low density species. Also, these studies have proved eDNA analysis to be useful in occupancy model and species' range delimitation (e.g., Hunter *et al*., 2015).

Persistence of eDNA in aquatic environment

Environmental DNA detection relies on the secretion of DNA by organisms and also on the rate of disintegration of these molecules (Takahara *et al*., 2013). In order to accurately determine the presence or absence of a species it is thus of paramount importance to know whether DNA molecules can remain in the environment and for how long prior to degradation (Dejean *et al.*, 2011). Knowing the persistence of DNA in the environment is essential when it comes to defining precisely the time limit of detection of an organism - i.e. what is the maximum interval between the presence of the organism and its DNA detection.

Studies investigating DNA degradation have determined that these molecules can be detected within 25 and 14 days in controlled and natural aquatic environments, respectively, and that the DNA detection rate was negatively correlated with time (Dejean *et al*., 2011). In a controlled laboratory experiment with *Pelobates fuscus* and *Triturus cristatus*, Thomsen *et al.* (2012) found that immediately after the animals are removed from the aquarium, a fast and continuous reduction in DNA concentrations was observed, with DNA becoming undetectable within one to two weeks. These results led the authors to suggest that eDNA samples found in the environment are contemporary to the species presence and the donor species occurrence in the sampling locality can be safely inferred. However, these studies were conducted in aquariums or lentic environments in the absence of water flow. In lotic environments, on the other hand, eDNA concentrations from salamanders were shown fall within hours after removal of the individuals, with DNA molecules not being detected after only 24 h (Pilliod *et al*., 2013b).

Therefore, a short interval of persistence of detectable amounts of DNA allows access to presence or absence of the species in a fine time scale. This information is critical to limit the incidence of false-positive errors in cases where the molecule is detected but the

organism no longer occupies the site, and to ensure the DNA identified belongs to an organism recently present in that given environment (Dejean *et al.*, 2012).

Advantages of using eDNA

Monitoring species using eDNA detection offers a number of potential advantages over traditional methods (Darling and Mahon, 2011). An environmental sample for DNA extraction is considerably simple to obtain, performed in a non-invasive and non-disruptive manner, and does not require the capture and removal of organisms from the environment (Jerde *et al*., 2011).

Monitoring species can be challenging when applying conventional sampling methods, particularly if the initial starting place to look for the species is not known or if they occur at low abundances (Wilson and Wright, 2013). The high sensitivity of eDNA has been helpful to guide locations for traditional search of individuals. For instance, directed by the discovery of carp eDNA in a pool in Illinois, United States, Jerde *et al.* (2011) was able to find one silver carp after 93 person-days of electrofishing effort.

Compared to traditional survey methods, obtaining eDNA is also considerably faster and less costly (Rees *et al.*, 2014a). In the same study with the Asian carp held in Chicago (Illinois, USA), only 0.174 person-days were necessary to get a positive detection with eDNA detection (Jerde *et al.*, 2011).

The detection rate of eDNA was also compared to that of conventional methodologies in aquatic environment (such as snorkel, "kick-netting" and electrofishing). eDNA resulted in detection of a greater number of species and in more locations than conventional methods (Jerde *et al*., 2011; Pilliod *et al*., 2013b; Tréguier *et al*., 2014). For instance, Dejean *et al.* (2012) detected bullfrog eDNA in 38 lakes in France, and in only

seven using visual and acoustic survey. In other study for amphibian survey in Mediterranean ponds, Valentini *et al.* (2016) estimated that it was necessary at least four successive visits to the field using traditional methods to obtain the same detection probability achieved by a single sampling using eDNA metabarcoding approach. This difference suggests that traditional methods may underestimate the species' occurrence and distribution, at least in the aquatic environment.

Restrictions on the use of eDNA

Despite the aforementioned advantages, the eDNA detection is not devoid of constraints. Lodge *et al.* (2012) and Taberlet *et al.* (2012) pointed hurdles to be overcome so that eDNA detection becomes more reliable as an ecological tool. For example, the efficiency of this approach depends on the development of molecular techniques, such as primer specificity and on the absence of amplification inhibitors in the sample. Primers not so specific might result in false-positive results - particularly if any successful amplification is interpreted as a positive result without further investigation through sequencing (Wilcox *et al*., 2013).

Additionally, DNA release in the environment - as well its persistence and detection - can be influenced in varying degrees by biotic and abiotic factors. Little is known on the importance of the species natural history (e.g. activity, life stages habitat) for interspecific and intraspecific difference in DNA secretion, and on the influence of climatic factors in DNA detection. A review by Barnes *et al.* (2014) indicated that environmental factors should be considered in studies sampling eDNA. Enzyme present in water, for example, can decompose DNA and so can microorganism activity, temperature, ultraviolet radiation and acidity. These factors can act synergistically or antagonistically to preserve or facilitate DNA degradation (Barnes *et al*, 2014; Pilliod *et al.*, 2013). Understanding eDNA

degradation is crucial if we are to make accurate statements about the presence of a species at a given location, since failing to detect eDNA will not be necessarily due to the species absence.

In summary, the literature exploring eDNA as a possible survey tool for aquatic species is slightly recent but escalating, with many studies investigating different aspects of the feasibility of eDNA on species detection. This includes a better understanding of how eDNA can be applied for community survey. To fill gaps in our knowledge on eDNA applicability, we sampled eDNA from four streams in the Atlantic Forest with well-known species compositions to test whether the results of eDNA analyses were consistent with those of traditional methodologies.

GENERAL REFERENCES

Barnes, M. A., Turner, C. R., Jerde, C. L., Renshaw, M. A, Chadderton, W. L., Lodge, D. M., 2014. Environmental conditions influence eDNA persistence in aquatic systems. Environmental Science & Technology, 48, 3, 1819-1827.

Darling, J. A., Mahon, A. R., 2011. From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. Environmental Research, 111, 978-988.

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., Miaud, C., 2011. Persistence of environmental DNA in freshwater ecosystems. PLoS One, 6, e23398.

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E., Miaud, C., 2012. Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*. Journal of Applied Ecology, 49, 953-959.

Deiner, K., Altermatt, F., 2014. Transport distance of invertebrate environmental DNA in a natural river. PLoS One, 9, e88786.

Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z., Knowler, D., Lévêque, C., Naiman, R. J., Prieur-Richard, A.-H., Soto, D., Stiassny, M. L. J. and Sullivan, C. A. 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. Biological Reviews, 81,163-182.

Ficetola, G. F., Miaud, C., Pompanon, F., Taberlet, P., 2008. Species detection using environmental DNA from water samples. Biology Letters, 4, 423-425.

Goldberg, C. S., Sepulveda, A., Ray, A., Baumgardt, J., Waits, L. P., 2013. Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). Freshwater Science, 32, 792-800.

Handelsman, J., Rondon, M. R., Brady, S. F., Clardy, J., Goodman, R. M., 1998. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chemistry and Biology, 5, 245-249.

Hofreiter, M., Mead, J. I., Martin, P., Poinar, H. N., 2003. Molecular caving. Current Biology, 13, 693-695.

Hopkins, G. W., Freckleton, R. P., 2002. Declines in the numbers of amateur and professional taxonomists: implications for conservation. Animal Conservation, 5, 245-249.

Hunter, M. E., Oyler-McCance, S. J., Dorazio, R. M., Fike, J. A., Smith, B. J., Hunter, C. T., Reed, R. N., Hart, K. M. 2015. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. PLoS One, 10, e0121655.

Jerde, C. L., Mahon, A. R., Chadderton, W. L., Lodge, D. M., 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation Letter, 4, 150-157.

Piaggio, A. J., Engeman, R. M., Hopken, M. W., Humphrey, J. S., Keacher, K. L., Bruce, W. E., Avery, M. L. 2013. Detecting an elusive invasive species: a diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. Molecular Ecology Resources, 14, 374-380.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., Waits, L. P., Richardson, J., 2013. Estimating occupancy and abundance of stream amphibians using eDNA from filtered water samples. Canadian Journal of Fisheries and Aquatic Science, 70, 1123-1130.

Lodge, D. M., Turner, C. R., Jerde, C. L., Barnes, M. A., Chadderton, L., Egan, S. P., Feder, J. L., Mahon, A. R., Pfrender, M. E. 2012. Conservation in a cup of water:

estimating biodiversity and population abundance from environmental DNA. Molecular Ecology, 21, 2555-2558.

MacKenzie, D. I., Nichols, J. D., Lachman, G. B., Droege, S., Royle, J. A., Langtimm, C. A., 2002. Estimating site occupancy rates when detection probabilities are less than one. Ecology, 83, 2248-2255.

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., Gough, K. C. 2014. The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. Journal of Applied Ecology, 51, 5, 1450-1459.

Ribeiro, M. C., Metzger, J. P., Martensen, A. C., Ponzoni, F. J., Hirota, M. M., 2009. The Brazilian Atlantic Forest: How much is left, and how is the remaining forest distributed? Implications for conservation. Biological Conservation, 142, 1141-1153.

Rödel, M., Ernst, R. 2004. Measuring and monitoring amphibian diversity in tropical forests. I. An evaluation of methods with recommendations for standardization. Ecotropica, 10, 1-14.

SBH - Sociedade Brasileira de Herpetologia, 2014. Brazilian amphibians - List of species. Electronic database accessible at http://www.sbherpetologia.org.br.

Schmidt, T. M., DeLong, E. F., Pace, N. R., 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. Journal of Bacteriology, 173, 4371-4378.

Secondi, J., Dejean, T., Valentini, A., Miaud, C., Audebaud, B., 2016. Detection of a global aquatic invasive amphibian, *Xenopus laevis*, using environmental DNA. Amphibia-Reptilia, 37, 131-136.

Silvano, D. L., Segalla, M. V., 2005. Conservação de anfíbios no Brasil. Megadiversidade, 1, 79-86.

Silveira, L. F., Beisiegel, B. de M., Curcio, F. F., Vadujo, P. H., Dixo, M., Verdade, V. K., Mattox, G. M. T., Cunningham, P. T. M., 2010. Para que servem os inventários de fauna? Estudos Avançados, 24, 173-207.

Stein, J. L., Marsh, T. L., Wu, K. Y., Shizuya, H., DeLong, E. F., 1996. Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment from a planktonic marine archaeon. Journal of Bacteriology, 178, 591-599.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., Willerslev, E., 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Molecular Ecology, 21, 2045-2050.

Takahara, T., Minamoto, T., Doi, H., 2013. Using environmental DNA to estimate the distribution of an invasive fish species in ponds. PLoS One, 8, e56584.

Thomsen, P., Kielgast, J., Iversen, L. L., Møller, P. R., Rasmussen, M., Willerslev, E., 2012. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLoS One, 7, e41732.

Tréguier, A., Paillisson, J. M., Dejean, T., Valentini, A., Schlaepfer, M. A., Roussel, J. M., 2014. Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkia* in freshwater ponds. Journal of Applied Ecology, 51, 871-879.

Valentini, A., Pompanon, F., Taberlet, P., 2009. DNA barcoding for ecologists. Trends in Ecology and Evolution, 24, 110-117.

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F., Gaboriaud, C., Jean, P., Poulet, N., Roset, N., Copp, G. H., Geniez, P., Pont, D., Argillier, C., Baudoin, J. M., Peroux, T., Crivelli, A.J., Olivier, A., Acqueberge, M., Le Brun, M., Moller, P. R., Willerslev, E., Dejean, T., 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Molecular Ecology, 25, 929-942.

Wilcox, T. M, McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley A. R., Schwartz, M. K., 2013. Robust detection of rare species using environmental DNA: the importance of primer specificity. PloS one, 8, e59520.

Wilson, C., Wright, E. 2013. Using environmental DNA (eDNA) as a tool in risk-based decision-making. Ontario Ministry of Natural Resources, Aquatic Research and Development Section, Aquatic Research Series, 2013- 01.

Willerslev, E., Cappellini, E., Boomsma, W., Nielsen, R., Martin, B., Brand, T.B., Hofreiter, M., Bunce, M., Poinar, H. N., Dahl-jensen, D., Johnsen, S., Steffensen, J. P., Bennike, O., 2007. Ancient biomolecules from deep ice cores reveal a forested Southern Greenland. Science, 317, 111-114.

Willerslev, E., Cooper, A., 2005. Ancient DNA. Proceedings Biological Sciences / The Royal Society, 272, 3-16.

Willerslev, E., Hansen, A. J., Binladen, J., Brand, T. B., Gilbert, M. T. P., Shapiro, B., Bunce, M., Wiuf, C., Gilichinsky, D. A., Cooper, A., 2003. Diverse plant and animal genetic records from Holocene and Pleistocene sediments. Science, 300, 791-795.

CHAPTER 1

SPATIAL AND TEMPORAL ABUNDANCE AND OCCURRENCE PATTERNS OF THREE TORRENT FROGS IN THE BRAZILIAN ATLANTIC FOREST

THAIS SASSO LOPES[1], LAURA R. V. ALENCAR[1], MARÍLIA P. GAIARSA[1], MARCIO MARTINS[1]

[1]*Departamento de Ecologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil, CEP 05508-090*

ABSTRACT

Ecological research and species management depend on accurate assessments of the species temporal and spatial distribution, which are determined by biotic and abiotic factors. Amphibians are extremely diverse in habitat use, ranging from forest-floor and arboreal species, to stream-dwellers. Here, we studied habitat use and variation in spatial-temporal abundance of three torrent frogs (*Cycloramphus boraceiensis, Hylodes asper* and *Hylodes phyllodes*) along four mountain streams in the Atlantic Forest of southeastern Brazil. We performed monthly visual survey from January 2007 to December 2010 and every two months in 2011. We searched for post-metamorphic frogs by conducting diurnal and nocturnal surveys within a 100-115 m transect per stream. We mapped each frog's location and characterized their microhabitat use by recording five environmental variables. A total of 6335 observations were made over the five years of field survey. All species showed similar habitat use and were found mainly on wet or humid rocks without moss or cover, and adjacent to flowing water. The abundance of *C. boraceiensis* and *H. asper* was significantly higher in the wet season, potentially reflecting a higher reproduction rate during this time of the year. Species abundance also varied along some of the streams. Mean abundance of *C. boraceiensis* was higher in the 75-100 m section in stream 1 and that of *H. asper* was higher in the 75-100 m section in stream 3. Only *H. phyllodes* were recorded on the stream 2 and its abundance was higher in the 50-75 m section in this stream. Environmental factors and availability of suitable habitats might be influencing these species occurrence along the streams. Throughout this study, we documented long-term abundance and occurrence patterns of three torrent frogs endemic to tropical streams, shown to vary due to environmental variables.

Keywords: Seasonal pattern, rheophilic habitat, microhabitat use, stream monitoring, *Cycloramphus, Hylodes*

INTRODUCTION

Long-term studies are crucial to detect and understand potential trends in the dynamics of natural population (Gibbons *et al.,* 2000). Changes in abundance over time can reveal population declines and the understanding of these changes have intrinsic scientific value and conservation implications (Bury, 2006). Moreover, effective conservation management requires spatial information on habitat use to better direct conservation decisions (Hartel *et al.,* 2011). However, the latest figures from the International Union for Conservation of Nature's (IUCN) showed that approximately 24% of known amphibians are classified as data-deficient species, which means they lack baseline data on temporal variation and spatial occurrence of their populations (IUCN, 2015). Therefore, habitat use, occurrence patterns and temporal abundance variation of many amphibians are poorly known, despite its importance from ecological and practical perspectives.

To understand the spatial distribution of species it is essential to comprehend how they use the available microhabitats (Gillespie *et al*., 2004). Specifically, "habitat use" can be defined as the way an organism uses a collection of physical and biological components (i.e., resources) available in a habitat (Hall *et al.*, 1997). Each species can occupy a variety of habitats for different purposes, like foraging, cover, nesting and other life history traits (Krausman, 1999). Furthermore, amphibians require specific microhabitats with appropriate abiotic conditions, which are known to influence a species distribution (e.g. Gascon, 1991; Leuven *et al*., 1986). Therefore, to understand species occurrence and to provide accurate management guidelines for amphibian conservation it is critical to monitor populations across a fine spatial scale.

Although many studies on amphibian spatial and temporal distribution have analyzed habitat-specific assemblages such as forest-floor, stream-edge and pond communities, few studies focused on variation in abundance and occurrence of stream-dwelling amphibians during long-term surveys (e.g., Duellman and Trueb, 1986; Van Sluys *et al.*, 2007). Here, we provide the results of a 5-year survey on habitat use, occurrence and seasonal variation in abundance of three stream-dwelling amphibians (*Cycloramphus boraceiensis*, *Hylodes asper* and *Hylodes phyllodes*), endemic to the Atlantic Forest of southeastern Brazil. Specifically, for each species, we aimed to: (i) investigate spatial distribution among and within streams, (ii) estimate how abundance varies over time and (iii) characterize differences in habitat use based on environmental variables.

MATERIAL AND METHODS

Study site

The study area is located within the Parque Estadual da Serra do Mar (PESM) - Núcleo Picinguaba, in the municipality of Ubatuba, state of São Paulo, southeastern Brazil (24°13'12.49" S, 47°22'4.71" W, 23°22'36.90" S, 44°44'19.07" W) (Figure 1). Núcleo Picinguaba is a protected area situated in the Atlantic Rainforest domain, along the north coast of the state of São Paulo. The climate is tropical (Koppen, 1948) with mean annual air temperature of 26.7 °C, high and relatively constant air humidity (monthly means around 85-90%), and an annual average rainfall of 2650 mm. The region has a warm and humid season with frequent rains from October to April (monthly rainfall 215-376 mm, with a peak in December and January and mean temperatures from 21.1 to 25.5 °C), and a drier and colder season from May to September (monthly rainfall 11-166 mm, with July as the driest month and mean temperatures from 18.4 to 20.5 °C) (Ruggeri *et al.*, 2015).

Fieldwork was carried out monthly from January 2007 to December 2010, and every other month in 2011, throughout a total of 54 months, comprising 147 days at the field. Visual survey of amphibians was performed twice a day (one nocturnal and one diurnal survey) within a ~100 m (95-115 m) transect parallel to the water flow along four rocky streams. We searched for post-metamorphic individuals while walking slowly upstream during 30 to 60 minutes, checking all visually accessible spots in the streambed. The streams in the sampled region are disconnected (i.e., each stream is a distinct unit). The streams are mostly covered by forest canopy and underlain by a mosaic of sand and rocks that create many short waterfalls. In the sampled region, streams vary in their width, elevation, water flow and structure as follow: *Stream 1* (23° 21' 15.2" S, 44° 46' 3.2" W) is the largest stream sampled (4-15 m wide), and it runs along a relatively flat area with many large pools and relatively few waterfalls; *Stream 2* (23° 21' 34.4" S, 44° 47' 3.2" W) is the smallest stream sampled (1-5 m wide, it ceases to flow during the driest periods of the driest years) and runs along a relatively flat area in the first 40 m, sloping for the rest of its length and finishing at a steep area; *Stream 3* (23° 21' 41.2" S, 44° 47' 15.3" W) has a smaller inclination than the other streams sampled, with fewer waterfalls and many large pools and its width varies from five to 10 m; *Stream 4* (23° 21' 53.7" S, 44° 48' 2.8" W) is the most sloping of the four streams, with many waterfalls and few large pools and its width varies from five to 10 m.

Torrent frogs

Cycloramphus boraceiensis, Hylodes asper and *H. phyllodes* are syntopic torrent frogs inhabiting shallow streams restricted to the Atlantic forest. These frogs are obligate stream dwellers, with most of its life stages occurring along the stream, as follows:

Cycloramphus boraceiensis (Heyer, 1983) occurs in primary or secondary forest in the Serra do Mar complex along the southeastern region of the state of Rio de Janeiro and northeastern part of the state of São Paulo. The species has rheophilic habits, as other congeneric species (Haddad *et al*., 2008). Adults of this species are commonly found in rocky surfaces next to small freshwater streams. Eggs are deposited outside the water in wet surfaces (rocks, branches, roots) on the streambed. Individuals of this species vocalize at night, usually in the first half of the night, but occasionally also by day (Hartmann *et al*., 2010). Male size can vary from 35 to 55 mm and female size varies from 52 to 59 mm (Heyer, 1983).

Hylodes asper (Müller, 1924) is endemic to the Atlantic forest and its distribution ranges from southern to southeastern Brazil, occurring from Santa Catarina to Rio de Janeiro states. Individuals are diurnal and are known for their territorial behavior, vocalization and visual displays (foot flagging) along rocky rivers and streams (Haddad and Giaretta, 1999). Individuals of this species and of *H. phyllodes* characteristically deposit their eggs in submerged chambers in streams (Hartmann *et al*., 2010).

Hylodes phyllodes (Heyer and Cocroft, 1986) is endemic to the Atlantic forest and is also known to inhabit mountain streams in primary and secondary forests of Serra do Mar, in the states of São Paulo and Rio de Janeiro. The species has rheophilic habits when adult, as other congeneric species (Haddad *et al*., 2008). From metamorphosis to reproductive maturity, froglets inhabit the leaf litter of forests adjacent to streams. Individuals are found active during the day, along streams or films of water on rocks (Heyer *et al*., 1990; Hartmann *et al*., 2006). Male size varies from 27.5 to 31.4 mm and females from 29 to 35.5 mm (Heyer and Cocroft, 1986).

Data Collection

We assessed the species spatial and temporal distribution by recording the date and time when each individual was seen and mapping its exact location using a stream map drawn on graph paper (Supplementary Figure I). We analyzed the recorded individual locations in Quantum GIS (v 2.10; QGIS, 2016) and counted the number of individuals of each species at every 25 m along the 100 m transect of each stream.

We also characterized the habitat of the three species recording the following variables for each individual where it was first seen: (i) surface type (rock, crevices, sand, leaf, pipe, tree branch, leaf litter, trunk, other), (ii) surface humidity (dry, humid, wet, film of water), (iii) moss (presence or absence), (iv) cover type (rock, leaf, branch, absence, other) and (v) distance (in cm) from the nearest water body, waterfall, pond, water flow and height above the water. We classified every individual as active if found during its active period of the day (e.g. *Hylodes asper* was active if found during the diurnal survey) and as inactive otherwise. By examining these environmental variables simultaneously we provide a detailed description of the habitat used by the torrent frogs analyzed in the present study.

