Dendrocephalus brasiliensis (Crustacea:Anostraca) hatching egg capacity in different water treatments

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Abstract

When considering the threats against biodiversity, biological invasion is an important element, as alien species alter the composition and functioning of the ecosystems, so much so that the invasion from exotic species is considered the top cause of global biodiversity loss. Considering how invasive species pose significant threats against local biodiversity, it's necessary to develop technologies that allow the use, including for ecotoxicological tests, of native species. *Dendrocephalus brasiliensis* (Crustacea: Anostraca) is a species that presents economical potential, with high nutritional value, so important in aquaculture, as well as high sensibility to several toxic substances, which allows its use as a scientific tool in toxicity studies. Therefore, it's important to study this species and develop new methodologies that could enable its use to the detriment of exotic species. Therefore, this thesis aimed to provide a new approach on how to perform hatching studies for *Dendrocephalus brasiliensis* species, evaluate the effects of using different mediums such as Dimethylsulfoxide (DMSO), Glycerol, Reconstituted (RW), and Natural water (NW) and their effects on the hatching rate; the effects of controlling/not the mediums pH, and the effect of saline buffers use over the cyst hatch. The results indicate the use of Natural and/or Reconstituted Water as a preferential medium for *Dendrocephalus brasiliensis* cultures, buffered (as a tool to provide hatching homogeneity) in a range of 7.3 to 8 (being 8 the recommended in the literature for the species), using cysts without previous dormancy break attempt (pre-treatment). Also, to automatize and facilitate cyst processing for *Dendrocephalus brasiliensis* using computer vision as a tool. To evaluate the viability of automating cyst recognition and counting using domain-specific object detection techniques based on computer vision. Then, we trained two state-of-the-art object detection methods, YOLOv3 (You Only Look Once) and Faster R-CNN (Region-based Convolutional Neural Networks), on the DBrasiliensis data set, which was also created for this study, to compare them under both cyst detection and counting tasks. We

concluded that the proposed approach using YOLOv3 is adequate to detect and count *Dendrocephalus brasiliensis* cysts. The stated results and considerations provided by this study allowed us to provide important considerations that can be applied to improve *Dendrocephalus brasiliensis* studies and production.
Keywords: Computer Vision, *Dendrocephalus brasiliensis*, resistance cyst hatch, Dimethilsulfoxyde, Glycerol, Hatching rate.

Resumo

A invasão biológica está entre os elementos mais importantes que ameaçam a biodiversidade, isso porque as espécies exóticas alteram a composição e o funcionamento dos ecossistemas, tanto que a invasão de espécies exóticas é considerada a principal causa da perda global de biodiversidade. Considerando como as espécies invasoras representam ameaças significativas à biodiversidade local, é necessário desenvolver tecnologias que permitam o uso, inclusive para testes ecotoxicológicos, de espécies nativas. *Dendrocephalus brasiliensis* (Crustacea: Anostraca) é uma espécie que apresenta potencial econômico, com alto valor nutricional tão importante na aquicultura, e alta sensibilidade a diversas substâncias tóxicas, o que permite seu uso como ferramenta científica em estudos de toxicidade. Portanto, é importante estudar esta espécie e desenvolver novas metodologias que possam viabilizar seu uso em detrimento de espécies exóticas. Assim, esta tese teve como objetivo fornecer uma nova abordagem de como realizar estudos de eclosão para espécies de *Dendrocephalus brasiliensis*. Avaliar os efeitos do uso de diferentes meios de cultivo, como soluções à base de Dimetilsulfóxido (DMSO), Glicerol, em comparação aos meios tradicionais como Água Natural (NW) e Reconstituída (RW), avaliando seus efeitos na taxa de eclosão; também os efeitos de realizar ou não o controle do pH dos meios, assim como, o efeito de tampões salinos sobre a eclosão dos cistos. Os resultados indicam o uso de Água Natural e/ou Reconstituída como meio preferencial para culturas de *Dendrocephalus brasiliensis*, com meio tamponado (apenas para favorecer a homogeneidade da eclosão) na faixa de 7,3 a 8 (sendo 8 o recomendado na literatura para a espécie), utilizando cistos sem tentativa prévia de quebra de dormência (pré-tratamento). Além disso, automatizar e facilitar o processamento de cistos de *Dendrocephalus brasiliensis* usando visão computacional como ferramenta. Avaliar a viabilidade de automatizar o reconhecimento e a contagem de cistos usando técnicas de detecção de objetos específicos de domínio com base em visão computacional. Em seguida, treinamos dois métodos