Data Analysis

First we summarized species presence and abundance variation per month in each stream. To further investigate species' spatial distribution we divided each stream transect in 25 m sections and tested for significant differences in mean number of observations per month in every section using Kruskal-Wallis tests.

We used a circular statistical analysis to assess whether abundance was randomly distributed along the year, or restricted to a particular month (Zar, 1999). Circular statistics allows us to examine the distribution of data plotted on a circular scale as compass directions or months of a year, where there is no "0" (zero) point and the axis is divided

equally into angles (Zar, 1999). Months were converted to angles (e.g., 0◦ = January to 330◦ = December, intervals of 30◦), and the mean number of individuals per 100 m every month was used in the calculation of each angle frequency. We analyzed each species separately, using monthly data from 2007 to 2010. We did not include data from 2011 because in this year we only sampled every other month. We calculated the mean angle (the month of the year when most of the data is concentrated) and measured the angular dispersion (*r*), which vary from 0 (data is uniformly dispersed) to 1 (data complete concentrated in one direction). We performed the Rayleigh's Uniformity test to evaluate if the data are distributed uniformly along the circular axis.

To investigate habitat use for each species, we searched for differences in the frequency of every categorical variable (variables 1 to 4 mentioned above) using a chi-square goodness-of-fit test. For this test, we considered active and inactive individuals separately. We performed all statistical tests described above in R (v 3.1.3; R Development Core Team, 2015). We also summarized the habitat characteristics in relation to the distance to water using a principal component analysis (PCA) to identify the most important factors in microhabitat use by each species. Only data for active individuals were included in the PCA. Prior to analysis, all distances were log (x + 1) transformed. Analysis was perfomed using PAST (3.x, Hammer *et al.*, 2001).

RESULTS

We visually recorded 6335 frogs throughout 54 months consisting of: 2335 *Cycloramphus boraceiensis*, 3149 *Hylodes asper* and 851 *Hylodes phyllodes.* The species were found in at least one of the streams in all sampled months. *Hylodes phyllodes* were found in all the four streams and were the only species observed in the stream 2. Both *C. boraceiensis* and *H.*

asper were found only in streams 1, 3 and 4. The mean abundance of each species varied along streams (Figure 2). In stream 1 the mean abundance of *C. boraceiensis* within the 75-100 m section was significantly higher than that of the other three sections of the stream ($p << 0.001$) (Figure 2). In stream 3 the mean abundance of *H. asper* at the 75-100 m section was significantly higher than in the other three sections ($p < 0.002$) (Figure 2). Finally, in stream 2 the mean abundance of *H. phyllodes* within the 50-75 m section was significantly higher than in the other three sections ($p << 0.01$) (Figure 2).

The number of active individuals visually found of each species varied among streams. The encounter rate of *C. boraceiensis* per visual survey ranged from 2-18 individuals/100 m in stream 1, 1-13 individuals/100 m in stream 3 and 6-52 individuals/100 m in stream 4 (Figure 3). *Hylodes asper* was the most common species at our sites with encounter rates ranging from 1-49 individuals/100 m in stream 1, 1-21 individuals/100 m in stream 3 and 1-36 individuals/100 m in stream 4 (Figure 4). Finally, the encounter rates of *H. phyllodes* ranged from 0-4 individuals/100 m in stream 1, 0-16 individuals/100 m in stream 2, 0-3 individuals/100 m in stream 3 and 0-9 individuals/100 m in stream 4 (Figure 5). *Hylodes phyllodes* were the only species that we did not visually observe every month (Figure 5).

Circular statistical results revealed that species' mean abundance was significantly seasonal for some of the years tested (Table 1). Among the years with significantly seasonal abundance, months with the highest mean abundance were: January (2009) and December (2010) for *C. boraceiensis*, March (2007, 2008 and 2009) and December (2010) for *H. asper* and August (2007) and December (2009) for *H. phyllodes*. The degree of seasonality (r) varied from 0.13 to 0.49 (Table 1).

Active individuals of *Cycloramphus boraceiensis* were found more often on humid or wet rocky surfaces, without moss, and without any cover (Table 2). Active individuals of

Hylodes asper were found more often on moist and rocky surfaces, without moss, and without any cover (Table 2). Active individuals of *Hylodes phyllodes* were found more often on moist and rocky surfaces without moss, and with rocky cover or without any cover (Table 2). Inactive individuals of *H. asper* were most commonly found on dry leaves, without moss, and covered with leaves or without cover (Table 3). Similarly, inactive individuals of *H. phyllodes* were commonly found on dry leaves, without moss or cover (Table 3). No data was available for inactive individuals of *C. boraceiensis*.

Among the active individuals of all species, *C. boraceiensis* were found closer to waterfalls (mean distance = 91.24 cm, Table 4), *H. asper* closer to ponds and to the water (mean distance of 43.12 cm and 11.45 cm, respectively; Table 4), and *H. phyllodes* were found farther away from waterfalls and water flow (mean distance of 198.15 cm and 179.58 cm, respectively; Table 4). Principal component analysis was performed on a total of 3097 frogs: 1399 *C. boraceiensis*, 1628 *H. asper* and 70 *H. phyllodes*. The first and second principal components explained 67.15% of total dispersion (Table 5). All distances measured showed important overlap among the three species, not discriminating species (Figure 6).

DISCUSSION

Our study revealed the variability in spatial and temporal distributions among each studied species as well as their species-specific microhabitat features. We demonstrate that torrent frogs can use rocks similarly along the streams as primary habitat, varying only in occurrence along and among streams, and in abundance during the months. Collectively, these data allow us to improve our understanding of species occurrence and to identify how

each species exploits stream habitats, which in turn can help steer conservation efforts of stream-dweller amphibians.

Hylodes phyllodes were the only species present in stream 2, the smallest stream sampled and the only one that ceases to flow during the driest periods, in accordance with previous observations (Heyer *et al.,* 1990). This species was also the only one found in all streams. Within streams 1, 2 and 3 one of the species was spatially segregated as it was found in higher abundance in different sections of the streams. These differences in frog occurrence may be related to differences in environmental conditions and, therefore, differences in the availability of suitable habitats between and within streams. For instance, a wide, flat area with several large ponds at the 75-100 m section of stream 3 is probably the main feature responsible for the high abundance of *H. asper* at this location. Parris and McCarthy (1999) found that habitat variables related to stream size and adjacent forest affected site suitability for different frog species at forest streams in southeast Queensland, Australia. Future studies should investigate additional stream environmental conditions to unravel their influence on species occurrence, such as stream width, catchment volume, water flow, extent of rocky surfaces and vegetation cover.

The three torrent frogs studied here are known to breed continuously, like many other amphibians in the tropical region. However, encounter rates were significantly higher in the wet season (October to March) than in the dry season (April to September) for *C. boraceiensis* and *H. asper*. The seasonal variation found for these two species may reflect the higher reproduction rate and juvenile recruitment in the wet season, and may correspond to seasonal patterns in temperature and precipitation, as suggested by Ruggeri *et al.* (2015). *Hylodes phyllodes* were the least common species during visual surveys. This may be due to a survey bias because *H. phyllodes* individuals are more difficult to record visually (pers. obs.), perhaps because of their small size.

In other amphibian assemblages habitat appears to be a major resource partitioned by post-metamorphic individuals (Toft, 1985). In relation to substrate and distances from the stream, however, the three torrent frogs studied here were most likely to occur at similar microhabitats, such as wet or moist rocks without moss and coverage. Almeida-Gomes *et al.* (2007) and Hartmann (2004) also recorded these species restricted to rocks on streams in the Atlantic forest. Yet, Hartmann (2004) found *C. boraceiensis* calling on rock crevices in the middle of the stream, *H. asper* exposed on rocks in the middle or edge of the stream and *H. phyllodes* always in shaded trunks or rocks at the edge of the streams. This transversal species distribution on rocks was not analyzed in our study, but *H. phyllodes* were found more distant from the water among the three species, being commonly found at stream margins (pers. obs.).

Most individuals were found next to the water, which may be due to the supposedly higher humidity closer to these locations. The availability of suitable moist microhabitats is important for most of post-metamorphic stages of amphibians (Wells, 2010). On a small scale, soil moisture and availability of moist retreat sites can be important determinants of the local distribution and abundance of amphibians (Wells, 2010). In addition, when frightened, individuals may dive in the stream and avoid being captured, as observed in both species of *Hylodes* studied (per. obs.) and in other stream-dwelling species (Gillespie and Hollis, 1996; Almeida-Gomes *et al.*, 2007).

Data on microhabitat use by amphibian adults are mostly limited to breeding sites and there are scarce data on inactive individuals. Similar to our results, Heyer *et al.* (1990) and Hatano *et al.* (2002) reported inactive specimens of *H.asper* and *H. fredi* (a closely related species of *H. phyllodes*) resting at night on leaves overhanging streams. The riparian vegetation coverage can be an important feature of microhabitat suitability for torrent frogs, as leaves along the stream banks may serve as resting habitats. Additional studies on the

adult dependence on riparian vegetation would be useful to clarify the dependence between the torrent frogs' occurrence and the presence of riparian vegetation (e.g. Parris, 2001).

Habitat loss is one of the main causes of worldwide amphibian population declines (Cushman, 2006). Furthermore, flowing freshwater, usually streams, is regarded as the second habitat most preferred by amphibians after forests (IUCN, 2015). Unfortunately, declines in freshwater biodiversity are far greater than in terrestrial ecosystems (Dudgeon *et al.*, 2006). Preventing population declines, nonetheless, requires baseline data, such as the species distribution and description of its habitat use at fine scales if one is to set conservations resolutions for effective management. For instance, this knowledge is extremely useful during the assessment of the conservation status of species (e.g., IUCN Red List of Threatened Species, IUCN, 2015) and to subsequently guide conservation initiatives. However, detailed long-term survey data and habitat information for amphibian species remains scarce (see Blaustein *et al.*, 1994; Houlahan *et al*, 2000 for a list of long-term studies with amphibian populations). Our study is one the few available that not only characterizes fine-scale microhabitat use by the species studied, but also explore long-term spatial and seasonal variations in population abundance. We believe our results can be applied to similar torrent frogs and that future studies should extend this approach.

ACKNOWLEDGEMENTS

This work was supported by São Paulo State Research Foundation (FAPESP, grants 2006/52057-2 and 2007/51478-7 to MPG, and grants 2006/58011-4 and 2010/50146-3 to MM). MM also thanks CNPq for a research fellowship. TSL received M.Sc. fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) and FAPESP

(grant 2014/06795-8). We thank all people who participated in the field work, gave helpful suggestions on the manuscript, and the Instituto Florestal for allowing our fieldwork in the Serra do Mar State Park.

REFERENCES

Almeida-Gomes, M., Hatano, F. H., Van Sluys, M., Rocha, C. F. D. 2007. Diet and microhabitat use by two Hylodinae species (Anura, Cycloramphidae) living in sympatry and syntopy in a Brazilian Atlantic Rainforest area. Iheringia Série Zool., 97, 27-30.

Blaustein, A. R., Wake, D. B., Sousa, W. P., 1994. Amphibian Declines: Judging stability, persistence, and susceptibility of populations to local and global extinctions. Conservation Biology, 8, 60-71.

Bury, R. B., 2006. Natural history, field ecology, conservation biology and wildlife management: time to connect the dots. Herpetological Conservation and Biology, 1, 56-61.

Cushman, S. A., 2006. Effects of habitat loss and fragmentation on amphibians: A review and prospectus. Biological Conservation, 128, 231-240.

Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z., Knowler, D., Lévêque, C., Naiman, R. J., Prieur-Richard, A.-H., Soto, D., Stiassny, M. L. J. and Sullivan, C. A., 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. Biological Reviews, 81, 163-182.

Duellman, W. E., Trueb, L., 1986. Biology of amphibians. JHU Press.

Gascon, C., 1991. Population and community-level analyses of species occurrences of central amazonian rainforest tadpoles. Ecology, 72, 1731-1746.

Gibbons, J. W., Scott, D. E., Ryan, T. J., Buhlmann, K. A., Tuberville, T. D., Metts, B. S., Greene, J. L., Mills, T., Leiden, Y., Poppy, S., Winne, C. T., 2000. The Global decline of reptiles, Déjà Vu amphibians. Bioscience. 50, 653-666.

Gillespie, G. R., Hollis, G. J., 1996. Distribution and habitat of the spotted tree frog, *Litoria spenceri* Dubois (Anura:Hylidae), and an assessment of potential causes of population declines. Wildlife Research, 23, 49-75.

Gillespie, G. R., Lockie, D., Scroggie, M. P., Iskandar, D. T., 2004. Habitat use by stream-breeding frogs in south-east Sulawesi, with some preliminary observations on community organization. Journal of Tropical Ecology, 20, 439-448.

Haddad, C. F. B., Giaretta, A. A., 1999. Visual and acoustic communication in the Brazilian Torrent Frog, *Hylodes asper* (Anura: Leptodactylidae). Herpetologica 55, 324-333.

Haddad, C. F. B., Toledo, L. F., Prado, C. P. A., 2008. Anfíbios da Mata Atlântica: guia dos anfíbios anuros da Mata Atlântica. São Paulo, Editora Neotropica, 244p.

Hall, L. S., Krausman, P. R., Morrison, M. L., 1997. The habitat concept and a plea for standard terminology. Wildlife Society Bulletin, 25, 173-182.

Hammer, Ø., Harper, D. A. T., Ryan, P. D., 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica, 4, 1. http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

Hartel, T., Băncilă, R., Cogălniceanu, D., 2011. Spatial and temporal variability of aquatic habitat use by amphibians in a hydrologically modified landscape. Freshwater Biology, 56, 2288-2298.

Hartmann, M. T., 2004. Biologia reprodutiva de uma comunidade de anuros (Amphibia) na Mata Atlântica (Picinguaba, Ubatuba, SP). Universidade Estadual Paulista "Julio de Mesquita Filho", Campus de Rio Claro, Brasil. Thesis.

Hartmann, M. T., Hartmann, P. A., Haddad, C. F. B., 2006. Repertório vocal de *Hylodes phyllodes* (Amphibia, Anura, Hylodidae). Papéis Avulsos de Zoologia (São Paulo), 46, 203-209.

Hartmann, M. T., Hartmann, P. A., Haddad, C. F. B., 2010. Reproductive modes and fecundity of an assemblage of anuran amphibians in the Atlantic rainforest, Brazil. Iheringia, Série Zoologia, 100, 207-215.

Hatano, F. H., Rocha, C. F. D., Van Sluys, M., 2002. Environmental factors affecting calling activity of a tropical diurnal frog (*Hylodes phyllodes*: Leptodactylidae). Journal of Herpetology, 36, 314-318.

Heyer, W. R., 1983. Variation and systematics of frogs of the genus *Cycloramphus* (Amphibia, Leptodactylidae). Arquivos de Zoologia, 25, 235-339.

Heyer, W. R., Cocroft, R. B., 1986. Descriptions of two new species of *Hylodes* from the Atlantic Forests of Brazil (Amphibia, Leptodactylidae). Proc. Biol. Soc. Washingt. 99, 100–109.

Heyer, W. R., Rand, A. S., Cruz, C. A. G. da, Peixoto, O. L., Nelson, C. E., 1990. Frogs of Boracéia. Arquivos de Zoologia, 31, 4, 231-410.

Houlahan, J. E., Findlay, C. S., Schmidt, B. R., Meyer, A. H., Kuzmin, S. L., 2000. Quantitative evidence for global amphibian population declines. Nature. 404, 752-755.

IUCN, Conservation International, and NatureServe. 2015. An Analysis of Amphibians on the 2015. IUCN Red List <www.iucnredlist.org/amphibians>.

Köppen, W., 1948. Climatologia: con un estudio de los climas de la tierra. Fondo de Cultura Econômica, México. 479p.

Krausman, P.R., 1999. Some basic principles of habitat use. *In* Launchbaugh, K. L., Sander, K. D. and Mosley, J. C., Grazing behavior of livestock and wildlife, University of Idaho Forest, Wildlife and Range Experimental Station bulletin 70, 85-90.

Leuven, R. S. E. W., Den Hartog, C., Christiaans, M. M. C., Heijligers, W. H. C., 1986. Effects of water acidification on the distribution pattern and the reproductive success of amphibians. Experientia, 42, 495-503.

Parris, K. M., McCarthy, M. A., 1999. What influences the structure of frog assemblages at forest streams? Austral Ecology, 24, 495-502.

Parris, K. M., 2001. Distribution, habitat requirements and conservation of the cascade treefrog (*Litoria pearsoniana*, Anura : Hylidae). Biological Conservation, 99, 285-292.

QGIS Development Team, 2016. QGIS: Geographic Information System. Open Source Geospatial Foundation Project. URL http://qgis.osgeo.org.

R Development Core Team, 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. URL http://www.R-project.org.

Ruggeri, J., Longo, A. V., Gaiarsa, M. P., Alencar, L. R. V., Lambertini, C., Leite, D. S., Carvalho-e-Silva, S. P., Zamudio, K.R., Toledo, L. F., Martins, M., 2015. Seasonal variation in population abundance and chytrid infection in stream-dwelling frogs of the Brazilian Atlantic Forest. PLoS One, 10, e0130554.

Toft, C.A., 1985. Resource partitioning in amphibians and reptiles. Copeia, 1, 1-21.

Van Sluys, M., Vrcibradic, D., Alves, M. A. S., Bergallo, H. G., Rocha, C. F. D., 2007. Ecological parameters of the leaf-litter frog community of an Atlantic Rainforest area at Ilha Grande, Rio de Janeiro state, Brazil. Austral Ecology, 32, 254–260.

Wells, K. D., 2010. The ecology and behavior of amphibians. University of Chicago Press.

Zar, J. H., 1999. Biostatistical Analysis. Prentice Hall, New Jersey, U.S.A, 718 pp.

FIGURES

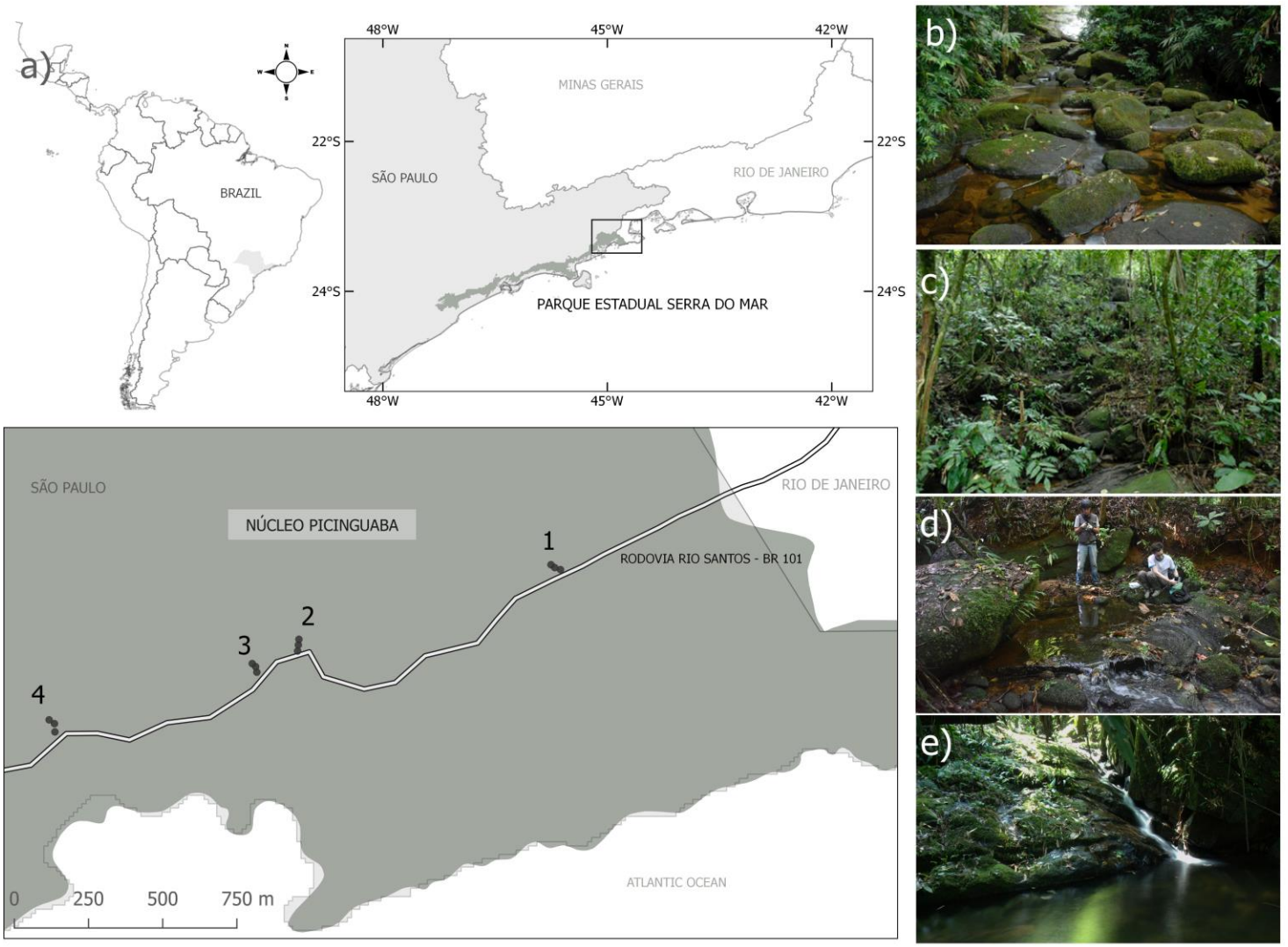


Figure 1. Study area. a) Maps showing the location of study site, Núcleo Picinguaba at the Parque Estadual Serra do Mar, São Paulo, southeastern Brazil. Numbers 1 to 4 indicate each stream sampled in the area, b) Stream 1, c) Stream 2, d) Stream 3, e) Stream 4. Photos by Marcio Martins.

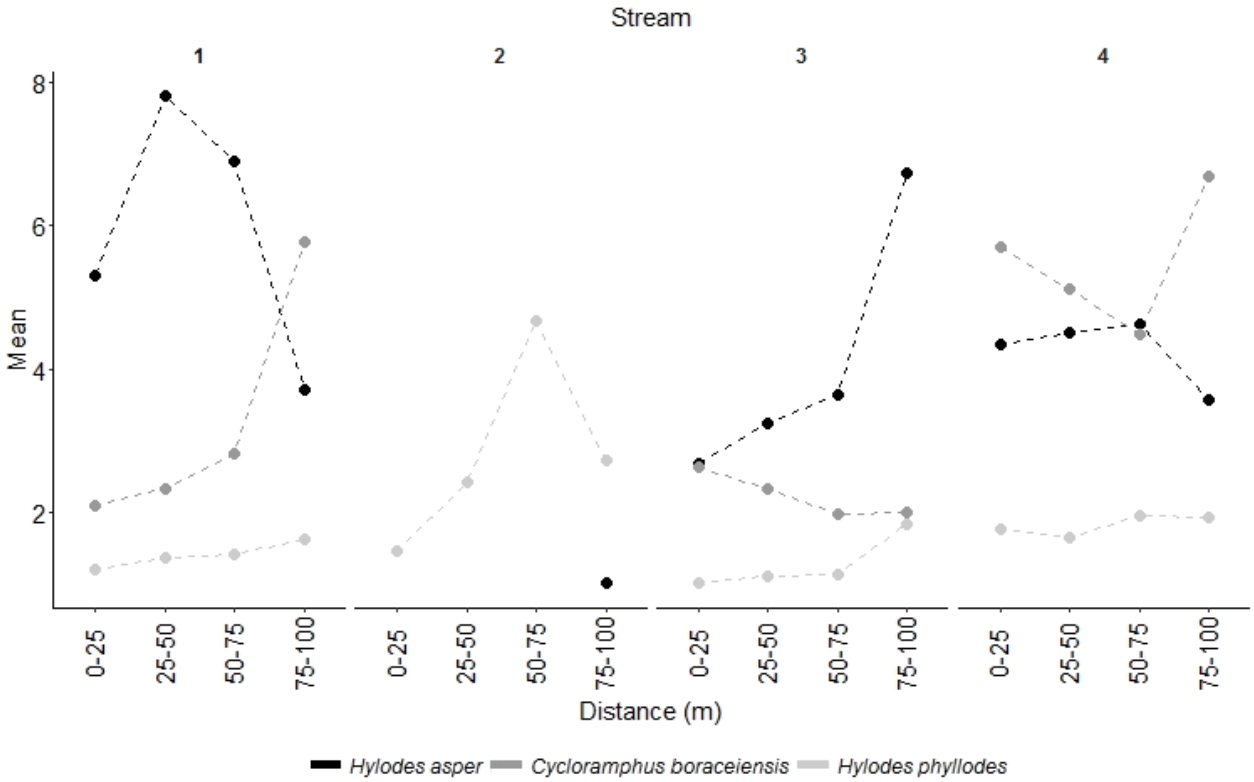


Figure 2. Mean encounter number during the 5 year study at every 25 m of each stream sampled.

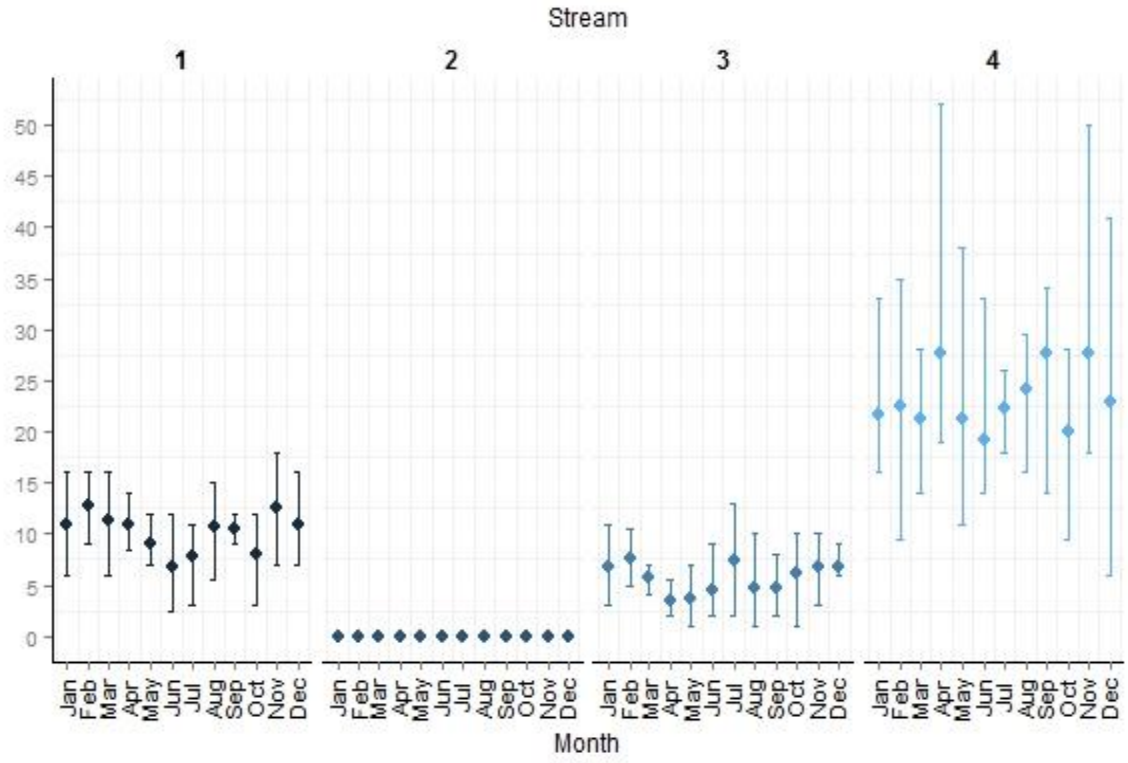


Figure 3. Mean encounter number by visual survey of *Cycloramphus boraceiensis* per month at each stream. Bars indicate maximum and minimum number found every month.

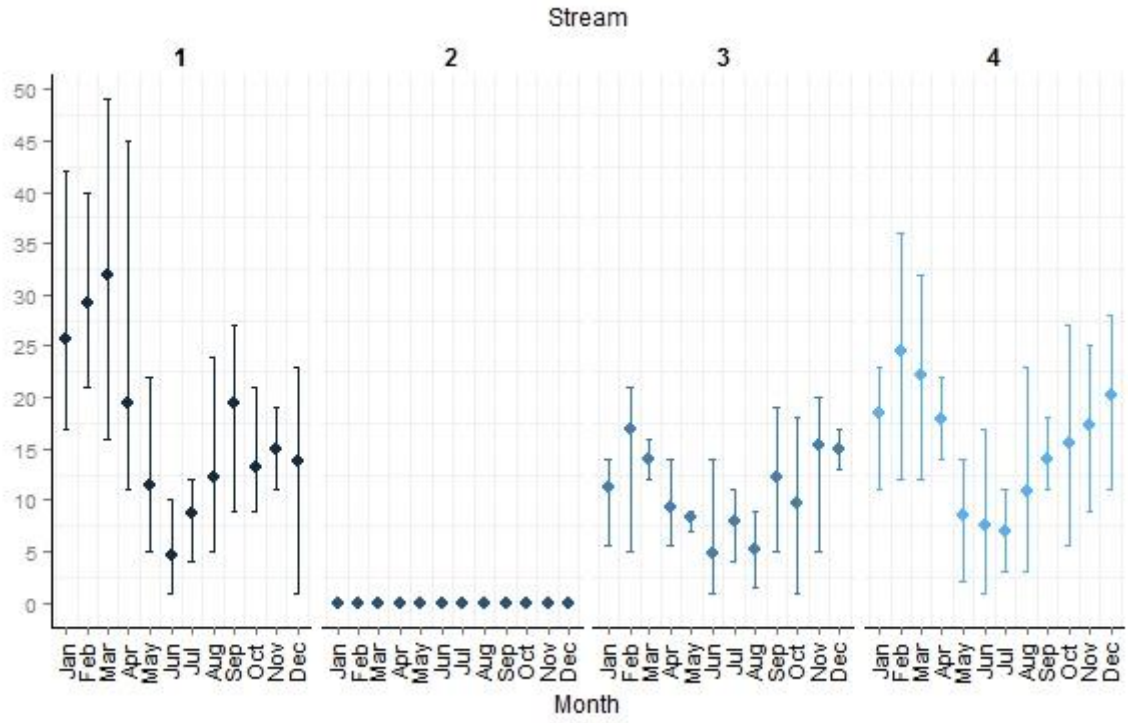


Figure 4. Mean encounter number by visual survey of *Hylodes asper* per month at each stream. Bars indicate maximum and minimum number found every month.

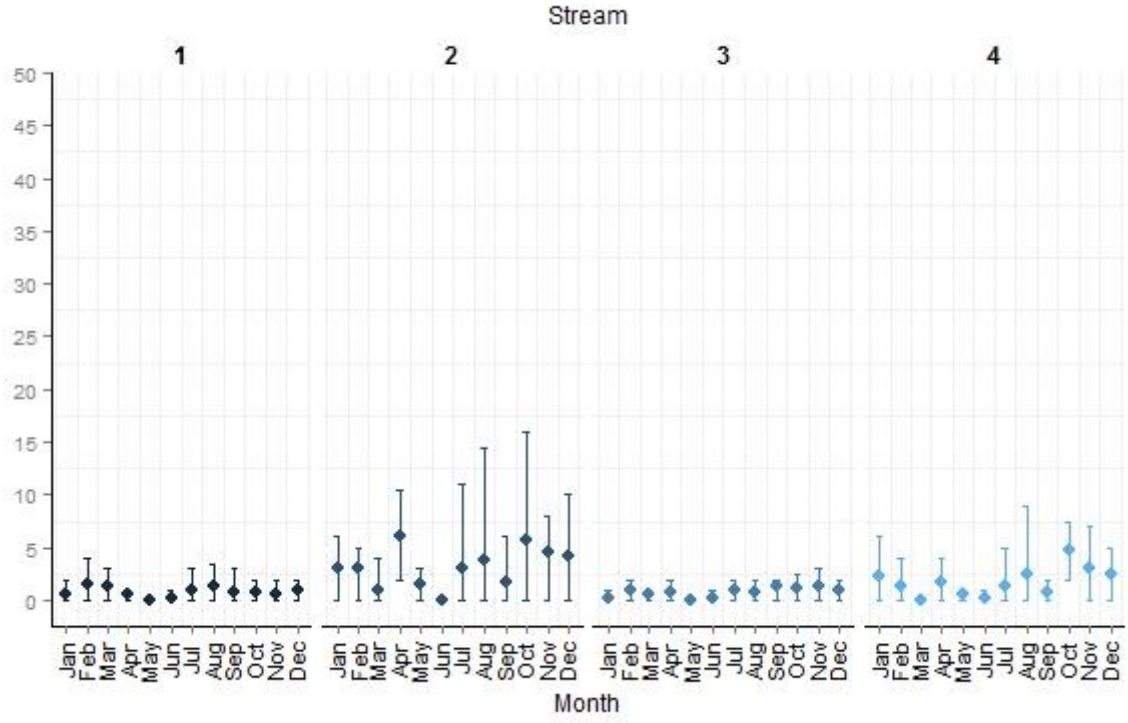


Figure 5. Mean encounter number by visual survey of *Hylodes phyllodes* per month at each stream. Bars indicate maximum and minimum number found every month.

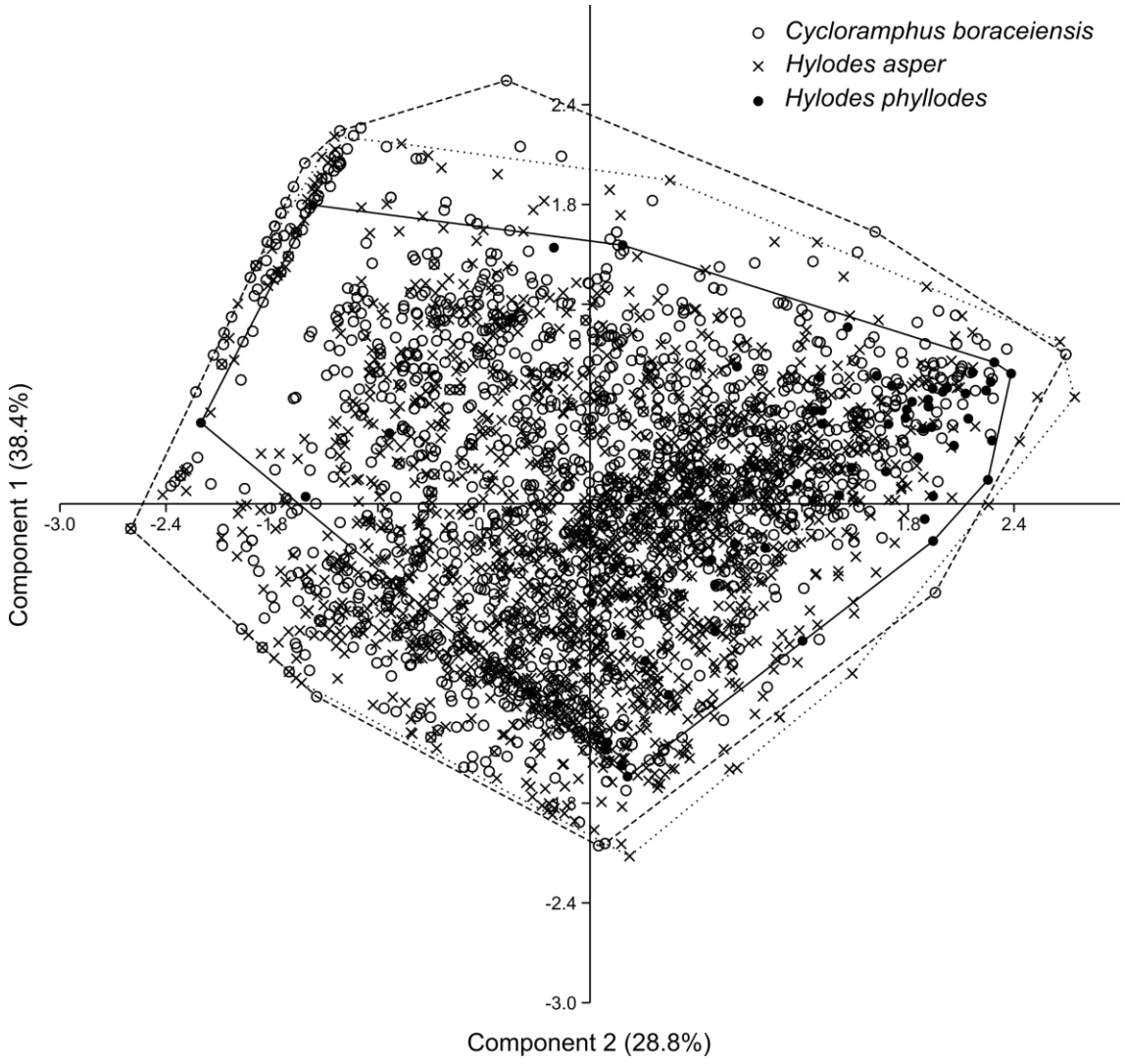


Figure 6. Biplot of PCA analysis based on measures of distance from water.

TABLES

Table 1. Results of circular statistical analysis testing for the occurrence of seasonality on abundance of *Cycloramphus boraceiensis, Hylodes asper* and *Hylodes phyllodes* from Núcleo Picinguaba, São Paulo, Brazil in four consecutive years. Mean angle indicates the month with the highest abundance when significant.

		Years			
		2007	2008	2009	2010
Mean angle (*a*)	*C. boraceiensis*	87.45°	61.43°	2.034° (Jan)	287.62 ° (Dec)
	H. asper	68.33° (Mar)	73.36° (Mar)	68.55° (Mar)	332.18° (Dec)
	H. phyllodes	227.5° (Aug)	358,43°	332.57° (Dec)	347.66°
Length of mean vector (*r*)	*C. boraceiensis*	0.135	0.21	0.229	0.267
	H. asper	0.262	0.255	0.213	0.289
	H. phyllodes	0.492	0.354	0.349	0.13
Rayleigh test of uniformity (*p*)	*C. boraceiensis*	0.858	0.7174	0.049	0.0098
	H. asper	<< 0.001	<< 0.001	<< 0.001	<< 0.001
	H. phyllodes	0.0196	0.1064	<< 0.001	0.4706

Table 2. Number of individuals active of *Cycloramphus boraceiensis*, *Hylodes asper* and *Hylodes phyllodes* at each habitat variable along the four streams sampled at Núcleo Picinguaba, São Paulo, Brazil (percentages in parentheses). Summary of chi-square goodness-of-fit test results is presented for each species (degrees of freedom in parentheses).

		Species		
		C. boraceiensis	*H. asper*	*H. phyllodes*
Surface type	Rock	1451 (88.5)	1612 (91.5)	89 (84.8)
	Crevices	141 (8.6)	4 (0.2)	0
	Sand	4 (0.2)	5 (0.3)	1 (0.95)
	Leaf	12 (0.7)	17 (0.96)	2 (1.9)
	Pipe	0	36 (2.0)	0
	Tree branch	0	23 (1.3)	1 (0.95)
	Leaf litter	5 (0.3)	1 (0.1)	0
	Trunk	25 (1.5)	52 (2.95)	11 (10.5)
	Other	2 (0.1)	11 (0.6)	1 (0.95)
X^2 (df)		10027.6 (8)	11544.9 (8)	584.9 (8)
p-value		<< 0.001	<< 0.001	<< 0.001
Surface humidity	Dry	134 (8.6)	316 (18.1)	34 (32.4)
	Humid	546 (35.1)	1035 (59.4)	58 (55.2)
	Wet	587 (37.7)	324 (18.6)	10 (9.5)
	Film of water	290 (18.6)	68 (3.9)	3 (2.9)
X^2 (df)		356.3 (3)	1196.03 (3)	71.35 (2)
p-value		<< 0.001	<< 0.001	<< 0.001
Moss	Presence	549 (36.3)	722 (41.3)	38 (37.6)
	Absence	962 (63.7)	1025 (58.7)	63 (62.4)
X^2(df)		112.88 (1)	52.55 (1)	6.18 (1)
p-value		<< 0.001	<< 0.001	0.013
Cover type	Rock	349 (23.5)	317 (18.5)	37 (35.2)
	Leaf	87 (5.8)	50 (2.9)	16 (15.2)
	Branch	20 (1.3)	16 (0.9)	4 (3.8)
	Absence	1013 (68.1)	1320 (77.01)	42 (40)
	Other	19 (1.3)	11 (0.6)	6 (5.7)
X^2(df)		2397.42 (4)	3670.4 (4)	58.86 (4)
p-value		<< 0.001	<< 0.001	<< 0.001

Table 3. Number of individuals inactive of *Hylodes asper* and *Hylodes phyllodes* at each habitat variable along the four streams sampled at Núcleo Picinguaba, São Paulo, Brazil (percentages in parentheses). Summary of chi-square goodness-of-fit test results is presented for each species (degrees of freedom in parentheses).