de detecção de objetos de última geração, YOLOv3 (You Only Look Once) e Faster R-CNN (Region-based Convolutional Neural Networks), no conjunto de dados DBrasiliensis, também criado para este estudo, para compará-los nas tarefas de detecção e contagem de cistos. Concluímos que a abordagem proposta usando YOLOv3 é adequada para detectar e contar cistos de *Dendrocephalus brasiliensis*. Os resultados, fornecidos por este estudo, nos permitiram oferecer considerações importantes que podem ser aplicadas para melhorar os estudos e a produção de *Dendrocephalus brasiliensis*.

Palavras Chave: Visão Computacional, *Dendrocephalus brasiliensis*, eclosão de cistos de resistência, Dimethilsulfoxido, Glicerol, Taxa de Eclosão.

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Chapter 1

General Introduction

The key element of a functioning ecosystem is biodiversity, since it characterizes how resilient to different kinds of pressures the ecosystem will be (Wardle, 2005). When considering the threats against biodiversity, biological invasion is an important element because alien species alter the composition and functioning of the ecosystems (Vitousek et al., 1997), so much so that the invasion from exotic species is considered the top cause of global biodiversity loss (Leprieur et al., 2008). This biodiversity crisis is linked to human actions that, deliberately or accidentally, introduce species outside their native range, harming the native species and disturbing the ecosystem's processes (Vitousek et al., 1997). Moving species is common practice for humans since the origin of agriculture and cattle raising (Macisaac et al., 2010), and the practice only increased as humanity developed technologies that allowed the conquest of geographical barriers, facilitating moving and trading around the world. And as much an area is introduced to non-native species higher will be the probability of some of them becoming invasive (Pyšek and Richardson, 2006).

Considering how invasive species pose significant threats against local biodiversity, it's necessary to develop technologies that allow the use of native species, independent of their finality. Some native species present characteristics that allow their use in several areas, presenting economic and scientific potential, thus the importance of studying its characteristics and developing new methods allowing its use to the detriment of exotic species. *Dendrocephalus brasiliensis* is a species that presents economical potential, with high nutritional value so important in aquaculture (da Cruz Daltro et al., 2021), as high sensibility to several toxic substances, which allows

its use as a scientific tool in toxicity studies (Santos et al., 2018). Therefore, it's important to surpass impediments for the complete applicability and use of that species. One of the difficulties of working with *Dendrocephalus brasiliensis* is its low hatching rate which, according to Lopes et al. (2007), is around 7% in natural conditions.

Considering this scenario, the present work aims to contribute to this challenge by providing a new approach to the methodology of hatching studies. Additionally, it aims to increase the hatching rate by studying the response to the use of Dimethylsulfoxide and Glycerol, different mediums (natural, reconstituted, and distilled water), and different pH ranges. The results of these studies are expected to provide important information that can be applied to improve studies for *Dendrocephalus brasiliensis* species, and also to improve its hatching rate.

1.1 General Objectives

The objective of this thesis is to provide a new approach to how to perform hatching studies for *Dendrocephalus brasiliensis* species, to evaluate the effects of using Dimethylsulfoxide and glycerol and their effects on the hatching rate, and the effects of variant pH in these solutions; comparing it with the hatch results of different mediums as distilled, reconstituted, and natural water. This thesis also aims to propose an automatized approach for the detection and counting of *Dendrocephalus brasiliensis* cysts from images to facilitate cyst processing.