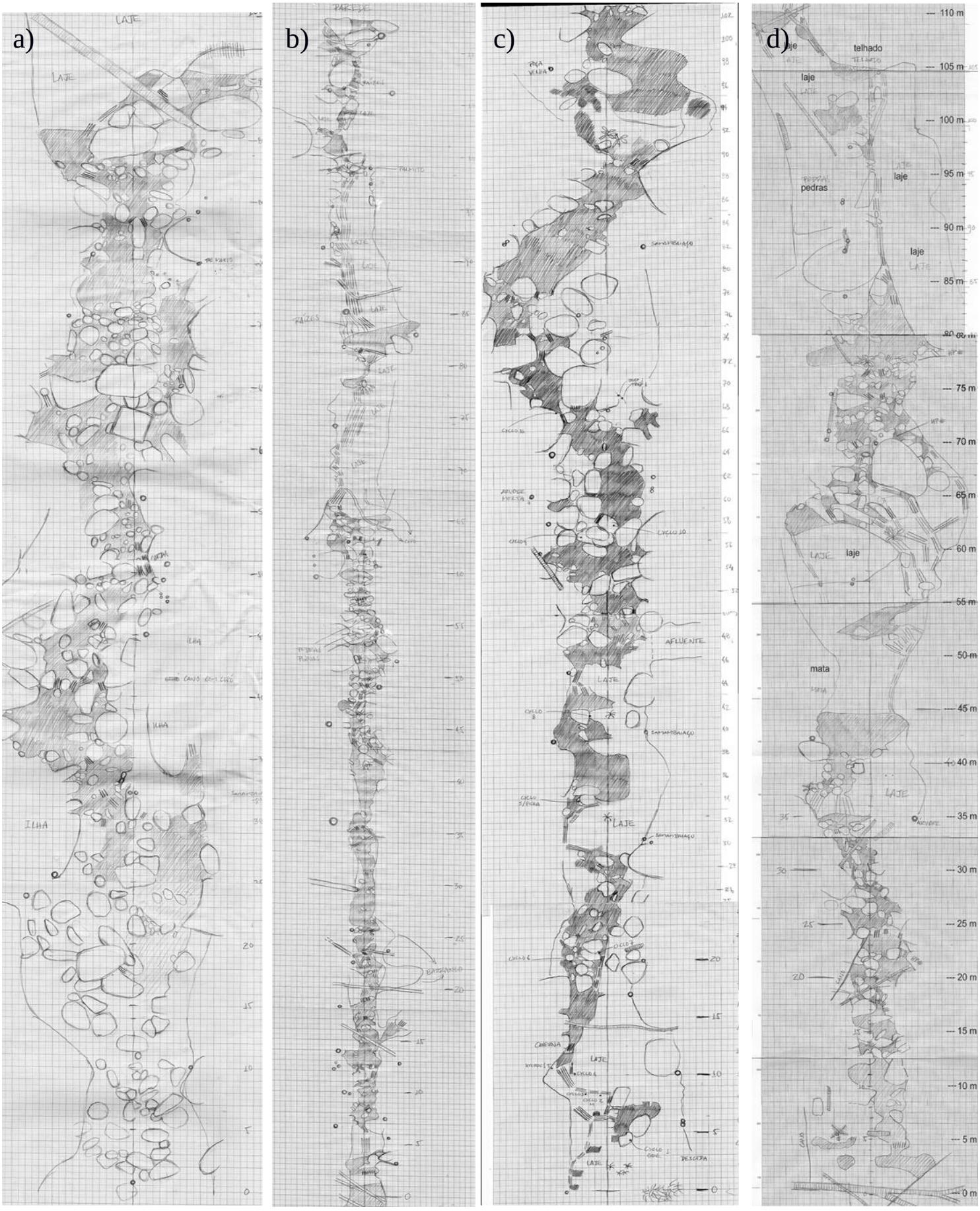
		Species	
		H. asper	*H. phyllodes*
Surface type	Rock	35 (7.9)	2 (1.0)
	Crevices	2 (0.5)	0
	Sand	0	0
	Leaf	338 (76.3)	193 (97.0)
	Pipe	9 (2.0)	0
	Tree branch	40 (9.0)	1 (0.5)
	Leaf litter	0	0
	Trunk	9 (2.0)	1 (0.5)
	Other	10 (2.3)	2 (1.0)
X^2(df)		1411 (6)	737.16 (4)
p-value		<< 0.001	<< 0.001
Surface humidity	Dry	170 (61.6)	90 (70.9)
	Humid	72 (26.1)	28 (22.0)
	Wet	34 (12.3)	7 (5.5)
	Film of water	0	2 (1.6)
X^2(df)		107.04 (2)	154.48 (3)
p-value		<< 0.001	<< 0.001
Moss	Presence	23 (10.1)	1 (0.9)
	Absence	205 (89.9)	106 (99.1)
X^2(df)		145.28 (1)	103.04 (1)
p-value		<< 0.001	<< 0.001
Cover type	Rock	39 (10.8)	6 (4.7)
	Leaf	149 (41.4)	32 (24.8)
	Branch	3 (0.8)	0
	Absence	158 (43.9)	90 (69.8)
	Other	11 (3.1)	1 (0.8)
X^2(df)		318 (4)	155.06 (3)
p-value		<< 0.001	<< 0.001

Table 4. Mean distances in cm from the nearest water body, waterfall and pond and height above the water for each species at the streams in Núcleo Picinguaba, São Paulo, Brazil. Ranges of distances and total number of observations (N) are indicated in parentheses.

	Distance (cm)				
Species	Water	Waterfall	Pond	Water flow	Height water
Cycloramphus boraceiensis	15.84 (0 – 400, N= 1522)	91.24 (0 – 1000, N=1534)	70.26 (0-600, N=1506)	65.62 (0-700, N=1527)	38.01 (0-300, N=1496)
Hylodes asper	11.45 (0-500, N=1728)	140.58 (0-800, N=1729)	43.12 (0-800, N=1721)	73.95 (0-800, N=1714)	21.91 (0-400, N=1728)
Hylodes phyllodes	56.58 (0-250, N=100)	198.15 (0-700, N=83)	110.2 (0-600, N=77)	179.58 (0-800, N=86)	36.30 (0-200, N=103)

Table 5. Results of the Principal Components Analysis on five variables on distance from the nearest water body, waterfall, pond, water flow and height above the water in streams at Núcleo Picinguaba, São Paulo, Brazil.

	PC1	PC2	PC3	PC4	PC5
Water	0.45058	0.20543	0.1478	0.34354	-0.78416
Waterfall	0.38951	-0.37412	-0.31196	0.68913	0.36891
Pond	0.35407	0.79067	-0.39236	-0.10425	0.29095
Water flow	0.69604	-0.38355	0.048335	-0.60343	0.044209
Height water	0.1882	0.21345	0.85121	0.17912	0.40297
Eigenvalue	0.907	0.68	0.306	0.283	0.187
% Variance	38.35	28.79	12.95	11.98	7.92



Supplementary Figure I - Graph paper maps of sampled stream at Núcleo Picinguaba, São Paulo, Brazil: a) stream 1, b) stream 2, c) stream 3, d) stream 4. Circles on maps represent rocks along the stream and the black area represents water flow.

CHAPTER 2

SPECIES DETECTION IN STREAM AMPHIBIAN COMMUNITIES USING ENVIRONMENTAL DNA

THAIS SASSO LOPES[1*], CARLA MARTINS LOPES[2], ALICE VALENTINI[3], TONY DEJEAN[3], KELLY ZAMUDIO[4], CÉLIO HADDAD[2], MARCIO MARTINS[1]

[1]Departamento de Ecologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, CEP 05508-090, Brazil;
[2]Departamento de Zooologia, Instituto de Biociências, Universidade Estadual Paulista, UNESP, Rio Claro, SP, CEP 13506-900, Brazil;
[3]SPYGEN, Savoie Technolac - BP 274, Le Bourget-du-Lac, 73375, France;
[4]Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY, 14853, USA.

ABSTRACT

Freshwater ecosystems support an important percentage of all known amphibian species. Yet, these ecosystems are experiencing a sharp decline in biodiversity while at the same time knowledge of species that depend on these environments is still scant. Environmental DNA (eDNA) detection is a new approach for monitoring aquatic vertebrates, and can provide unprecedented and reliable information on species occurrence in freshwater ecosystems. To determine whether the analysis of eDNA can accurately inform amphibian community composition in the tropics we compared data from a five year traditional field survey with those of eDNA analysis collected in streams in the Atlantic forest, of southeastern Brazil. Twelve water samples were filtered from four streams. We used eDNA metabarcoding with a universal amphibian primer of a mitochondrial marker (12S). We recorded ten species through traditional visual-acoustic survey and nine species through the eDNA metabarcoding approach. All species detected with metabarcoding matched the known amphibian found during traditional surveys. *Proceratophrys appendiculata* was the only species found through the traditional survey but not in the metabarcoding analysis. On the other hand, we identified *Bokermannohyla* sp. aff *circumdata* and *Aplastodiscus eugenioi* in eDNA samples, which were previously detected only by call surveys. Three species (*Cycloramphus boraceiensis, Hylodes asper* and *Hylodes phyllodes*) with the highest dependence on aquatic habitat and highest constancy indices were the ones with highest positive detections in eDNA samples, suggesting an important role of the species natural history on eDNA detection from stream water samples. Our results are encouraging for the use of eDNA sampling and metabarcoding as a reliable method for assessing community diversity in tropical streams. Our study is the first effort to detect species using eDNA in the Atlantic forest, in addition to demonstrating that this technique can be successfully applied in Neotropical forest streams.

Keywords: Metabarcoding, biodiversity assessment, 12S, community sampling, high throughput sequencing

INTRODUCTION

The Atlantic forest of eastern Brazil is home of one of the highest diversity of amphibians on Earth, harboring over 500 amphibian species (ca. 7% of all known amphibians), 88% of which are endemics (Haddad *et al*., 2013). On the other hand, the Atlantic forest is one of the most threatened ecosystems in the Neotropical region (e.g., Myers *et al*., 2000) with only 12.5% of its original vegetation cover remaining (SOS Mata Atlântica, 2015). As a consequence of this extensive habitat loss, as well as of other less obvious threats (see Eterovick *et al*., 2005), about 87.5% of threatened anuran species in Brazil are from Atlantic forest (ICMBio, 2016).

A great part of the Atlantic forest, especially along the coast of eastern Brazil, occurs on mountainous terrains where a multitude of streams flow (Morellato and Haddad, 2000). These freshwater environments house an astounding diversity of amphibians, including some species that have their life histories tightly connected to streams, with most or all life stages depending on lotic waters (examples are the anuran families Hylodidae, Cycloramphidae, Centrolenidae) (Haddad *et al*., 2013). The few well documented frog declines in Brazil include species closely associated to streams (see a review in Eterovick *et al.,* 2005) and almost 23% of the Atlantic forest amphibians that present rheophilic habit lack baseline data and are listed as Data-Deficient species (Haddad *et al*., 2013). Describing the occurrence of stream frogs from this hotspot of biodiversity is, therefore, crucial for the conservation of many species of amphibian. However, field techniques traditionally applied

to reveal amphibians occurrence require an enormous amount of fieldwork and comes with a set of constraints (e.g., Heyer *et al.*, 1993).

Recently, a promising alternative approach for monitoring species, the analysis of environmental DNA (eDNA), became available. The term eDNA refers to the nuclear or mitochondrial DNA released by an organism in the environment, such as water, soil, or even air (Pilliod *et al*., 2013). This DNA is shed into the environment from sloughed skin, spores, feces, secretions or gametes, and can be then sampled for sequencing and species identification (Bohmann *et al.,* 2014). The use of eDNA has recently gained wide spread attention because it is allowing researchers to detect species even at low abundances (Dejean *et al*., 2011). In the past five years the analysis of eDNA began to be employed to a wide phylogenetic range, targeting species of amphibians, fishes, mammals, reptiles, arthropods, and mollusks (Supplementary Material List I).

Although the number of ecological studies applying eDNA analysis to detect species is increasing, few studies have actually sampled eDNA from aquatic environments to perform broad community surveys. The majority of the research effort has focused on a particular species using a species-specific marker. However, universal primers can be used instead, if the goal is to assess a broader range of biodiversity. The use of DNA-based identification of multispecies with universal primers is known as "DNA metabarcoding" (Taberlet *et al*., 2012). To fill gaps in our knowledge on eDNA applicability for community survey, we performed eDNA metabarcoding to investigate the amphibian community composition in four streams in Atlantic forest, Brazil. We compared amphibian biodiversity assessed by using eDNA metabarcoding with that obtained through traditional field methods (i.e. visual and calling survey). We also tested whether eDNA detection depends on habitat use or constancy of species in traditional survey. Findings from this study will

improve our current understanding of eDNA usefulness for tropical amphibians survey and for broader ecological questions.

MATERIAL AND METHODS

Study Site

The study area is located within the Parque Estadual da Serra do Mar (PESM) - Núcleo Picinguaba, municipality of Ubatuba, state of São Paulo, southeastern Brazil (24°13'12.49" S, 47°22'4.71" W, 23°22'36.90" S, 44°44'19.07" W) (Figure 1). Núcleo Picinguaba is a protected area situated in the Atlantic forest domain, along the north coast of the state of São Paulo (Figure 1). The climate is tropical (Koppen, 1948) with mean annual air temperature of 26.72 °C, high and relatively constant air humidity (monthly means around 85-90%), and an annual average rainfall of 2650 mm (CIIAGRO, 2016). The region has a warm and humid season with frequent rains from October to April (monthly rainfall 215-376 mm, with a peak in December and January and mean temperatures from 21.1 to 25.5 °C), and a drier and colder season from May to September (monthly rainfall 11-166 mm, with July as the driest month and mean temperatures from 18.4 to 20.5 °C) (Ruggeri *et al.*, 2015).

We collected data from four freshwater mountain streams that are not connected (i.e., the four streams are distinct units). The streams are mostly covered by forest canopy and underlain by a mosaic of rocks and sand. In the sampled region, streams vary in their width, elevation, water flow, and structure as follows: *Stream 1* (23° 21' 15.2" S, 44° 46' 3.2" W) is the largest stream sampled (4-15 m wide) and runs along a relatively flat area with many large pools and relatively few waterfalls; *Stream 2* (23° 21' 34.4" S, 44° 47' 3.2" W) is the smallest stream sampled (1-5 m wide, it ceases to flow during the driest periods of

the driest years), runs along a relatively flat area in the first 40 m, sloping for the rest of its length and finishing at a steep area; *Stream 3* (23° 21' 41.2" S, 44° 47' 15.3" W) has a smaller inclination than the other streams sampled, with fewer waterfalls and many large pools, and its width varies from 5 to 10 m; *Stream 4* (23° 21' 53.7" S, 44° 48' 2.8" W) is the most sloping of the four streams, with many waterfalls and few large pools, and its width varies from 5 to 10 m.

Traditional survey

Fieldwork was carried out monthly from January 2007 to December 2011, except in 2011 when data was taken every other month, for a total of 54 months, comprising 147 days at the field. Visual and calling surveys were performed twice a day (one diurnal and one nocturnal survey) within a 95-115 m transect parallel to the water flow along the four streams. We searched for post-metamorphic individuals while walking slowly upstream during 30 to 60 minutes, checking all visually accessible spots in the streambed. We assessed the species spatial distribution by recording the date and time when each individual was seen and mapping its exact location using a stream map drawn on graph paper (Supplementary Figure I).

Water Parameters

During sample surveys, we took physical and water quality parameters *in situ* to characterize each stream. We measured dissolved oxygen and water temperature at all sampling sites using YSI 55 Dissolved Oxygen and Temperature Meter. We measured water speed with a Mechanical Flowmeter (G.O. Environmental) at all sampling sites with enough water depth for instrument placement. We used a Labcon Test to measure water pH once at every first sampling site in each stream.

Detection Protocol for eDNA

Field sampling

Collections of eDNA samples were performed during four days. Each stream was sampled on a different day, from April 21st to 26th 2015. A 100 m transect was defined upstream at each stream and water samples were collected in three locations. The first sampling site was at 0 m, the second at the middle of the transect (~50 m), and the third at the end of the transect (~100 m). Samplings were first performed at the most downstream site (0 m) and proceeded upstream (~100 m) to avoid contamination and water perturbation by previous sampling.

We sampled eDNA by filtering the water column through a disposable capsule (Envirochek HV 1 µm, Pall Corporation, Ann Arbor, MI, USA) using a peristaltic pump (1.60 L min^{-1}, model 410, Solinst, Canada). We collected the water directly from the surface of the stream using a plastic hose and measured the discharge water in a graduated flask. At each sampling site we filtered 60 L of water. Moreover, after the sampling of each stream we filtered 5 L distilled water to serve as negative control and assess possible sources of contamination from the handling procedures in the field. To avoid contamination across samples, we used new hoses for each filtration and nitrile gloves during all the process. At the end of each filtration, the capsule was filled with a buffer solution to prevent DNA degradation and stored at room temperature. We had a total of four filters for each stream.

Molecular Analyses

After filtering the water at the field, eDNA analyses involved three key steps performed in the laboratory: DNA extraction of the sample, amplification of a target gene via polymerase

chain reaction (PCR), and next-generation sequencing. For these laboratory procedures, capsules were sent to Spygen (Le Bourget du Lac, France). All DNA extraction and PCR procedures were done in "DNA-free" rooms, equipped with positive air pressure, UV treatment, and frequent air renewal. Laboratory personnel wore full protective clothing (disposable coveralls, hood, mask, laboratory-specific shoes, overshoes, and two pair of gloves) in order to prevent any source of external contamination.

To recover the DNA contained in each filter, the capsules were left at 56 °C for two hours and stirred for five minutes as described in Valentini *et al.* (2016). The buffer inside each capsule was then transferred to three 50 ml tubes. In total, we retrieved 120 ml per capsule. The tubes were centrifuged at 15,000 g for 15 min and the supernatant was carefully discarded leaving 15 ml of liquid at the bottom of the tubes. Later, 33 ml of ethanol and 1.5 ml of 3 M sodium acetate were added to each tube and then centrifuged again at 15,000g for 15 minutes and at 6 °C. The supernatant was discarded once more. After these steps, 360 µL of ATL Buffer of the DNeasy Blood & Tissue Extraction Kit (Qiagen, Germany) were added to the first tube, the tube was vortexed and the supernatant was transferred to the second tube. This operation was repeated for all tubes. The supernatant in the third tube was transferred to a 2 mL tube and the DNA extraction was performed following the manufacturer's instructions. Two negative extraction controls were added and they were amplified and sequenced in the same way and in parallel to the samples to monitor reagents or external products contamination.

A short fragment (~52 bp) of the 12S mitochondrial gene was amplified via PCR using the universal Batrachia 12S primer L3541/H3596 (Table 1, see Valentini *et al.,* 2016 and Supplementary Figure IV for primer tests). Each amplification reaction consisted of 3 µL of DNA extract as template, 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA), 10 mM of Tris-HCl, 50 mM of KCl, 2.5 mM of MgCl2, 0.2

mM of each dNTP, 0.2 µM of the Batrachia primers (L3541/H3596), 4 µM of human blocking primers for the Batrachia primers (Valentini *et al.*, 2016) and 0.2 µg/µL of bovine serum albumin (BSA, Roche Diagnostic, Basel, Switzerland) for a final reaction volume of 25 µL. Blocking primers for human DNA were used to avoid undesired amplification and increase the specificity of the amplicons (see Wilcox *et al*., 2014). The PCR mixture was denatured at 95 °C for 10 min, followed by 50 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C, followed by a final elongation at 72°C for 7 min, in a room dedicated only to amplified DNA, physically separated from the DNA extraction room. We replicated each capsule in 12 independent PCR reactions. Three negative PCR controls containing ultrapure water, also with 12 replicates were analyzed in parallel to the samples. To distinguish eDNA sequences from different samples, primers were 5' labelled with a unique ~8 pb long tag pair (with at least three differences between tags) to assign sequences to the respective stream, sample location, and PCR replicate. After amplification, the samples were titrated using capillary electrophoresis (QIAxcel; Qiagen GmbH, Hilden, Germany) and purified using a MinElute PCR purification kit (Qiagen GmbH, Hilden, Germany). Before sequencing, purified DNA was titrated again using capillary electrophoresis. The purified PCR products were pooled in equal volumes, to achieve an expected sequencing depth of 300,000 reads. Library preparation and sequencing were performed at Fasteris facilities (Geneva, Switzerland). Libraries were prepared using Metafast protocol (https://www.fasteris.com/dna/?q=content/metafast-protocol-amplicon-metagenomic-analysis). A pair-end sequencing (2x125 bp) was carried out using an Illumina Hiseq 2500 sequencer (Illumina, San Diego, CA, USA) with HiSeq SBS Kit v4 (Illumina, San Diego, CA, USA) following the manufacturer's instructions. In total, two HiSeq runs were performed.

Species Reference Database

In order to identify species whose eDNA was collected in water samples, we built a 12S reference database of anuran species. The reference database was composed of (i) a local database with sequences from all anuran species known to occur at Núcleo Picinguaba, and (ii) anuran species sequences from the European Molecular Biology Laboratory (EMBL) database. The list of anuran species in the local database was based on Hartmann's (2004) amphibian composition list for the same region (Table 2).

To build the local sequence database, we obtained tissue samples from one to three specimens collected at Picinguaba or a close locality and pertaining to the scientific collection of Célio F. B. Haddad (CFBHT), UNESP, Rio Claro, São Paulo, Brazil. A total of 54 tissue samples were used for DNA extraction performed following a modified protocol from Sambrook and Russel (2001). Isolated DNA was amplified by PCR with three different primer sets: tVal/MVZ59, 148/H978 and MVZ59-FH/tVal-AL (Table 1), following the protocols described in Palumbi *et al.* (1991). PCR products were run on 2% agarose gel, visualized under ultraviolet light, and then purified using Exonuclease I and Shrimp Alkaline Phosphatase (ThermoFischer Scientific). DNA sequencing by capillary electrophoresis was done by Macrogen Inc. (Seoul, Republic of South Korea) and chromatograms were visually inspected, edited and assembled into consensus sequences using Codon Code Aligner 5.1.5. Using the software ecoPCR (http://pythonhosted.org/OBITools/scripts/ecoPCR.html), we trimmed the metabarcode region with the universal Batrachia primer pair L3541/H3596 (Table 1) allowing a maximum of two base mismatches per prime. Finally, as the universal Batrachia primers are expected to amplify a 52 bp segment, we excluded any sequence smaller than 40 bp or longer than 60 bp (thresholds were arbitrary). The finalized local database contained a total of 36 anuran species from 10 families (Table 2). We were not able to obtain sequences from

three species *(Bokermannohyla circumdata*, *Brachycephalus hermogenesi* and *Myersiella microps*) due to low quality sequences.

We also obtained from the *vrt124* release (standard sequences) of the EMBL database (ftp://ftp.ebi.ac.uk/pub/databases/embl/) sequences only for anuran species corresponding to the metabarcode region using the software ecoPCR. We trimmed the metabarcode region with the universal Batrachia primer pair L3541/H3596 (Table 1) and selected only sequences with length from 20 to 100 bp, allowing a maximum of two base mismatches per prime. In total, we retained 3425 sequences from 47 anuran families (Supplementary Table II). Finally, sequences from both EMBL database and the local database were merged to compose the complete species reference database.

Sequence analysis and filtering

Sequences resulting from the water samples were first analyzed to recover the sequences reads. In order to assemble forward and reverse DNA strands and construct a consensus sequence we used the program *illuminapairedend*, from the software OBITools (http://metabarcoding.org/obitools) as described in Boyer *et al.* (2016). We used the program *ngsfilter* (OBITools) to keep only sequences properly identified by the primers (2 bp mismatches per primer allowed) and molecular tags (no mismatch allowed) implemented in the PCR process, and assign each sequence record to the corresponding sample. Replicated reads were grouped into unique sequences, keeping their count per sample, using the *obiuniq* program. We considered in the following analysis only sequence records having a length longer than 20 bp and more than ten counts (*obigrep*, OBITools). To detect PCR errors and identify chimeric sequences we classified each sequence as *head, internal* or *singleton* using the program *obiclean*. Sequences with higher reads counts were labeled as *head* and were considered as true sequences. Sequences that differed from head sequences

by more than one base pair and with less than 50% of the *head* count were labeled as *internal* and considered as erroneous sequences. Sequences with no relation to other sequences were labeled as *singleton* (Boyer *et al.* 2016).

The next step was to assign sequences to a corresponding taxon in order to get the complete list of taxa associated to each sample. Taxonomic assignation of query sequences was performed with the program *ecotag* (OBITools) based on sequence similarity with the species reference database previously compiled. When the query sequence has equal similarity to two or more reference sequences, than *ecotag* assigns the query sequence to the last common taxon among the reference sequences identified. Supplementary Figure V represents a workflow of the sequences analysis for better comprehension.

Further filtering analysis were performed using the program R (v 3.1.3; R Development Core Team, 2015). Samples from the second sampling point were not retained in further analysis because no DNA was detected in any sample from the four streams. To avoid sequences that may represent errors from PCR procedures, sequence records with frequency below 0.001 for each PCR replicate were discarded. Sequences labeled as *internal* must correspond to PCR substitution and errors and were also omitted. Only sequences with high identity percentage, i.e. sequences that match with more than 96% to the sequences from the reference database, were kept. Subsequently, we discarded sequences with low frequency (< 0.3% per sample per *molecular operational taxonomic units* - MOTU) analyzing each run separately. Cross-contamination sources were verified by comparing the ratio between the count of sequences among all samples (N = 278) and among control samples (field, DNA extraction and PCR negative controls; N = 72). Sequences with ratio among control samples higher than all samples were removed. One species not known to occur at the Atlantic forest (*Scinax ruber*) is probable consequence of contamination and was not considered in further analyses. Finally, we considered the

species presence if at least one PCR replicate had positive detection among the 12 PCR replicates.

Sequence conflict occurred for six species in the complete reference database due to mislabeled sequences in the EMBL database. Identical sequences for *Thoropa miliaris* and *Thoropa taophora* were deposited in the public database before the recent taxonomic revision of the group (Feio *et al.*, 2006). When a query sequence was similar to these species sequences it was assigned to the genus *Thoropa*, the last common taxon. In this case, we manually edited and assumed the query sequence to be *Thoropa taophora* as *Thoropa miliaris* does not occurs in the study area. Similar taxonomic misidentification happened with *Phasmahyla guttata* and *Phasmahyla cruzi* sequences and *Bokermannohyla hylax* and with *Bokermannohyla* sp. (aff. *circumdata*) sequences, which lack recent taxonomic revision and for which query sequences were assigned to the latter in both cases.

Data Analyses

We calculated Jaccard's index to measure species composition similarity between traditional survey results for the month of April and eDNA metabarcoding results for each stream. Jaccard's index is a binary coefficient that deals only with presence and absence data, not considering abundance of the species found (Krebs, 1998). The Jaccard's index is calculated for every pair of locations and defined as $S_j = \frac{a}{(a+b+c)}$, where *a* is the number of species shared between two locations, and *b* and *c* are the numbers of species unique to each location (Krebs, 1998). Higher values of *Sj* show a higher similarity between the location pair.

To assess the constancy of species in relation to monthly samplings, we used the formula C = P x 100 / N (based on Silveira-Netto *et al*. 1976), where: P = number of

sampling months containing a certain species; N = total number of months sampled. The Constancy index (C) ranges between 0 and 100% for each species and were calculated using visual and acoustic data for all months sampled (N = 54). The results were grouped into the following categories: (i) constant species, present in over 50% of the samples, (ii) accessory species, present in 25% to 50% of samples, (iii) accidental species, present in less than 25% of samples, and (iv) absent species if the species was never recorded and C = 0% (based on Silveira-Netto *et al*. 1976). We used linear regressions to assess the relationship between the constancy index calculated for each species at each stream and the mean between the proportions of positive PCR replicates of that species obtained at sampling point 1 and point 3 of each stream.

We searched the literature for data on habitat for all life stages of each species. We coded egg, larval, and adult habitats as exclusively terrestrial or arboreal (value = 0), occupying ponds (value = 0), or exclusively riparian (value = 1). We calculated a non-rank aquatic habitat index (Hi) to each species (based on Lips *et al.*, 2003) which quantifies the presence of the species in aquatic environments and serves as a relative measure of DNA contribution into the water. We used linear regressions to assess the relationship between the habitat index calculated for each species and positive PCR replicates. We calculated the positive PCR replicates for each species with the mean of the proportion positive of PCR replicates at the sampling point 1 and point 3 at each stream. All analyses described above were performed in R (v 3.1.3; R Development Core Team, 2015).

RESULTS

We observed a small variation in physical and water quality measurements among sites and streams sampled. Water temperatures ranged from 20.4 °C to 20.9 °C, dissolved oxygen in water from 4.43 to 7.23 mg/L, and pH from 6.5 to 7.0 (Supplementary Table III).

During the five years of visual and calling surveys, we found a total of ten amphibian species comprising five families and nine genera: Hylidae (4 genera, 4 species), Cycloramphidae (1 genus, 1 species), Centrolenidae (1 genus, 1 species), Hylodidae (1 genus, 2 species), and Leptodactylidae (2 genera, 2 species) (Supplementary Figure II and S. Table I). *Bokermannohyla circumdata* was considered accidental along the four streams (C < 14.81). *Cycloramphus boraceiensis* and *Hylodes asper* were found constantly in streams 1, 3 and 4 (C > 98.15), not occurring in the stream 2, although one juvenile of *H. asper* was visually recorded only once in the stream 2. *Hylodes phyllodes* was the only species considered constant among the four streams (C ≥ 75.93). *Phasmahyla cruzi* was only observed in stream 4 where it was considered an accessory species (C = 31.48). *Scinax trapicheiroi* was found only in streams 2, 3 and 4, being constant only in the stream 3 (C = 70.37). *Thoropa taophora* was accidental in streams 1, 2 and 3 (C < 18.52), but accessory in stream 4 (C = 25.93). Both *Proceratophrys appendiculata* and *Aplastodiscus eugenioi* were accidental species, observed in only one month, the former in streams 1 and 4, the latter in the stream 3. Finally, *Vitreorana uranoscopa* was only present in streams 1 and 3, being accessory (C = 40.71) in the stream 1 and accidental in the stream 3 (C = 7.41) (Table 3). From 2007 to 2011, only eight species were recorded during surveys performed on April (Supplementary Figure III). *Bokermannohyla circumdata* was only visually recorded on April 2011 in the stream 3 and on April 2008 in the stream 4. *Phasmahyla cruzi* was only visually recorded on April 2008 and 2009 in the stream 4. *Scinax trapicheiroi* was visually

recorded twice (2008 and 2009) in the stream 2. In stream 3, *S. trapicheiroi* was visually recorded in three non-consecutive years (2008, 2009 and 2011) and in acoustic survey only once (April, 2007). *Thoropa taophora* was visually recorded twice (2009 and 2011) in the stream 2 and only once in streams 3 and 4. *Vitreorana uranoscopa* was only recorded in acoustic survey and in two non-consecutive years (2007 and 2011) in the stream 1. Finally, *C. boraceiensis*, *H. asper* and *H. phyllodes* were observed in all five years (Supplementary Figure III).

A total of 10,104,512 sequence reads were obtained from all capsules before the filtering process. After the filtering steps, we recovered 1,286,149 sequence reads. We were able to detect amphibians eDNA in the four streams sampled. All field controls, DNA extraction and PCR negative controls showed no sequence reads in the end of the cleaning process. We could detect a total of nine species representing eight genera of amphibians, all previously observed at some point in the 5-year traditional survey (Figure 2).

Species composition described by eDNA method had high similarity with species composition described by traditional survey results for the stream 1 and 4 (S = 0.8 and 0.833, respectively). In stream 1, we recovered DNA from a species known to use it, *T. taophora*, but whose presence had not been recorded in previous traditional survey in April (Figure 2). We also found *V. uranoscopa* DNA in stream 1, a species that was visually detected in only two years (2007 and 2008) during traditional surveys and acoustically recorded only in April of 2007 and 2011 (Figure 2, Supplementary Figure III). Similarly, in stream 4, the similarity observed was not complete because we failed to detect DNA from *B. circumdata*. On the other hand, species composition described by eDNA method had low similarity with traditional survey results in streams 2 and 3 (S = 0.33 and 0.571, respectively). In the stream 2, we detected *H. phyllodes* DNA, but we were unable to detect

DNA from *S. trapicheiroi* and *T. taophora,* two species visually observed in two years of traditional survey for the same period (April of 2008 and 2009, April of 2009 and 2011, respectively). In stream 3, we detected DNA of six species, including one not observed in the traditional survey on April (*Aplastodiscus eugenioi*). We also detected *Bokermannohyla* sp. (aff. *circumdata*) DNA, where *B. circumdata* were only visually recorded on April 2011. However, we were unable to detect DNA from *T. taophora*, a species visually observed only in one year of traditional survey (April of 2008) (Figure 2).

The proportion of positive replicates ranged from 0.08 (1/12 PCR replicates) to 1.0 (12/12 PCR replicates). The aquatic habitat index ranged from 1 to 3 (Table 4) and explained almost two thirds of the variation in eDNA detection rate (i.e., proportion of positive PCR replicates) ($r^2 = 0.5787$, $p << 0.001$) (Figure 3). A similar result was obtained for the constancy index ($r^2 = 0.581$, $p << 0.001$) (Figure 3). Three species (*C. boraceiensis, H. asper* and *H. phyllodes*) with the highest Hi and the highest C also had a high proportion of positive detection among eDNA samples.

DISCUSSION

In the present study, we tested and confirmed the reliability of eDNA analysis in amphibian detection along tropical streams, and showed that this new molecular tool is able to sample communities by comparing its results to a five-year traditional survey. Our results also allowed us to illustrate the effects of the different stream habitats explored by amphibians in eDNA detection and the power of the eDNA analysis to detect elusive species that escaped traditional surveys.

We confirmed the efficiency of the eDNA analysis when assessing amphibian community composition along four streams in the Atlantic forest, Brazil. In general, the

species composition detected by eDNA metabarcoding had high similarity to that obtained with traditional surveys. Exceptions were due to eDNA detection of species not recovered in traditional survey or to the non-detection by eDNA analyses of species rarely observed in traditional surveys. For instance, in stream 2 the similarity was not higher because we recovered *Bokermannohyla* sp. sp. (aff. *circumdata*) and *Bokermannohyla* DNA, a genus previously undetected by traditional survey. Perhaps these differences between the results from eDNA metabarcoding and those from traditional surveys may reflect the fact that life stages not quantified in the latter (e.g., tadpoles) may have contributed to the DNA available in the water. Alternatively, these species could occur upstream, too far from our transect to be detected by their calls, but close enough to contribute with detectable eDNA in our sampling points downstream.

Our study revealed that eDNA metabarcoding performs better or similarly to visual and calling survey in most cases, capable of detecting species found only once during five years of traditional survey. For instance, in stream 3, we detected DNA of *A. eugenioi*, a species considered accidental, observed only once in traditional survey. Previous studies also compared eDNA analysis with traditional survey results. Thomsen *et al.* (2012) noticed that eDNA analysis recovered fish diversity from seawater samples better than or equal to other nine traditional methods. Similarly, Dejean *et al.* (2012) found bullfrog eDNA in five times more places than visual and calling surveys together. Although, Tréguier *et al.* (2014) found a higher detection rate with eDNA analysis, when the authors compared it to traditional survey, crayfish eDNA was detected only in 59% of the ponds where the species presence was confirmed by trapping. According to Roussel *et al.* (2015) the idea that eDNA analysis is more efficient than traditional surveys is fast becoming commonplace although it is based on just a few comparative studies where species abundance is not always reported. We believe our study contributes to confirm that the performance of eDNA metabarcoding

is equivalent to that of traditional survey for biodiversity monitoring, taking into account not abundance, but natural history and constancy of observation in traditional survey.

When investigating how the reproductive biology impacted the efficacy of using eDNA as a species detection tool, we found a positive relationship between eDNA detection and the aquatic habitat index, which estimates the degree of dependence of a species at different life stages on a riparian environment. As expected, we did not recover DNA from exclusively terrestrial species with direct development (i.e., not dependent on water bodies) and whose distributions overlapped the sampling locations, such as *Haddadus binotatus, Ischnocnema parva* and *Brachycephalus hermogenesi* (the former two were observed accidentally in some streams during the five year traditional survey). All sampled eDNA matched amphibian species with at least one phase of its life associated with the streams' watercourse. For instance, adults of *Aplastodiscus eugenioi* are arboreal, their eggs occur in subterranean constructed nests, and the tadpoles occupy ponds and streams (Hartmann *et al.*, 2010). Adults of *V. uranoscopa* and *P. cruzi* are also arboreal, and their eggs are laid on leaves hanging over lotic water where tadpoles drop into (Costa *et al.*, 2010; Hartmann *et al.*, 2010). Eggs and tadpoles of *S. trapicheiroi* can be found in lotic water, while calling males are mainly found on shrubby vegetation along streams (Hartmann *et al.*, 2010). *Hylodes asper* and *H. phyllodes* lay their eggs in subaquatic chambers and tadpoles develop in ponds along the streams, while eggs of *C. boraceiensis* and *T. taophora* are semiterrestrial with tadpoles living on wet rocks along streams (Hartmann *et al.*, 2010). Unlike the species loosely associated to the streams (e.g., *Aplastodiscus eugenioi*), *C. boraceiensis* and *H. asper* use streams as their primary habitat across life stages and are found only on rocks along streams. In *H. phyllodes* the growing from metamorphosis to sexual maturity occurs in the leaf litter adjacent to the streams and postmetamorphic individuals of *T. taophora* may be found far from streams, but always close to wet rocks.

Not surprisingly, the latter four species were more constant in our traditional survey and had their eDNA found in higher proportions in water samples. In the case of more seasonal species, whose adults are found in the streams only during the breeding season, such as *V. uranoscopa* and *S. trapicheiroi*, tadpoles can stay for months in the water and are likely the main contributors of eDNA to water samples.

Analyses of eDNA have almost only been applied to temperate areas (Hoffman *et al.*, 2016). Our study consists of the first effort to detect species from the Atlantic forest using eDNA from aquatic samples. We demonstrated that eDNA metabarcoding can be successfully applied in Atlantic forest streams where water temperature can be higher and which harbors high anuran richness. Furthermore, few studies used eDNA from freshwater to detect community assemblages and to identify multiple species from one sample. Minamoto *et al.* (2012) were the first to report the detection of multiple vertebrate species by amplifying fish eDNA with degenerated primers. Evans *et al.* (2016) later measured the species richness of fish and amphibian using eDNA metabarcoding in experimental conditions and showed that some primers can accurately identify species assemblages with differing species densities. Kelly *et al.* (2014) and Shaw *et al*. (2016) were able to detect fish communities in controlled aquatic settings and in a river, respectively, using primers for vertebrate-specific fragments from mitochondrial genes. Finally, Valentini *et al.* (2016) applied the same universal primer pair for Batrachia to survey amphibian species in a wide range of aquatic ecosystems in Europe. Here, we also demonstrated the value of eDNA detection for monitoring vertebrate communities and not only single targeted species. Our four days sampling of eDNA was able to efficiently characterize amphibian communities and identified species assemblages with differing species composition, including species not tightly associated to stream.

The downside of working with tropical amphibians is that some tropical species are poorly represented or absent in public sequence databases and taxonomic errors in online databases can jeopardize the assignment of many sequences (Hoffman *et al.*, 2016). Shaw *et al*. (2016), for instance, could not detect some taxa from freshwater eDNA samples using 12S or 16S primers due to a lack of reference sequence data on the NCBI database. We circumvented this situation by building a local sequence reference database using specimens collected from Núcleo Picinguaba, Brazil. This is an important stage on bioinformatics analyses, and future studies on eDNA should also count on a local sequence reference database to improve eDNA results, although this demands previous knowledge on species composition on the study area, as was the case of our study.

General knowledge on species occurrence in the study area is also fundamental to avoid mistaken identifications. In our study, we detected *Scinax ruber* a species native to other regions in South America and species with mislabeled taxonomic names in the reference database that were manually removed or corrected in further analyses. Therefore, special caution must be taken when interpreting eDNA metabarcoding results for community studies.

Finally, freshwater ecosystems are essential for a high percentage of the world's amphibian species. Despite their value, many streams are being severely damaged by different threats and declines in freshwater biodiversity are far greater than in terrestrial ecosystems (Dudgeon *et al.*, 2006). Effective monitoring of species associated to these environments is therefore essential for conservation actions. In light of the added sensitivity of NGS technology, eDNA metabarcoding is potentially suitable for addressing ecological issues. Many studies pointed out the eDNA analyses as an advantageous tool across various freshwater ecosystems (e.g., Thomsen *et al.*, 2012), such as in early detection of invasive species and in surveillance of endangered ones. Our study has now shed additional light on

the use of this new approach to community monitoring and we encourage the use of eDNA metabarcoding as a reliable way to assess community diversity in streams, enhancing its application in future ecological research.

ACKNOWLEDGEMENTS

This work was supported by São Paulo State Research Foundation (FAPESP, grants #2006/58011-4, #2010/50146-3, and #2013/50741-7). Scholarships were provided by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) and FAPESP (grant 2014/06795-8) to T.S.L. during the development of this project. M.M. and C.F.B.H. thank CNPq for research fellowships. We thank all persons who participated in the field work, gave helpful suggestions on the manuscript, and the Instituto Florestal for research and collecting permits (260108 – 009.752/2014). CFBH is grateful to CNPq for a research fellowship.

REFERENCES

Barnes, M. A., Turner, C. R., Jerde, C. L., Renshaw, M. A, Chadderton, W. L., Lodge, D. M., 2014. Environmental Conditions Influence eDNA Persistence in Aquatic Systems. Environmental Science & Technology, 48, 3, 1819-1827

Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., Yu, D. W., Bruyn, M., 2014. Environmental DNA for wildlife biology and biodiversity monitoring. Trends in Ecology and Evolution, 29, 6, 358-367.

Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P., Coissac, E., 2016. OBITools: a Unix-inspired software package for DNA metabarcoding. Molecular Ecology Resources, 16, 176-182.

CIIAGRO, Centro integrado de informações agrometeorológicas, 2016. http://www.ciiagro.sp.gov.br/ciiagroonline.

Costa, P. N., Carvalho-e-Silva, M. P. T., Flaskman, A., 2010 Egg clutch and larval development of *Phasmahyla cruzi* (Carvalho-e-Silva, Silva and Carvalho-e-Silva, 2009) (Amphibia, Anura, Hylidae). Herpetology Notes, 3, 221-228.

Darling, J. A., Mahon, A. R., 2011. From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. Environmental Research, 111, 978-988.

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., Miaud, C., 2011. Persistence of environmental DNA in freshwater ecosystems. PLoS One, 6, e23398.

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E., Miaud, C., 2012. Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*. Journal of Applied Ecology, 49, 953-959.

Dudgeon, D., Arthington, A. H., Gessner, M.O., Kawabata, Z., Knowler, D., Lévêque, C., Naiman, R. ., Prieur-Richard, A.-H., Soto, D., Stiassny, M. L. J. and Sullivan, C. A., 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. Biological Reviews, 81, 163-182.

Eterovick, P. C., Carnaval, A. O. de Q., Borges-Nojosa, D. M., Silvano, D. L., Segalla, M. V., Sazima, I., 2005. Amphibian declines in Brazil : an overview. Biotropica, 37, 2, 166-179.

Evans, N. T., Olds, B. P., Renshaw, M. A., Turner, C. R., Li, Y., Jerde, C. L., Mahon, A. R., Pfrender, M. E, Lamberti, G. A., Lodge, D. M. 2016. Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding. Molecular Ecology Resources, 16, 29-41.

Feio, R. N., Napoli, M. F., Caramaschi, U. 2006. Considerações taxonômicas sobre *Thoropa miliaris* (Spix, 1824), com revalidação e redescrição de *Thoropa taophora* (Miranda-Ribeiro, 1923) (Amphibia, Anura, Leptodactylidae). Arquivos do Museu Nacional, Rio de Janeiro, 64, 41-60.

Ficetola, G. F., Miaud, C., Pompanon, F., Taberlet, P., 2008. Species detection using environmental DNA from water samples. Biology Letters, 4, 423-425.

Goebel, A. M., Donnelly, J. M., Atz, M. E., 1999. PCR primers and amplification methods for 12S ribosomal DNA, the control region, cytochrome oxidase 1, and cytochrome b

in bufonids and other frogs, and an overview of PCR primers which have amplified DNA in amphibians successfully. Molecular Phylogenetics and Evolution, 11, 163-199.

Goldberg, C. S., Sepulveda, A., Ray, A., Baumgardt, J., Waits, L. P., 2013. Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). Freshwater Science, 32, 792-800.

Graybeal, A., 1997. Phylogenetic relationships of bufonid frogs and tests of alternate macroevolutionary hypotheses characterizing their radiation. Zoological Journal of the Linnean Society, 119, 297-338.

Haddad, C. F. B., Toledo, L. F., Prado, C. P. A., Loebmann, D., Gasparini, J. L., Sazima, I., 2013. Guia dos anfíbios da Mata Atlântica: diversidade e biologia. Anolis Books, 544 pp.

Hartmann, M. T. 2004. Biologia reprodutiva de uma comunidade de anuros (Amphibia) na Mata Atlântica (Picinguaba, Ubatuba, SP). Thesis - Universidade Estadual Paulista "Julio de Mesquita Filho", Campus de Rio Claro, Brasil.

Hartmann, M. T., Hartmann, P. A., Haddad, C. F. B., 2010. Reproductive modes and fecundity of an assemblage of anuran amphibians in the Atlantic rainforest, Brazil. Iheringia, Série Zool. 100, 207-215.

Heyer, R., Maureen, A. D., Mercedes, F., Roy, M., 1993. Measuring and monitoring biological diversity: standard methods for amphibians. Smithsonian Institution.

Hoffmann, C., Schubert, G., Calvignac-Spencer, S., 2016. Aquatic biodiversity assessment for the lazy. Molecular Ecology, 25, 846-848.

Hunter, M. E., Oyler-McCance, S. J., Dorazio, R. M., Fike, J. A., Smith, B. J., Hunter, C. T., Reed, R. N., Hart, K. M., 2015. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive burmese pythons. PLoS One, 10, e0121655.