In order to reach the objectives, 3 specific aims were established:

1. To evaluate the viability of a different approach for *Dendrocephalus brasiliensis* cyst hatching studies and the differences in the hatching rate observed when compared with the traditional methodology in Chapter 3. The experiments described in Chapter 3 were designed to differ from the traditional methodology using known amounts of cysts in all experiments and not quantified per gram, as largely adopted in these tests. Cysts varied in size, presence of dirt (even after the cleaning process), and also the presence of hatched cysts. All those variables could amount to a difference from the real amount of the hatching for the species;

2. To evaluate the viability of automating cyst recognizing and counting using domain-specific object detection techniques based on computer vision Chapter 2. The experiments described in chapter 3 were designated based on previous works, such as Jalal et al. (2020), that use such technology to detect and classify fish in underwater videos; and Mohamed et al. (2020) that achieved satisfactory results detecting and tracking fish underwater.

3. To analyze the effects of Dimethylsulfoxide and Glycerol, with and without the pH variables, on the hatching rate when compared to other culture mediums in Chapter 3. The experiments described in Chapter 3 were designed based on tests performed previously on another Anostraca, the *Thamnocephalus platyurus* species, by Murugan and Dumont (1995). The authors observed positive results, such as an increase in hatching rate and the absence of toxicity.

The work is divided into four chapters: Chapter 1 presents a general introduction to all the topics covered in the thesis; Chapter 2 presents the results from the automating cyst recognizing and counting based on computer vision; Chapter 3 presents the results, structured as a scientific article, of the new approach to hatching studies and for the substances and conditions tested aiming to increase the hatching rate of *Dendrocephalus brasiliensis*; Chapter 4 presents, briefly, all the conclusions obtained. The description of each chapter is summarized below:

Chapter 1: General introduction: Contextualization of the work describing extensively the *Dendrocephalus brasiliensis* species characteristics, and addressing its economical and scientific importance. It also presents the difficulties that prevent its more extensive use as a possible tool to address these issues.

Chapter 2: The paper "Recognizing and counting *Dendrocephalus brasiliensis* (Crustacea: Anostraca) cysts using deep learning" was published in the PLoS ONE journal is presented. We propose an automatized approach for the detection and counting of *Dendrocephalus brasiliensis* cysts from images captured by a digital microscope. For this purpose, we built the DBrasiliensis dataset, a repository with 246 images containing 5141 cysts of *Dendrocephalus brasiliensis*.

Chapter 3: The manuscript "The key factors for performing hatching tests with *Dendrocephalus brasiliensis* (Crustacea: Anostraca) species" is presented. We presented a new protocol to be used as the methodology in hatching studies for *Dendrocephalus brasiliensis* species, the study aimed to improve the accuracy of the results in such studies. Also, the effects of Dimethylsulfoxide and Glycerol were studied aiming to increase the hatching rate for the species.

Chapter 4: Discussion and concluding remarks: This chapter discusses the results obtained, in an integrated way, summarizing the main highlights of the work.

1.2 Literature Review

1.2.1 Anostraca Generalities

Among crustaceans, the order Anostraca, commonly known as fairy shrimps, stands out due to being adapted to adverse environments. The freshwater Anostraca are found in semi-arid areas, which present striking periods of drought and posterior rainy season, resulting in temporary lakes and pools that constitute the fairy shrimp habitat (BELK and COLE., 1975). According to Rogers (2013), there are more than 700 Anostraca taxa, with 407 valid families, genera, and species. The number of new annually described Anostraca does not decrease since the 1940 decade and, until the presented study, there were 353 valid species names recognized, which represents a more than 20% increase since the publication of Brtek (2002) when 285 species were recognized; the endemicity is also high with the Anostraca, around 56% known species described to be found from ten or fewer localities (Rogers, 2013).