ICMBio, Instituto de Conservação Chico Mendes da Biodiversidade, 2016. http://www.icmbio.gov.br/portal/faunabrasileira/lista-de-especies.

IUCN, Conservation International, and NatureServe. 2012. An Analysis of Amphibians on the 2012. IUCN Red List <www.iucnredlist.org/amphibians>.

Jerde, C. L., Mahon, A. R., Chadderton, W. L., Lodge, D. M., 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation Letter, 4, 150-157.

Kelly, R. P., Port, J. A., Yamahara, K. M., Crowder, L. B., 2014. Using Environmental DNA to Census Marine Fishes in a Large Mesocosm. PLoS One, 9, e86175.

Lips, K. R., Reeve, J. D., Witters, L. R., 2003. Ecological traits predicting Amphibian population declines in Central America. Conservation Biology, 17, 1078-1088.

Lodge, D. M., Turner, C.R., Jerde, C. L., Barnes, M. A., Chadderton, L., Egan, S. P., Feder, J. L., Mahon, A. R., Pfrender, M. E., 2012. Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA. Molecular Ecology, 21, 2555-2558.

Myers, N., Mittermeier, R. A., Mittermeier, C. G., Fonseca, G. A. B. da, Kent, J., 2000. Biodiversity hotspots for conservation priorities. Nature, 403, 72.

Minamoto, T., Yamanaka, H., Takahara, T., Honjo, M. N., Kawabata, Z., 2012 Surveillance of fish species composition using environmental DNA. Limnology, 13, 193-197.

Morellato, P., Haddad, C. F. B., 2000. Introduction : The Brazilian Atlantic Forest. Biotropica. 32, 4,786-792.

Palumbi, S. R., Martin, A., McMillan, W. O., Stice, L., Grabowski, G., 1991. The simple fool's guide to PCR. Priv Publ Doc Compil by S. Palumbi, version 2.

Piaggio, A. J., Engeman, R. M., Hopken, M. W., Humphrey, J. S., Keacher, K. L., Bruce, W. E., Avery, M. L. 2013. Detecting an elusive invasive species: a diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. Molecular Ecology Resources, 14, 374-380.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., Waits, L. P., 2013a. Factors influencing detection of eDNA from a stream-dwelling amphibian. Molecular Ecology Resources, 14, 1,109-116.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., Waits, L. P., Richardson, J., 2013b. Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Canadian Journal of Fisheries and Aquatic Sciences, 70, 1123-1130.

R Development Core Team, 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. URL http://www.R-project.org

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., Gough, K. C., 2014. The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. Journal of Applied Ecology, 51, 5, 1450-1459.

Roussel, J-M., Paillisson, J-M., Tréguier, A., Petit, E., 2015 The downside of eDNA as a survey tool in water bodies. Journal of Applied Ecology, 52, 823-826.

Ruggeri, J., Longo, A. V., Gaiarsa, M. P., Alencar, L. R. V., Lambertini, C., Leite, D. S., Carvalho-e-Silva, S. P., Zamudio, K. R., Toledo, L. F., Martins, M. 2015. Seasonal variation in population abundance and chytrid infection in stream-dwelling frogs of the Brazilian Atlantic Forest. PLoS One, 10, e0130554.

Sambrook J, Russel D.W., 2001. Rapid isolation of yeast DNA. *In: Molecular cloning, a laboratory manual* (eds Sambrook J, Russel DW), pp. 631–632. Cold Spring Harbor, New York.

Secondi, J., Dejean, T., Valentini, A., Miaud, C., Audebaud, B. 2016. Detection of a global aquatic invasive amphibian, *Xenopus laevis*, using environmental DNA. *Amphibia-Reptilia*, 37, 131-136.

Shaw, J. L. A., Clarke, L. J., Wedderburn, S. D., Barnes, T. C., Weyrich, L. S., Cooper, A., 2016. Comparison of environmental DNA metabarcoding and conventional fish survey methods in a river system. Biological Conservation, 197, 131-138.

Silveira-Netto, S., Nakano, O., Barbin, D., Villa-Nova, N. A., 1976. Manual de ecologia dos insetos. São Paulo, Agronômica Ceres, 419 pp.

SOS Mata Atlântica, 2015. Atlas dos remanescentes florestais da Mata Atlântica (2013-1014).

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., Willerslev, E., 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Molecular Ecology, 21, 2045-2050.

Takahara, T., Minamoto, T., Doi, H., 2013. Using environmental DNA to estimate the distribution of an invasive fish species in ponds. PLoS One, 8, e56584.

Thomsen, P., Kielgast, J., Iversen, L. L., Møller, P. R., Rasmussen, M., Willerslev, E. 2012. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLoS One, 7, e41732.

Tréguier, A., Paillisson, J. M., Dejean, T., Valentini, A., Schlaepfer, M. A., Roussel, J. M., 2014. Environmental DNA surveillance for invertebrate species: advantages and

technical limitations to detect invasive crayfish *Procambarus clarkia* in freshwater ponds. Journal of Applied Ecology, 51, 871-879.

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F., Gaboriaud, C., Jean, P., Poulet, N., Roset, N., Copp, G. H., Geniez, P., Pont, D., Argillier, C., Baudoin, J. M., Peroux, T., Crivelli, A. ., Olivier, A., Acqueberge, M., Le Brun, M., Moller, P. R., Willerslev, E., Dejean, T., 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Molecular Ecology, 25, 929-942.

Wiens, J. J., Fetzner, J. W., Parkinson, C. L., Reeder, T. W., 2005. Hylid frog phylogeny and sampling strategies for speciose clades. Systematic Biology, 54, 778-807.

Wilcox, T. M, McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley A. R., Schwartz, M. K., 2013. Robust detection of rare species using environmental DNA: the importance of primer specificity. PloS one, 8, e59520.

Wilcox, T. M, Schwartz, M. K., McKelvey, K. S., Young, M. K., Lowe, W. H., 2014. A blocking primer increases specificity in environmental DNA detection of bull trout (*Salvelinus confluentus*). Conservation Genetics Resources, 6,283-284.

Wilson, C., Wright, E., 2013. Using environmental DNA (eDNA) as a tool in risk-based decision-making. Ontario Ministry of Natural Resources, Aquatic Research and Development Section, Aquatic Research Series, Report 2013- 01.

FIGURES

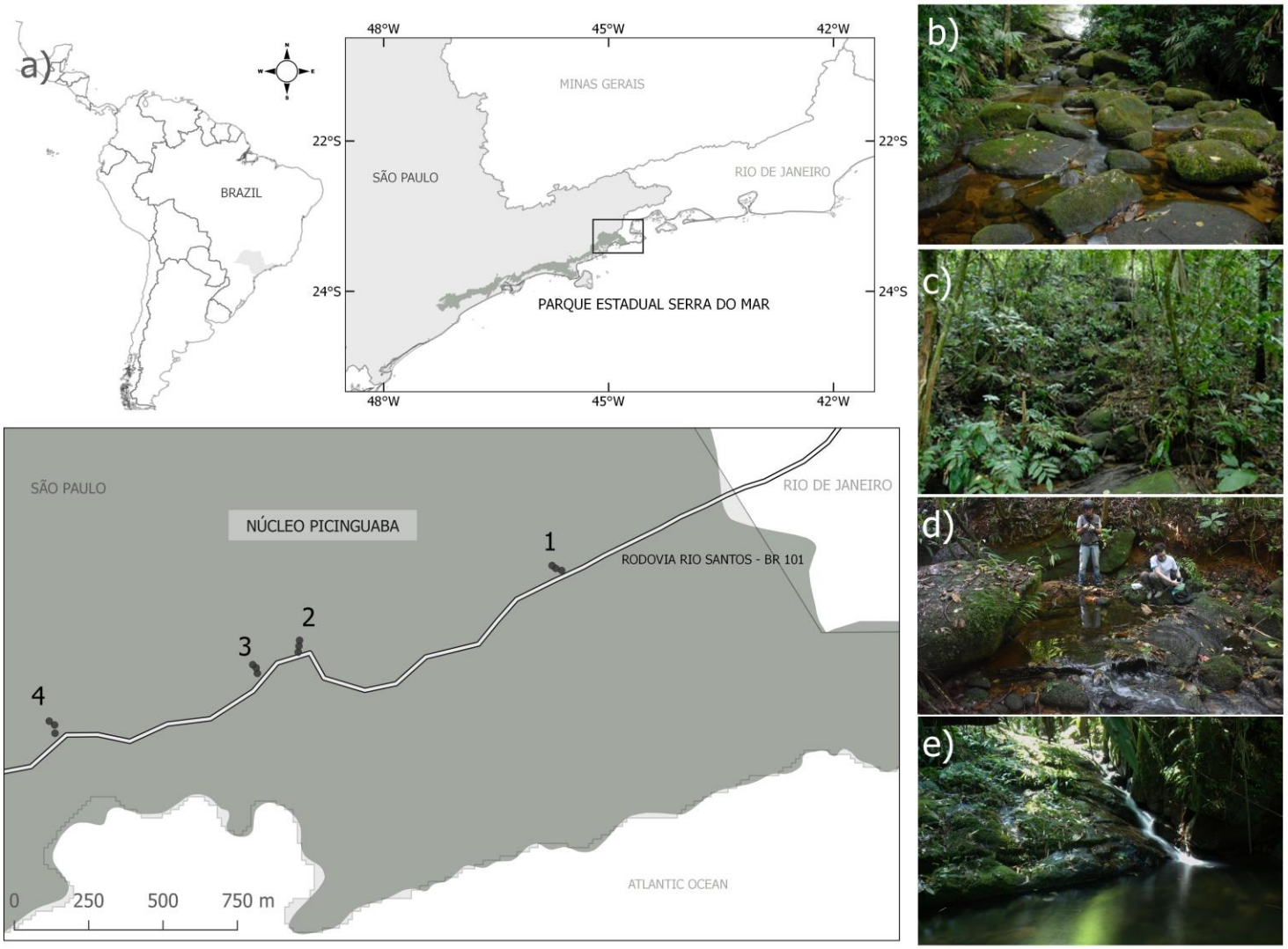


Figure 1. Study area. a) Maps showing the location of study site, Núcleo Picinguaba at the Parque Estadual Serra do Mar, São Paulo, southeastern Brazil. Numbers 1 to 4 indicate each stream sampled in the area. Black circles represent the sampling points in each stream, being point 1 closer to the Rodovia Rio Santos. The streams at the right column are: b) Stream 1, c) Stream 2, d) Stream 3, e) Stream 4. Photos by Marcio Martins.

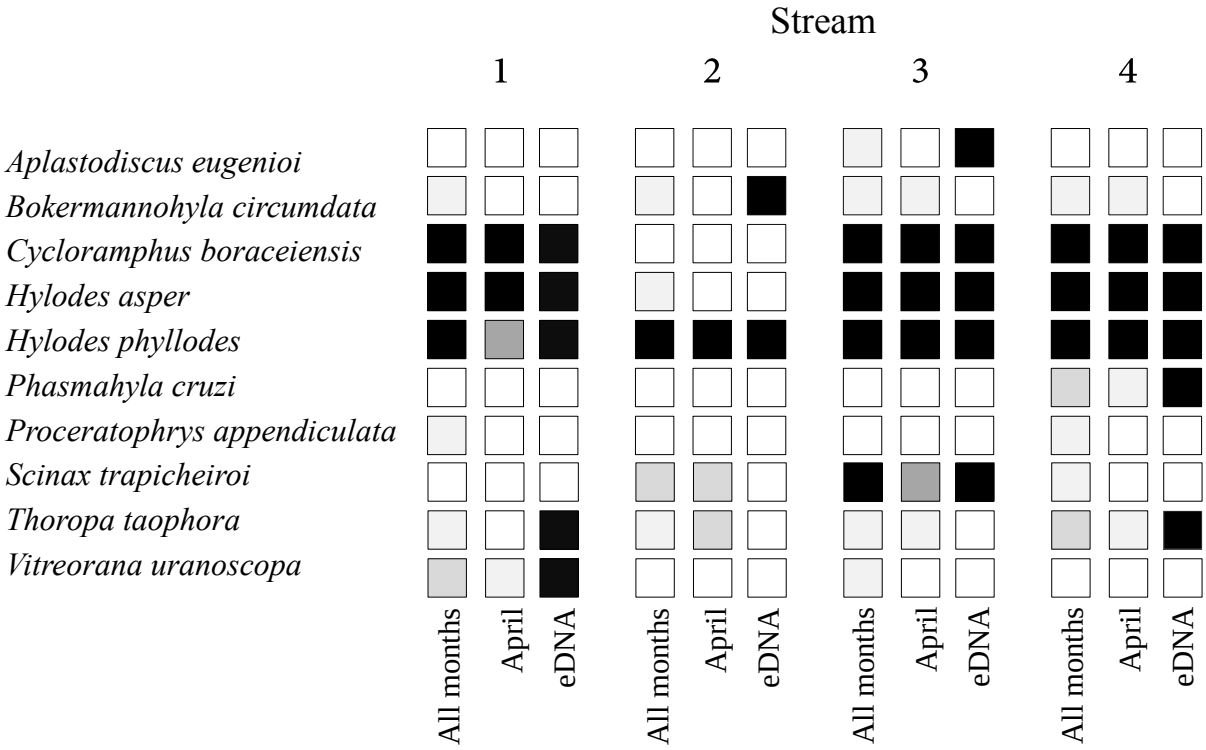


Figure 2. Species found through traditional methods (visual and calling surveys; compiling all 54 months of sampling from 2007 to 2011 or only sampling in April) and eDNA sampling in April 2014 in each stream at Núcleo Picinguaba, São Paulo, Brazil. The colors in the squares represent an increase in constancy, from white to black.

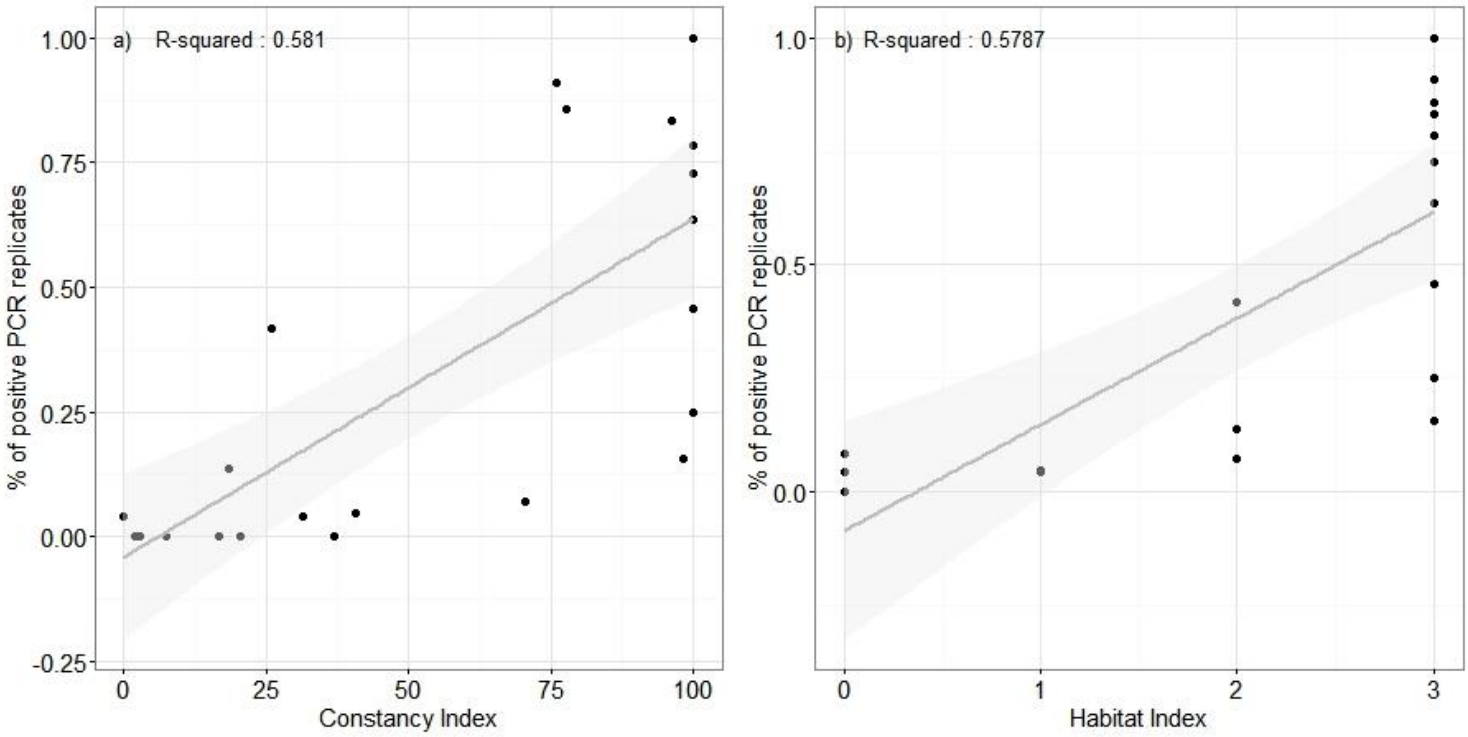


Figure 3. Relationship between a) mean proportion of positive PCR replicates and Constancy index calculated for each species at each stream, b) mean proportion of positive PCR replicates and Habitat index. Gray area and line indicates 95% CI and regression line, respectively.

TABLES

Table 1. Primers used in this study.

Primer	Sequence	Source
MVZ 59	5’-ATAGCACGTAAAAYGCTDAGATG-3’	Graybeal, 1997
tVAL	5’-TGTAAGCGARAGGCTTTKGTTAAGCT-3’	Wiens *et al.*, 2005
148	-	M.L. Lyra, unpublished data
12SF-H	5’-CTTGGCTCGTAGTTCCCTGGCG-3’	Goebel *et al.*, 1999
12SA-L	5’-AAACTGGGATTAGATACCCCACTAT-3’	Palumbi *et al*., 1991
H978	-	M.L. Lyra, unpublished data
L3541	5’-ACACCGCCCGTCACCCT-3’	Valentini *et al.* 2016
H3596	5’-GTAYACTTACCATGTTACGACTT-3’	Valentini *et al.* 2016

Table 2. Names and voucher identification (ID) of species with known occurrence at the Núcleo Picinguaba, São Paulo, Brazil, included in the local sequence reference database. CFBHT stands for Célio F. B. Haddad Tissue collection.

Species	ID
Brachycephalidae	
Brachycephalus hermogenesi	*
Ischnocnema bolbodactyla	CFBHT11603, CFBHT4495
Ischnocnema parva	CFBHT17536, CFBHT17534
Ischnocnema sp. (aff. *guentheri)*	CFBHT3807, CFBHT5998
Bufonidae	
Dendrophryniscus brevipollicatus	CFBHT17545, CFBHT17535
Rhinella icterica	CFBHT3639, CFBHT3640
Rhinella ornata	CFBHT3626, CFBHT3627
Centrolenidae	
Vitreorana uranoscopa	CFBHT1257, CFBHT1251
Craugastoridae	
Haddadus binotatus	CFBHT8915, CFBHT9724
Cycloramphidae	
Cycloramphus boraceiensis	CFBHT23, CFBHT2218, CFBHT2220
Thoropa taophora	CFBHT393, CFBHT398
Hylidae	
Aplastodiscus eugenioi	CFBHT7353, CFBHT10726
Bokermannohyla circumdata	*
Dendropsophus berthalutzae	CFBHT465, CFBHT6008
Dendropsophus elegans	CFBHT6694, CFBHT8209
Dendropsophus giesleri	CFBHT2
Dendropsophus minutus	CFBHT14647, CFBHT14648
Hypsiboas albomarginatus	CFBHT10267, CFBHT10735
Hypsiboas faber	CFBHT17542, CFBHT460
Hypsiboas semilineatus	CFBHT2577, CFBHT3178
Itapotihyla langsdorffii	CFBHT284, CFBHT8224
Phasmahyla cruzi	CFBHT281
Scinax tymbamirim	CFBHT2586, CFBHT8208
Scinax angrensis	CFBHT8899, CFBHT1399
Scinax argyreornatus	CFBHT2776, CFBHT6711
Scinax eurydice	CFBHT7, CFBHT6692
Scinax hayii	CFBHT3543, CFBHT10269
Scinax trapicheiroi	CFBHT389
Trachycephalus mesophaeus	CFBHT17575, CFBHT2768
Hylodidae	
Hylodes asper	CFBHT3810, CFBHT1488
Hylodes phyllodes	CFBHT249, CFBHT7913
Leptodactylidae	
Leptodactylus latrans	CFBHT42, CFBHT5704

Physalaemus atlanticus	CFBHT1398, CFBHT5699
Microhylidae	
Arcovomer passarellii	CFBHT7847, CFBHT466
Chiasmocleis carvalhoi	CFBHT73, CFBHT76
Myersiella microps	*
Odontophrynidae	
Macrogenioglottus alipioi	CFBHT280, CFBHT318
Proceratophrys appendiculata	CFBHT10264, CFBHT28

* Not sequenced.

Table 3. Dajoz constancy (C) in % for each species in each stream in Núcleo Picinguaba, São Paulo, Brazil. Species were classified as: (i) constant species, if C ≥ 50%, (ii) accessory species, if 25% ≥ C < 50%, (iii) accidental species, if C < 25 %, (iv) absent if C = 0 %.

Species	Stream			
	1	2	3	4
Aplastodiscus eugenioi	0	0	1.85	0
Bokermannohyla circumdata	1.85	5.56	14.81	5.56
Cycloramphus boraceiensis	100	0	98.15	100
Hylodes asper	100	1.85	100	100
Hylodes phyllodes	75.93	100	77.78	96.3
Phasmahyla cruzi	0	0	0	31.48
Proceratophrys appendiculata	1.85	0	0	1.85
Scinax trapicheiroi	0	37.04	70.37	7.41
Thoropa taophora	18.52	16.67	20.37	25.93
Vitreorana uranoscopa	40.74	0	7.41	0

Table 4. Aquatic habitat index (Hi) calculated for each species.

Species	Life stage			Hi
	Egg	Tadpole	Adult	
Aplastodiscus eugenioi	0	1	0	1
Bokermannohyla circumdata	1	1	0	2
Cycloramphus boraceiensis	1	1	1	3
Hylodes asper	1	1	1	3
Hylodes phyllodes	1	1	1	3
Phasmahyla cruzi	0	1	0	1
Proceratophrys appendiculata	1	0	0	1
Scinax trapicheiroi	1	1	0	2
Thoropa taophora	1	1	0	2
Vitreorana uranoscopa	0	1	0	1

SUPPLEMENTARY MATERIAL

Supplementary List I – Recent studies on eDNA from freshwater environments.

Author (Year)	**Title**	**Animal**	**Species**	**Environment**
Barnes *et al.*(2014)	Environmental Conditions Influence eDNA Persistence in Aquatic Systems.	fish	Common Carp	Mesocosm - aquarium
Collins *et al.*(2013)	Something in the water: biosecurity monitoring of ornamental fish imports using environmental DNA	fish	*Danio rerio* (zebrafish)	20L containers
Davy *et al.* (2015)	Development and Validation of Environmental DNA (eDNA) Markers for Detection of Freshwater Turtles	reptile	Blanding's Turtle (*Emydoidea blandingii*), Spotted Turtle (*Clemmys guttata*), Wood Turtle (*Glyptemys insculpta*), Painted Turtle (*Chrysemys picta*), Northern Map Turtle (Graptemys geographica) Eastern Musk Turtle (*Sternotherus odoratus*),Snapping Turtle (*Chelydra serpentina*) Eastern Spiny Softshell (*Apalone spinifera*) Red-eared Slider (*Trachemys scripta*)	Aquaria and man-made outdoor pond at Scales Nature Park in Orillia, Ontario
Deiner *et al.* (2014)	Transport distance of invertebrate environmental DNA in a natural river.	invertebrate	*Daphnia longispina* and *Unio tumidus*	Lake Greifensee e outflowing River Glatt
Dejean *et al.* (2011)	Persistence of environmental DNA in freshwater ecosystems	amphibian, fish	American bullfrog (*Rana catesbeiana=Lithobates catesbeianus*) and the Siberian sturgeon (*Acipenser baerii*)	900ml glass beakers and ponds of dimensions 12 m2 and 0.40 m deep

Dejean *et al.* (2012)	Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American *bullfrog Lithobates catesbeianus*	amphibian	American bullfrog (*Lithobates catesbeianus*)	48 fishery ponds south-west of France
Eichmiller *et al.* (2014)	The relationship between the distribution of common carp and their environmental DNA in Small Lake	fish	*Cyprinus carpio* (common carp)	Lake Staring, in the Upper Mississippi river Basin
Ficetola *et al.* (2008)	Species detection using environmental DNA from water samples	amphibian	American bullfrog *(Lithobates catesbeianus*)	Aquariums (3L) – Ponds (0.1–1 ha surface area)
Fukumoto *et al.* (2015)	A basin-scale application of environmental DNA assessment for rare endemic species and closely related exotic species in rivers: a case study of giant salamanders in Japan	amphibian	Japanese giant salamander *Andrias japonicus*, Chinese giant salamander *Andrias davidianus*	37 sites in the Katsura River system (approximately 1000 km2 in area), Japan
Goldberg *et al.* (2011)	Molecular detection of vertebrates in stream water—A demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders	amphibian	Idaho giant salamander (*Dicamptodon aterrimus*) and Rocky Mountain tailed frog (*Ascaphus montanus*)	Streams (1–2nd order)
Goldberg *et al.* (2013)	Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*)	invertebrate	New Zealand mudsnail (*Potamopyrgus antipodarum*)	1.5L plastic containers and Portneuf River in Idaho
Hunter *et al.* (2015)	Environmental DNA (eDNA) Sampling Improves Occurrence and Detection estimates of Invasive Burmese Pythons	reptile	Burmese python (*Python molurus bivittatus*), Northern African python (*P. sebae*), boa constrictor (*Boa constrictor*), and the green (*Eunectes murinus*) and yellow an- aconda (*E. notaeus*)	7 L or 14 L container and water body in the field
Jane *et al.* (2015)	Distance, flow, and PCR inhibition: eDNA dynamics in two headwater streams	fish	Brook trout (*Salvelinus fontinalis*)	Two stream in Massassuchetts

Janosik *et al.* (2015)	Environmental DNA as an effective tool for detection of imperiled fishes	fish	Slackwater darter (*Etheostoma boschungi*)	Streams in Alabama and Tennessee
Jerde *et al.* (2011)	"Sight-unseen" detection of rare aquatic species using environmental DNA	fish	Asian carp: Big headed carp (*Hypophthichthys nobilis*) and Silver carp (*H. molitrix*)	Chicago area waterway (Large river/canal complex)
Jerde *et al.* (2013)	Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance program	fish	Asian carp	Great Lakes
Laramie *et al.* (2014)	Characterizing the distribution of an endangered salmonid using environmental DNA analysis	fish	Chinook salmon (*Oncorhynchus tshawytscha*)	Methow and Okanogan Sub-basins of the Upper Columbia River
Mächler *et al.* (2015)	Utility of environmental DNA for monitoring rare and indicator macroinvertebrate species	invertebrate	*Ancylus fluviatilis, Asellus aquaticus, Baetis buceratus, Crangonyx pseudogracilis,and Gammarus pulex*	River and lake habitats in the canton of Zurich in the northeastern part of Switzerland
Mahon *et al.* (2013)	Validation of eDNA surveillance sensitivity for detection of Asian carps in controlled and field experiments.	fish	Bighead (*Hypophthalmichthys nobilis*), silver carp (H. molitrix), common carp (Cyprinus carpio), goldfish (Carassius auratus), black carp (Mylpharyngodon piceus)	2.6 river-mile stretch of the Little Calumet River, Chicago
Maruyama *et al.* (2014)	The Release Rate of Environmental DNA from Juvenile and Adult Fish	fish	Bluegill sunfish (*Lepomis macrochirus*)	Artificial water container
Minamoto *et al.* (2011)	Surveillance of fish species composition using environmental DNA	fish	*Nipponocypris temminckii, Cyprinidae; Oryzias latipes, Adrianichthyidae; Lepomis macrochirus, Centrarchidae; Odontobutis obscura, Odontobutidae; and Pelteobagrus nudiceps, Bagridae*	Aquarium (40 L)

Moyer *et al.* (2014)	Assessing Environmental DNA Detection in Controlled Lentic Systems	fish	African jewelfish (*Hemichromis letourneuxi*)	Artificial ponds
Piaggio *et al.* (2013)	Detecting an elusive invasive species: a diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA	reptile	Burmese python (*Python bivittatus*)	Aquarium (90L) and undeterminate sites
Pilliod *et al.* (2013)	Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples	amphibian	Rocky Mountain tailed frogs (*Ascaphus montanus*) and Idaho giant salamanders (*Dicamptodon aterrimus*)	13 streams in the South Fork Salmon River Sub-basin, Idaho
Pilliod *et al.* (2014)	Factors influencing detection of eDNA from a stream-dwelling amphibian	amphibian	Idaho giant salamander (*Dicamptodon aterrimus*)	Aquarium (3.78 L)
Rees *et al.* (2014)	The application of eDNA for monitoring of the Great Crested Newt in the UK	amphibian	Great crested newt (*Triturus cristatus*)	38 Ponds
Santas *et al.* (2013)	Noninvasive method for a Statewide survey of Eastern Hellbenders *Cryptobranchus alleganiensis* using environmental DNA	amphibian	Hellbenders	Creeks in Ohio and Kentucky
Sigsgaard *et al.* (2014)	Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples	fish	The European weather loach (*Misgurnus fossilis*)	ten localities, 54 triplicate samples were taken, Denmark (ponds,bogs and channels)
Strickler *et al.* (2015)	Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms	amphibian	American bullfrog tadpoles	Polypropylene microcosms

Takahara *et al.* (2012)	Estimation of fish biomass using environmental DNA	fish	Common Carp (*Cyprinus carpio)*	Aquariums (30 × 45 × 25 cm), Ponds, Freshwater lagoon
Thomsen *et al.* (2012a)	Monitoring endangered freshwater biodiversity using environmental DNA	amphibian, fish, mammal, bird, invertebrate	Common spadefoot (*Pelobates fuscus*), Great crested newt (*Triturus cristatus*) European weather loach (*Misgurnus fossilis*), Eurasian otter (*Lutra lutra*), White-faced darter (Leucorrhinia pectoralis) and Tadpole shrimp (*Lepidurus apus*)	Ponds, Lakes and Streams
Thomsen *et al.* (2012b)	Detection of a diverse marine fish fauna using environmental DNA from seawater samples	fish	Marine fish biodiversity	Seawater
Tréguier *et al.* (2014)	Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds	invertebrate	Red swamp crayfish (*Procambarus clarkii*)	158 ponds in a French Nature Park
Wilcox *et al.* (2013)	Robust Detection of Rare Species Using Environmental DNA: The Importance of Primer Specificity	fish	Brook trout (*Salvelinus fontinalis*) and bull trout *(S. confluentus*)	Two streams in west-central Montana
Wilson *et al.* (2014)	Tracking ghosts: combined electrofishing and environmental DNA surveillance efforts for Asian carps in Ontario Waters of Lake Erie	fish	Asian carp: Big headed carp (Hypophthichthys nobilis), Silver carp (*H. molitrix*) and grass carp (*Ctenopharyngodon idella*)	Western Lake Erie and tributaries

REFERENCES

Barnes, M. A., Turner, C. R., Jerde, C. L., Renshaw, M. A, Chadderton, W. L., Lodge, D.M., 2014. Environmental conditions influence eDNA persistence in aquatic systems. Environmental Science and Technology, 48, 1819-1827.

Collins, R. A., Armstrong, K. F., Holyoake, A. J., Keeling, S., 2013. Something in the water: biosecurity monitoring of ornamental fish imports using environmental DNA. Biological Invasions, 15, 1209 - 1215.

Davy, C. M., Kidd, A. G., Wilson, C. C., 2015. Development and validation of environmental DNA (eDNA) markers for detection of freshwater turtles. PLoS One, 10, e0130965.

Deiner, K., Altermatt, F., 2014. Transport distance of invertebrate environmental DNA in a natural river. PLoS One 9, e88786.

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., Miaud, C., 2011. Persistence of environmental DNA in freshwater ecosystems. PLoS One, 6, e23398.

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E., Miaud, C., 2012. Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*. Journal of Applied Ecology, 49, 953-959.

Eichmiller, J.J., Bajer, P.G., Sorensen, P.W. 2014. The relationship between the distribution of common carp and their environmental DNA in a small lake. PLoS One, 9, e112611.

Ficetola, G.F., Miaud, C., Pompanon, F., Taberlet, P., 2008. Species detection using environmental DNA from water samples. Biology Letters, 4, 423-425.

Fukumoto, S., Ushimaru, A., Minamoto, T., 2015. A basin-scale application of environmental DNA assessment for rare endemic species and closely related exotic species in rivers: a case study of giant salamanders in Japan. Journal of Applied Ecology, 52, 358-365.

Goldberg, C.S., Pilliod, D.S., Arkle, R.S., Waits, L.P., 2011. Molecular detection of vertebrates in stream water: a demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. PLoS One, 6, e22746.

Goldberg, C.S., Sepulveda, A., Ray, A., Baumgardt, J., Waits, L.P., 2013. Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). Freshwater Science, 32, 792-800.

Hunter, M.E., Oyler-McCance, S.J., Dorazio, R.M., Fike, J. A., Smith, B.J., Hunter, C.T., Reed, R. N., Hart, K. M. 2015. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. PLoS One, 10, e0121655.

Jane, S. F., Wilcox, T. M., McKelvey, K. S., Young, M. K., Schwartz, M. K., Lowe, W. H., Letcher, B. H., Whiteley, A. R. 2015. Distance, flow, and PCR inhibition: eDNA dynamics in two headwater streams. Molecular Ecology Resources, 15, 216-227

Janosik, A. M., Johnston, C. E. 2015. Environmental DNA as an effective tool for detection of imperiled fishes. Environmental Biology of Fishes, 98, 1889–1893.

Jerde, C. L., Mahon, A. R., Chadderton, W. L., Lodge, D. M., 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation Letter, 4, 150-157.

Jerde, C. L., Chadderton, W. L., Mahon, A. R., Renshaw, M. A., Corush, J., Budny, M. L., Mysorekar, S., Lodge, D. M., 2013. Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance program. Canadian Journal of Fisheries and Aquatic Science, 70, 522-526.

Laramie, M. B., Pilliod, D. S., Goldberg, C. S., 2015. Characterizing the distribution of an endangered salmonid using environmental DNA analysis. Biology Conservation, 183, 29-37.

Mahon, A. R., Jerde, C. L., Galaska, M., Bergner, J. L., Chadderton, W. L., Lodge, D. M., Hunter, M. E., Nico, L. G., 2013. Validation of eDNA surveillance sensitivity for detection of Asian carps in controlled and field experiments. PLoS One, 8, e58316.

Maruyama, A., Nakamura, K., Yamanaka, H., Kondoh, M., Minamoto, T., 2014. The release rate of environmental DNA from juvenile and adult fish. PLoS One, 9, e114639.

Minamoto, T., Yamanaka, H., Takahara, T., Honjo, M. N., Kawabata, Z., 2012 Surveillance of fish species composition using environmental DNA. Limnology, 13, 193-197.

Moyer, G. R., Díaz-Ferguson, E., Hill, J. E., Shea, C., 2014. Assessing environmental DNA detection in controlled lentic systems. PloS One, 9, e103767.

Piaggio, A. J., Engeman, R. M., Hopken, M. W., Humphrey, J. S., Keacher, K. L., Bruce, W. E., Avery, M. L., 2013. Detecting an elusive invasive species: a diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. Moecular Ecology Resources, 14, 374-380.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., Waits, L. P., Richardson, J., 2013. Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Canadians Journal of Fisheries and Aquatic Science, 70, 1123-1130.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., Waits, L. P., 2014. Factors influencing detection of eDNA from a stream-dwelling amphibian. Molecular Ecology Resources, 14, 109-116.

Rees, H. C., Bishop, K., Middleditch, D. J., Patmore, J. R. M., Maddison, B. C., Gough, K. C., 2014. The application of eDNA for monitoring of the Great Crested Newt in the UK. Ecology and Evolution, 4, 4023-4032.

Santas, A. J., Persaud, T., Wolfe, B. A., Bauman, J. M., 2013. Noninvasive method for a Statewide Survey of Eastern hellbenders *Cryptobranchus alleganiensis* using Environmental DNA. International Journal of Zoology, 2013, 1-6.

Sigsgaard, E. E., Carl, H., Møller, P. R., Thomsen, P. F., 2014. Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. Biological Conservation, 183, 46-52.

Strickler, K. M., Fremier, A. K., Goldberg, C. S., 2015. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biological Conservation, 183, 85-92.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., Kawabata, Z., 2012. Estimation of fish biomass using environmental DNA. PLoS One, 7, e35868.

Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L., Willerslev, E., 2012a. Monitoring endangered freshwater biodiversity using environmental DNA. Molecular Ecology, 21, 2565-2573.

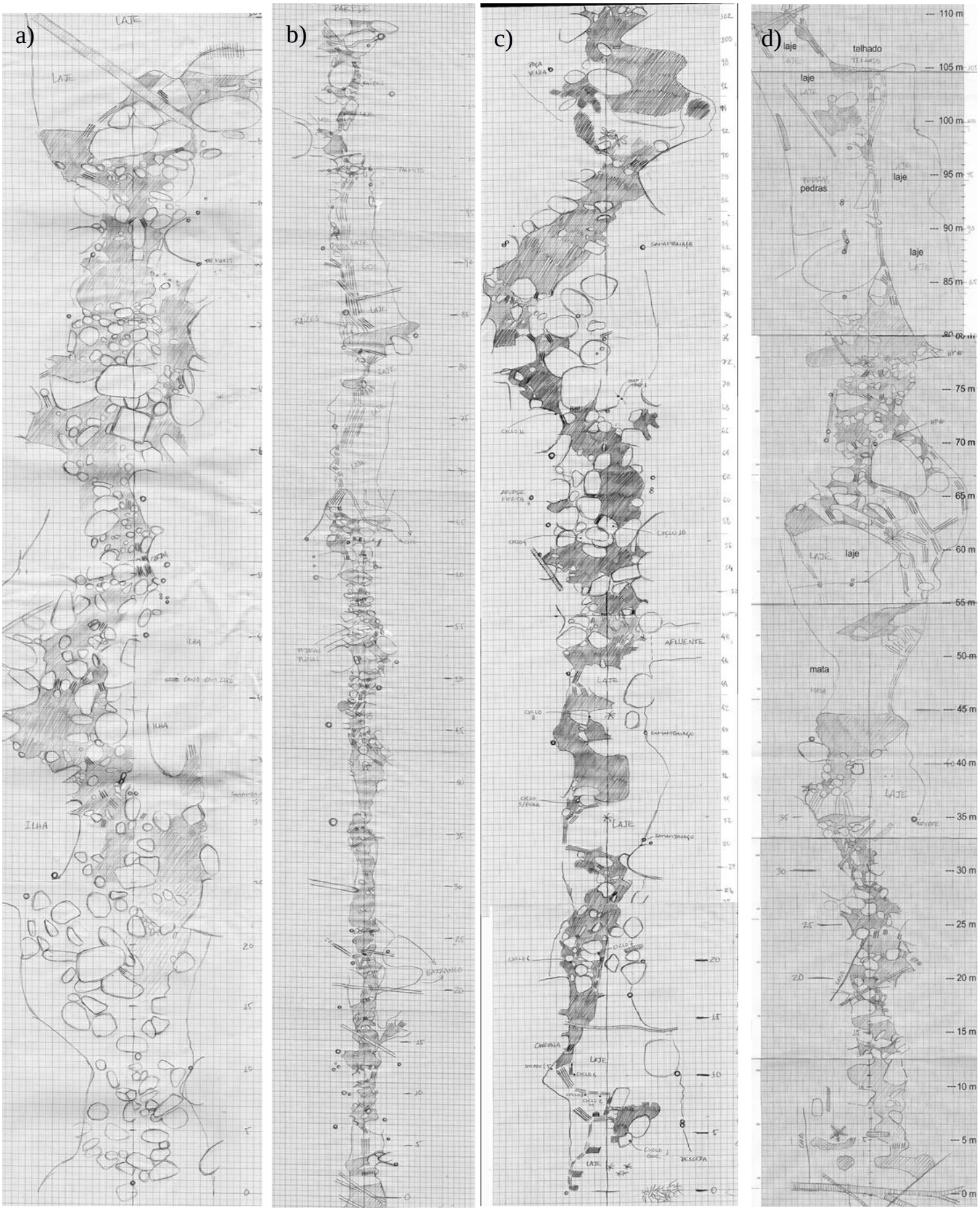
Thomsen, P. F., Kielgast, J., Iversen, L. L., Møller, P.R., Rasmussen, M., Willerslev, E., 2012b. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLoS One, 7, e41732.

Tréguier, A., Paillisson, J. M., Dejean, T., Valentini, A., Schlaepfer, M. A., Roussel, J. M., 2014. Environmental DNA surveillance for invertebrate species: advantages and

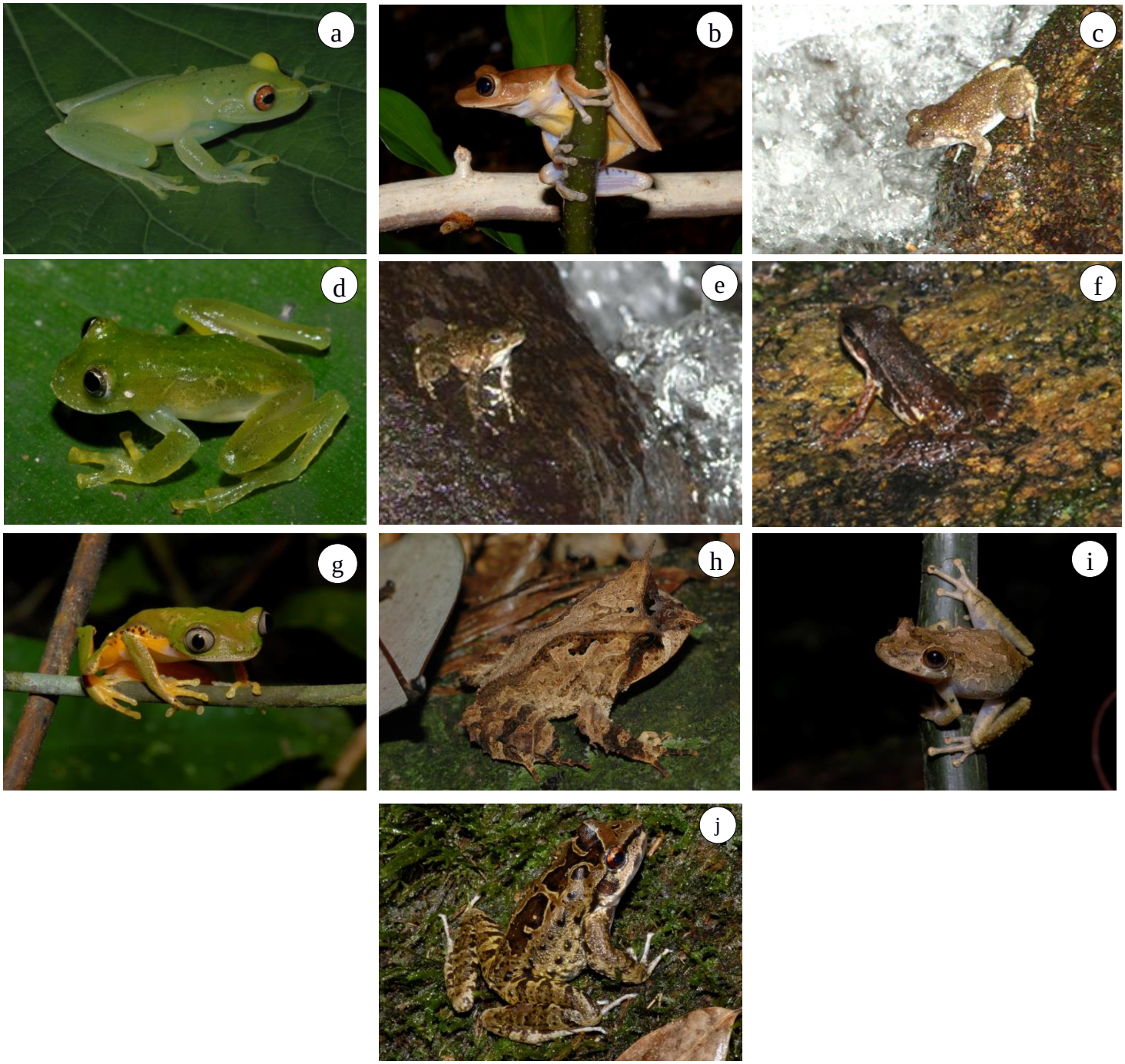
technical limitations to detect invasive crayfish *Procambarus clarkia* in freshwater ponds. Journal of Applied Ecology, 51, 871-879.