1.2.2 Dendrocephalus brasiliensis

In Brazil, three genera from the Anostraca order, are known to populate the temporary freshwater pools including *Dendrocephalus* Daday, 1908 (Thamnocephalidae). The *Dendrocephalus* genera present as characteristic the frontal appendage (only found in males) a long and complex cephalic extension while the females can be recognized by their brood pouch (Chaves et al., 2011). Among the Dendrocephalus genera, the *Dendrocephalus brasiliensis* species stands out because of a set of characteristics, as important from commercial as scientific points of view. This species can be found in semi-arid regions including several Brazilian states. The systematic of the *Dendrocephalus brasiliensis* species are:

- Class Branchiopoda
- Subclass Sarsostraca
- Order Anostraca **Sars, 1867**
- Family Thamnocephalidae **Linder, 1941**
- Genus ***Dendrocephalus*** Daday, 1908
- Subgenus ***Dendrocephalus*** Daday, 1908
- ***Dendrocephalus (Dendrocephalus) brasiliensis*** **Pesta, 1921**

1.2.3 Distribution of Dendrocephalus brasiliensis

Dendrocephalus brasiliensis is common in different states of Brazil and its geographical distribution extends from Northern Argentina (César, 1989; César et al., 2004) to the Piauí state (Rabet and Thiéry, 1996). The author in question made the first record of that species in Brazil in the Bahia and Piauí states. Lutz (1929) wrongfully registered the occurrence in Macaiba, Rio Grande do Norte-RN, in 1929, as *Dendrocephalus* ornatus, being corrected by Linder (1941), that verified that the described species was *Dendrocephalus brasiliensis*. The species was also found in Ceará (Freita et al., 2017), Paraíba e Minas Gerais (Mai et al., 2008). To this date, the species was also recorded in São Paulo (Mai et al., 2008) and Alagoas (de Paiva Barros-Alves et al., 2016), but both cases have humans as the suspect for the introduction since it's associated with fish-breeding tank activities. However, the state of Alagoas actually presents the possibility of natural distribution, not proven so far. The presence of the species can be observed in the presented maps (Figure 1.1 and Figure 1.2).

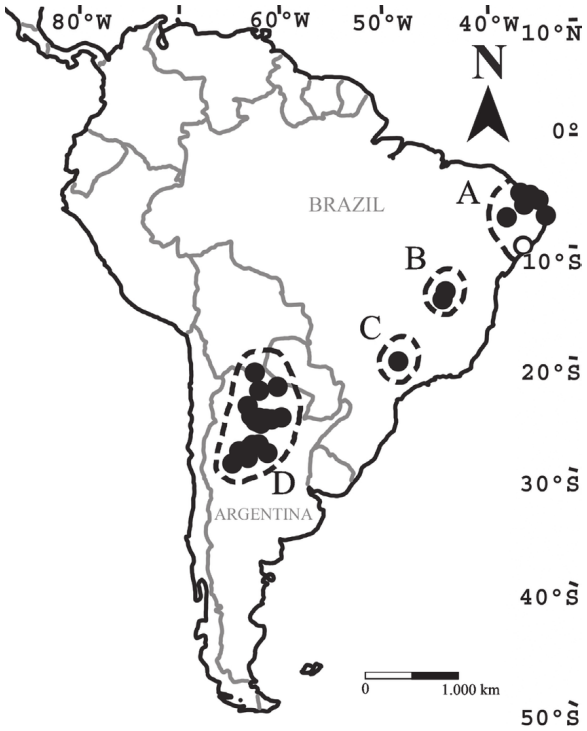

Figure 1.1: Map of distribution of *Dendrocephalus brasiliensis* until the year of 2016 B from (de Paiva Barros-Alves et al., 2016). With (A) representing the Piauí, Ceará, Rio Grande do Norte and Paraíba states; (B) Bahia and Minas Gerais states; (C) São Paulo state; And (D) northern Argentina.