Wilcox, T. M, McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley A. R., Schwartz, M. K., 2013. Robust detection of rare species using environmental DNA: the importance of primer specificity. PloS one, 8, e59520.

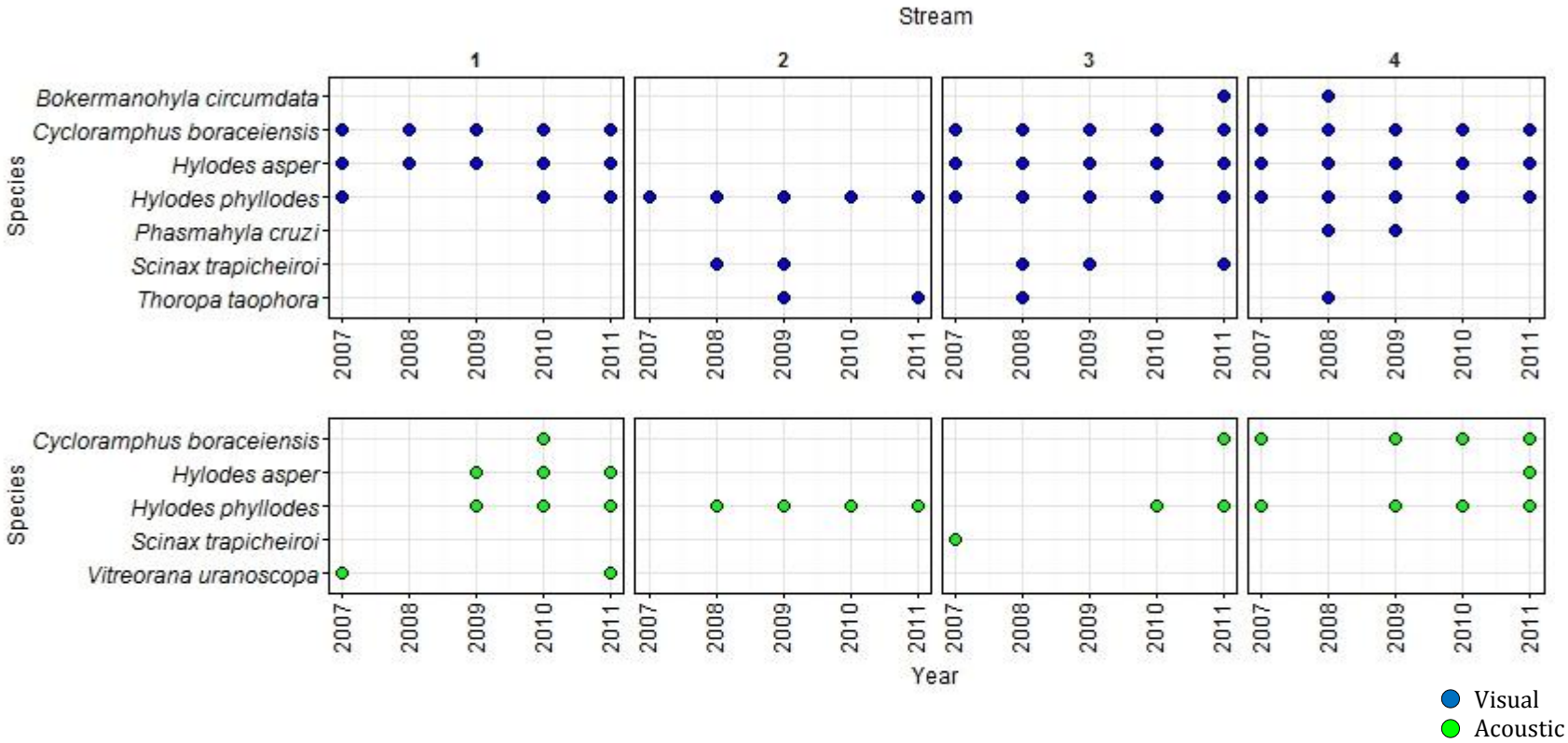
Wilson, C., Wright, E., Bronnenhuber, J., MacDonald, F., Belore, M., Locke, B., 2014. Tracking ghosts: combined electrofishing and environmental DNA surveillance efforts for Asian carps in Ontario Waters of Lake Erie. Management of Biological Invasions, 5,225-231.



Supplementary Figure I - Graph paper maps of sampled stream at Núcleo Picinguaba, São Paulo, Brazil: a) stream 1, b) stream 2, c) stream 3, d) stream 4. Circles on maps represent rocks along the stream and the black area represents water flow.

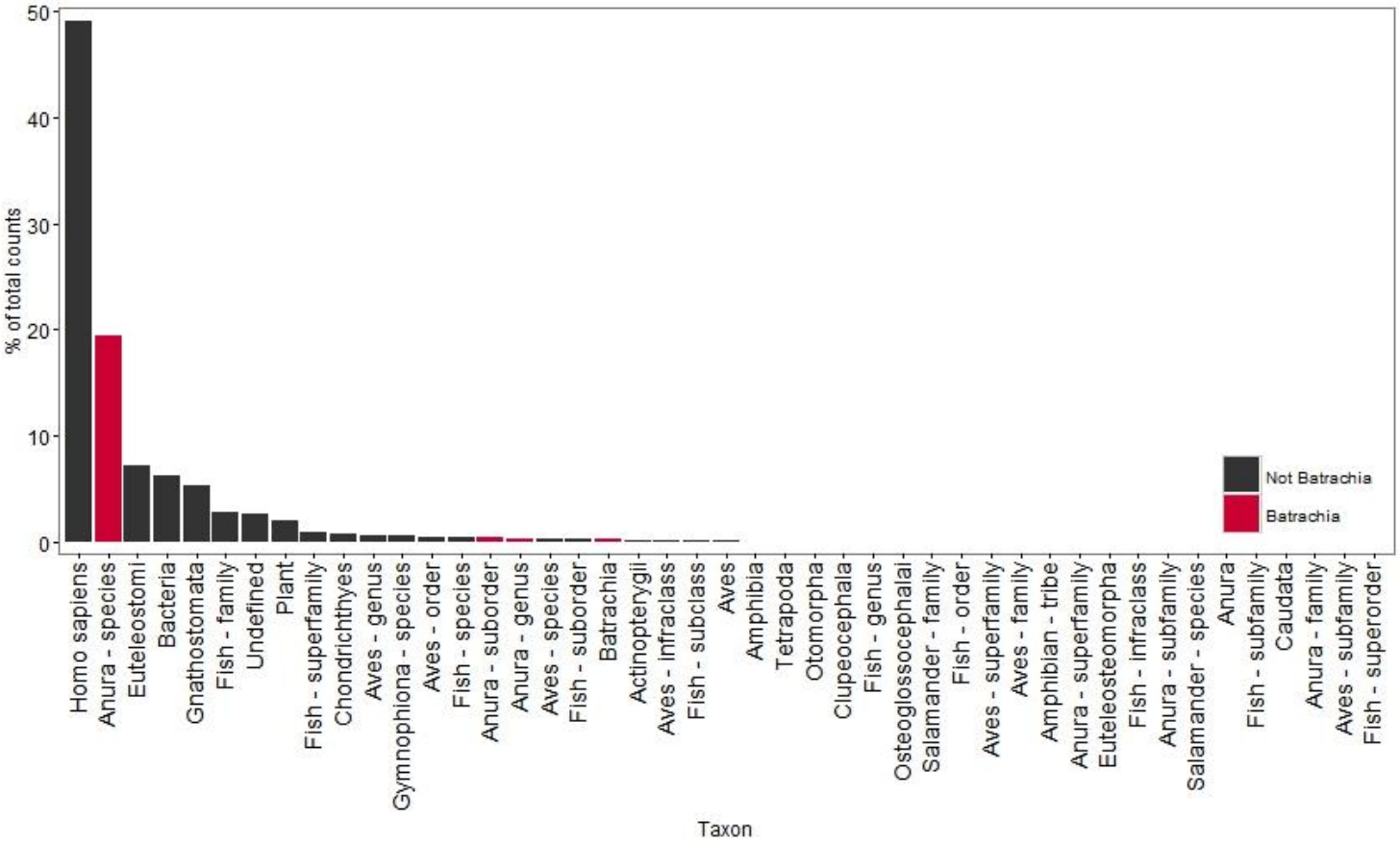


Supplementary Figure II - Amphibians recorded in Núcleo Picinguaba, São Paulo, Brazil. a) *Aplastodicus eugenioi* Photo by Célio F. B. Haddad, b) *Bokermannohyla circumdata*, c) *Cycloramphus boraceiensi*, d) *Vitreorana uranoscopa*, e) *Hylodes asper*, f) *Hylodes phyllodes*, g) *Phasmahyla cruzi*, h) *Procetaphrys appendiculata*, i) *Scinax trapicheroi*, j) *Thoropa taophora*. Photos by Marcio Martins.

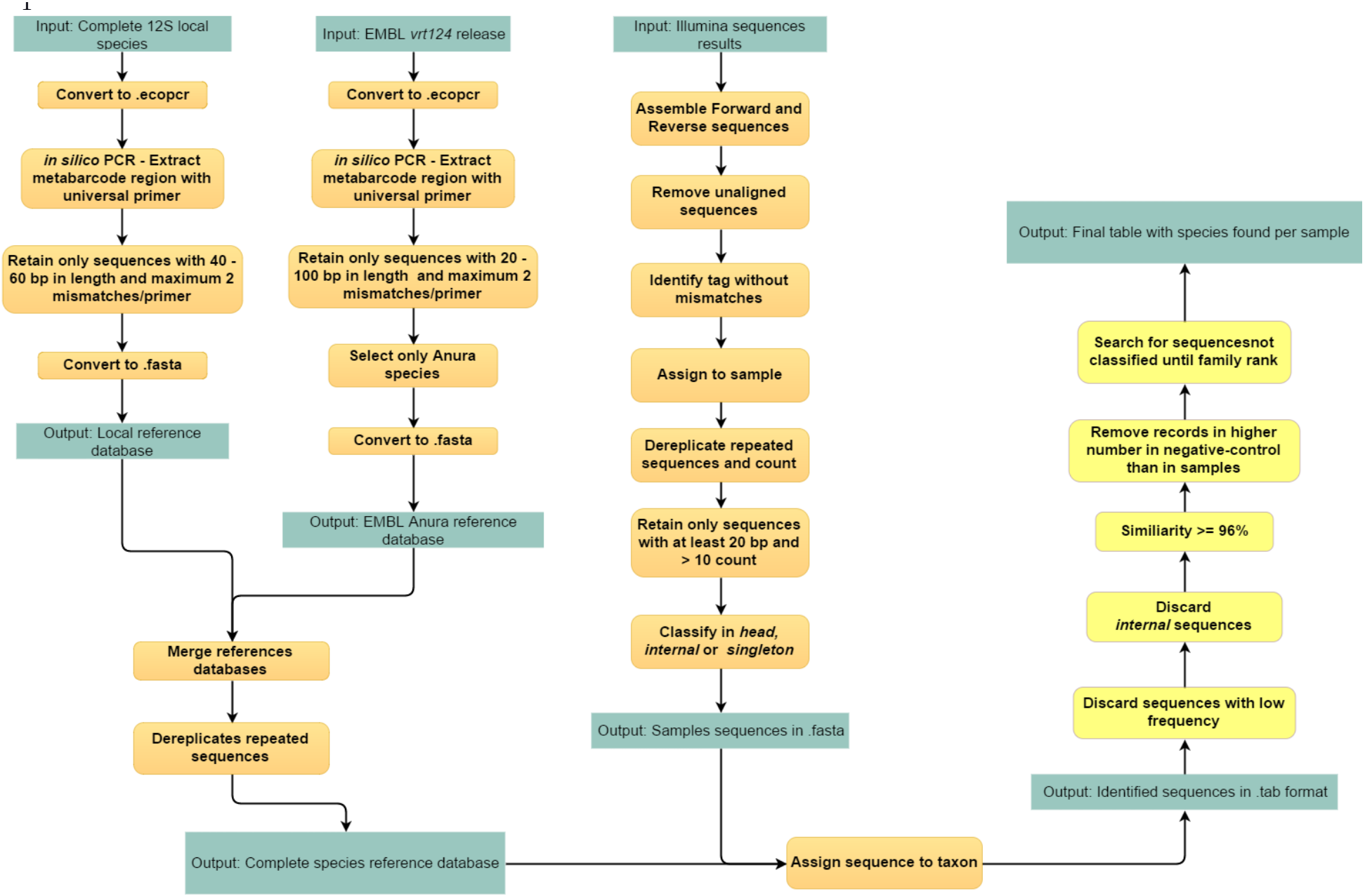


Supplementary Figure III - Species found in each stream during surveys performed on April for each year in Núcleo Picinguaba, São Paulo, Brazil. Blue and green indicate visual and acoustic survey, respectively.

Supplementary Figure IV – Although the universal primer pair for Batrachia group used on this study was able to distinguish two congeneric species (*Hylodes asper* and *Hylodes phyllodes*) from our samples, many 12S sequences belonged to other vertebrates groups. We performed a preliminary analysis with the metabarcoding results and the *vtr124* release from EMBL plus the local database. In this analysis, in order to identify the taxa present in our samples we included all Chordata species available in the *vtr124* release from EMBL database and performed BLAST to identify human sequences. In our study, we used this same release but we filtered the database and retained only Anuran species. Almost 49% of the reads count was human sequences, followed by Anuran species with 19.4%. Sequences belonging to Batrachia (in red) counted for 20.26% of all sequences. However, in this preliminary analysis, we did not follow with similarity control or error filtration in species identification and we not excluded sequences with less than 0.1% in frequency, as performed in our study. The non-specificity of the primer could lead to non-Batrachia DNA sequences saturating the amplification process, preventing detection of low abundance anuran taxa.



Supplementary Figure V – Workflow of eDNA informatics analyses. Orange boxes are steps done using OBITools and ecoPCR. Yellow boxes are steps performed in custom R scripts.



Supplementary Table I - Species found in each stream and each year during visually (V), acoustic (A) survey or eDNA analysis in Núcleo Picinguaba, São Paulo, Brazil.

Species	Stream								Year										eDNA
	1		2		3		4		2007		2008		2009		2010		2011		
	V	A	V	A	V	A	V	A	V	A	V	A	V	A	V	A	V	A	
Aplastodiscus eugenioi						x								x					x
Bokermannohyla circumdata	x				x	x	x	x		x	x		x				x	x	?
Cycloramphus boraceiensis	x	x			x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Hylodes asper	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Hylodes phyllodes	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Phasmahyla cruzi							x		x		x		x				x		x
Proceratophrys appendiculata	x						x		x		x								
Scinax trapicheiroi			x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Thoropa taophora	x		x		x		x		x		x		x		x		x		x
Vitreorana uranoscopa	x	x				x			x	x	x	x		x		x		x	x

Supplementary Table II – Anuran families retained from EMBL database. The number of sequences containing the metabarcode region and with length between 20 to 100 bp is indicated to each family.

Family	Number of sequences
Allophrynidae	3
Alsodidae	35
Alytidae	18
Arthroleptidae	82
Ascaphidae	1
Batrachylidae	2
Bombinatoridae	18
Brachycephalidae	55
Brevicipitidae	6
Bufonidae	254
Calyptocephalellidae	2
Centrolenidae	100
Ceratobatrachidae	12
Ceratophryidae	18
Ceuthomantidae	2
Craugastoridae	36
Cycloramphidae	4
Dendrobatidae	256
Dicroglossidae	208
Eleutherodactylidae	68
Heleophrynidae	3
Hemiphractidae	40
Hemisotidae	3
Hylidae	491
Hylodidae	4
Hyperoliidae	31
Leiopelmatidae	3
Leptodactylidae	157
Mantellidae	177
Megophryidae	108
Micrixalidae	2
Microhylidae	317
Myobatrachidae	46
Nyctibatrachidae	4
Pelobatidae	7
Pelodytidae	3
Petropedetidae	12
Phrynobatrachidae	112
Pipidae	39
Ptychadenidae	10
Pyxicephalidae	23
Ranidae	255
Ranixalidae	3
Rhacophoridae	236
Rhinodermatidae	3
Rhinophrynidae	3
Strabomantidae	152

Supplementary Table III – Description of water parameters in the four sampled streams at Núcleo Picinguaba, São Paulo, Brazil

Stream	Field Sample	Dissolved Oxygen (mg/L)	Water Temperature (°C)	pH	Discharge (cm/s)
1	P1	6.08	20.6		-
	P2	5.38	20.5		-
	P3	5.56	20.4		24.56
				6.6	
2	P1	5.08	20.8		Slow water discharge ~ pond
	P2	5.33	20.9		Slow water discharge ~ pond
	P3	4.43	20.9		-
				6.5	
3	P1	4.97	20.6		Fast water discharge ~ 13.34
	P2	5.25	20.6		Fast water discharge ~ 20.44
	P3	5.18	20.6		~ Pond
				6.8	
4	P1	5.35	20.6		Slow water discharge ~ pond
	P2	6.58	20.7		13.14
	P3	7.23	20.8		Slow water discharge
				7	

GENERAL CONCLUSION

Habitat use, abundance and occurrence data are crucial to the success of conservation initiatives and serve as material for various research areas. Our studies contribute to the understanding of Brazilian amphibians and encourage the use of eDNA metabarcoding to evaluate the diversity of amphibian communities in tropical streams.

The Atlantic Forest has an important diversity of microenvironments and harbors a particular richness in amphibian species which explore a variety of habitats. Some terrestrial species breed in streams and migrate to environments such as leaf litter or trees in other life stage. On the other hand, torrent frogs reside most of the time in lotic environments, as the case of the species studied, *Cycloramphus boraceiensis, Hylodes asper* and *H. phyllodes*, mainly found inhabiting wet rocks near the water. This behavior apparently contributed to a greater chance of eDNA detection from the water samples for these species. Environmental DNA from species with other habits was also successfully detected, nonetheless, in a lesser extent.

Our study also showed that traditional sampling and eDNA metabarcoding can be complementary. Through eDNA analysis it was possible to detect amphibian diversity in tropical streams in a non-invasively manner. Through visual survey, we gathered information regarding the use of habitat and species abundance. Thus, a combination of both methods will be potentially useful for future ecological studies. However, it is important to note that most of the traditional methods applied for amphibians meet the expected effectiveness for only a few species, habits or locations and cannot provide extensive information of the species ecology, like the species' microhabitat use (Rödel and Ernst, 2004). Therefore, the choice of the traditional method should reflect the objectives of the study and be one that provides a complete picture of the ecology of

amphibians, complementing the use of eDNA. In addition to difference in purposes, different survey methods implies in different survey effort. In this study it was necessary only a single visit for eDNA sampling to gather information on amphibian communities, which was obtained through a 5-year survey using traditional methods. Hence, as a survey methodology for species occurrence, eDNA metabarcoding is capable of optimizing considerable time effort.

Finally, through traditional survey we described abundance variation over a long-term study. Estimate the abundance or density of species accurately is not yet possible through eDNA analysis. This is because DNA secretion might vary between life stages or between species and might be not clearly related to the organism biomass. Klymus *et al*. (2015), for example, found that a single individual may secrete DNA in quantities varying from zero to hundreds of thousands of copies within a few weeks. Moreover, it is not known how activity, metabolism, seasonality and stress affect the eDNA production. Another impairment to abundance estimation is the PCR procedures or sequencing bias towards the more abundant DNA copies, which in turn may conceal the abundance estimation of less abundant species.

GENERAL REFERENCES

Klymus, K. E., Richter, C. A., Chapman, D. C., Paukert, C., 2015. Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. Biological Conservation, 183, 77-84.

Rödel, M., Ernst, R., 2004. Measuring and monitoring amphibian diversity in tropical forests. I. An evaluation of methods with recommendations for standardization. Ecotropica, 10, 1-14.

EDUCATION

2015 -2015 Tropical Biology: An Ecological Approach
Organization for Tropical Studies - Costa Rica

2014 - Present M.Sc. – Universidade de São Paulo, São Paulo, Brazil
Departamento de Ecologia - Instituto de Biociências
Grant: Fundação de Amparo à Pesquisa do Estado de São Paulo

2013 - 2013 Laboratory assistant - Museu de Zoologia da Universidade de São Paulo
Laboratório e Coleção de Herpetologia
DTI-C Fellowship, Cnpq

2011 - 2012 Internship in *Genétique de Population et Ecologie des communautés*.
Université Paris-Sud 11, Paris-Sud 11, Orsay, France

2010 - 2012 Undergraduate - *Licenciatura*
Universidade de São Paulo, São Paulo, Brazil

2008 - 2013 B.Sc. in Biology - Universidade de São Paulo, São Paulo, Brazil
Title: Biologia Reprodutiva e hábitos alimentares de serpentes da tribo Xenodontini na América do Sul
Advisor: Márcio Roberto Costa Martins
Grant: Fundação de Amparo à Pesquisa do Estado de São Paulo