1.2.4 Morphological characteristics of Dendrocephalus brasiliensis

The *Dendrocephalus* Daday (1908) generus presents great similarity, but the frontal appendage morphology provides good characteristics that enable the separation of all Brazilian species (Rabet and Thiéry, 1996). Although, Chaves et al. (2011) presented in their work a new form to identify utilizing the number of spines, Figure 1.3, being 0 for *Dendrocephalus brasiliensis*, 1 (each side) for *Dendrocephalus orientalis*, and more than one on each side for *Dendrocephalus goiasenses*, *thyeryi*, and *sp.nov* at the anterior edge of arms of the frontal appendage. The identification utilizing Dendrocephalus Daday (1908) females' characteristics isn't possible because of their similarities. The differentiation between females/males is easy since the male presents the frontal appendage that is inexistent in the females, and the last is easily recognizable since exhibits a brood pouch, Figure 1.4.

1.2.5 Reproduction of Dendrocephalus brasiliensis

The reproduction of *Dendrocephalus brasiliensis* have been the subject of studies, for example da Silva et al. (2013), that investigate the species reproductive strategy by means of Amplified Fragment Length Polymorphism (AFLP) markers and found forty polymorphic markers with 35% from the paternal origin and 65% were maternally inherited. Therefore, the results showed paternal inheritance revealing sexual reproduction by the species, at least under the experimental conditions of the study; but Lopes et al. (2011), also investigating the reproductive behavior of *Dendrocephalus brasiliensis*, concluded that the species reproduction is sexed. Another interesting fact discovered by da Silva et al. (2013) is that *Dendrocephalus brasiliensis* species presents sex

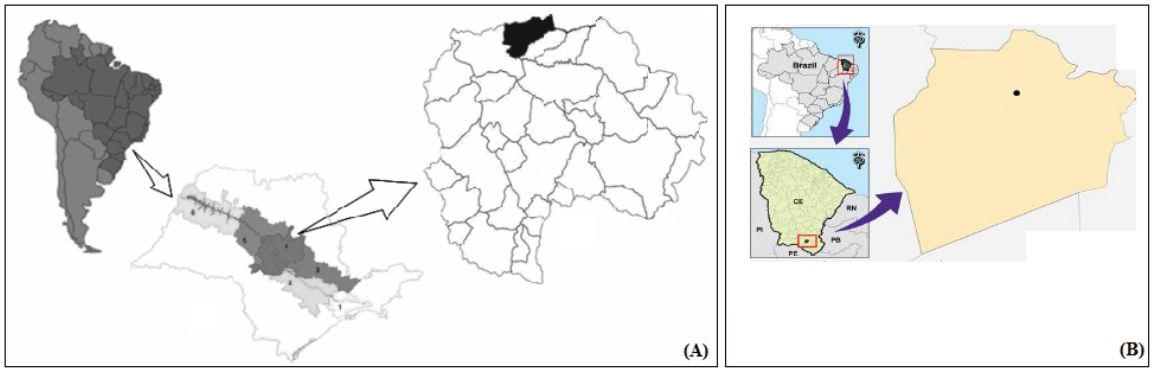

Figure 1.2: Modified map **A** shows the area where *Dendrocephalus brasiliensis* was found after being introduced in São Paulo state (Mai et al., 2008). Modified Map **B** describes the extended distribution of *Dendrocephalus brasiliensis* to the municipality of Juazeiro do Norte, northeastern Brazil, in the year 2017 from (Freita et al., 2017).

changes after 72 hours of grouping same-sex individuals, indicating, according to the authors, the possibility of a sex density-dependent behavior in the species.

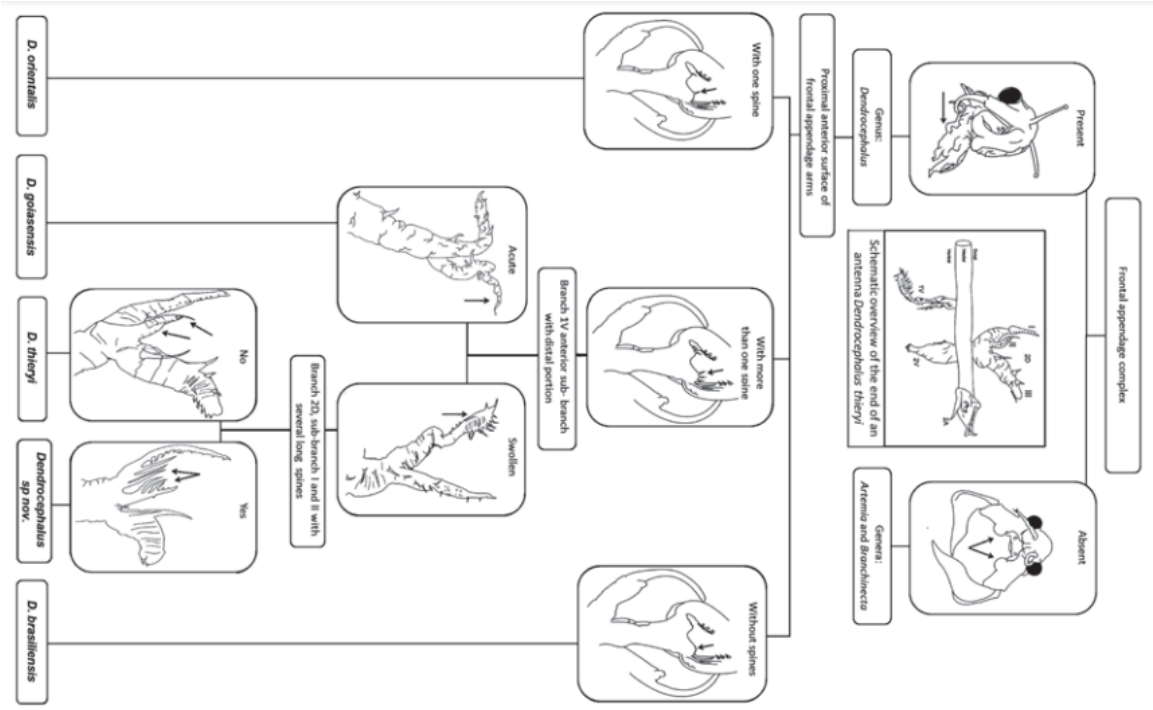

Figure 1.3: Illustration, from Chaves et al. (2011), showing the number of spines used as identification key for Brazilian Dendrocephalus species.

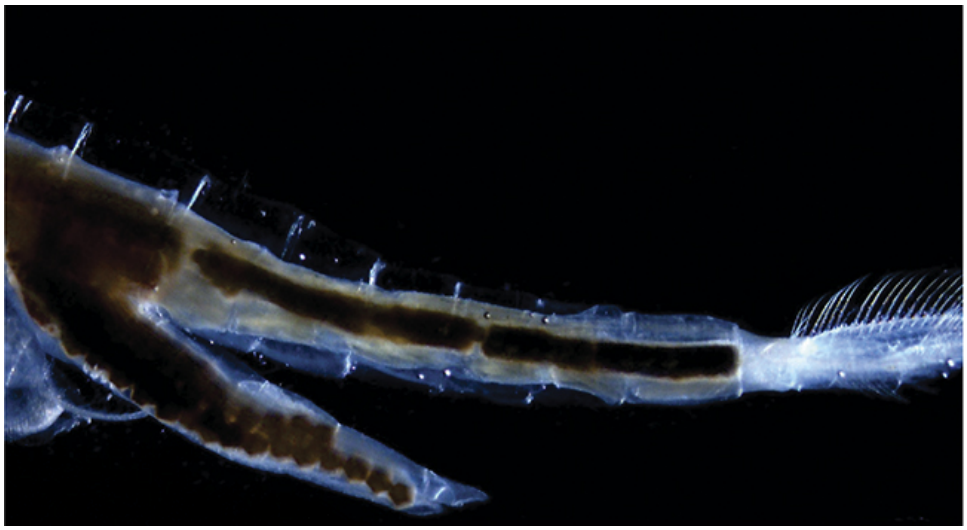

Figure 1.4: Brood Pouch of *Dendrocephalus brasiliensis* female adapted from de Paiva Barros-Alves et al. (2016).

1.3 Potentialities for Dendrocephalus brasiliensis species

1.3.1 Economical Potential of Dendrocephalus brasiliensis

The *Dendrocephalus brasiliensis* species presents several characteristics that make it stands out because of its economic potential. Studies show that, in multiple forms (cyst, nauplii, or as adult individuals), the species present a high nutritional value and can be used as a substitute food source in aquaculture. An example can be shown in Lopes et al. (2007) studies that aimed to develop a methodology to produce cysts of *Dendrocephalus brasiliensis* with the intent to enable its use as an alternative to *Artemia sp.* as a food source for shrimp and fingerlings. The authors concluded that the inoculation of cysts in the ponds increases the cyst production. Lopes et al. (2008a) continued to develop a methodology for the production of biomass of *Dendrocephalus brasiliensis* with the purpose of creating an alternative source of food to *Artemia sp.* because of its high prices; their results suggest an average biomass production of 1.863 kg/ha/year. Therefore, they recommend the production of *Dendrocephalus brasiliensis* biomass using their methodology of inoculating 2g of cysts per $2000m^2$.

Other authors, such as da Cruz Daltro et al. (2021), analyzed the effect of using the species as the first exogenous feed source for fish (tambaqui species), at the postlarvae phase, and concluded that the fish larvae fed with only *Dendrocephalus brasiliensis* had a better final weight, daily weight gain, and the best specific growth, indicating good potential as live food for the postlarvae phase of the tambaqui species. Yaflaar and Oliveira (2003), as another example, evaluated the performance of *Dendrocephalus brasiliensis* as food for *Litopenaeus vannamei* larvae and post-larvae. Their experiments resulted in significant differences among the 5 feeds tested and the treatments with *Dendrocephalus brasiliensis* nauplii may be useful in combination with live *Artemia sp.* nauplii, as live food for the *Litopenaeus vannamei* shrimp larvae.

Studies with *Dendrocephalus brasiliensis* species are vital since they provide data about possible use applications. Negative results are also crucial since show the species' limitations that might be the subject of future studies. One example is Albinati et al. (2005) that tested if *Dendrocephalus brasiliensis* could be used as an attractant on feed intake by Peacock bass (*Cichla ocellaris*) fingerlings since the fish species naturally does not accept dry feeds; they tested four rations (commercial fish feed formulation and the experimental diets that included dry and powder *Dendrocephalus brasiliensis*) and concluded that there wasn't a significant difference to feed intake, suggesting that the inclusion of *Dendrocephalus brasiliensis* in feed does not contribute to the development of Peacock bass fingerlings. These results, positive or negative, are important to provide new information and knowledge about the specific characteristics and potentiality of

the species.

1.3.2 Ecotoxicological Potential of Dendrocephalus brasiliensis

Dendrocephalus brasiliensis also presents important features to science as analyzed by Santos et al. (2018) that evaluate the sensitivity of *Dendrocephalus brasiliensis*, compared with *Daphnia magna*, as alternative test species for monitoring contaminants in tropical and subtropical freshwaters. They chose the species because they are adapted to the specificities of the ecosystems of tropical and subtropical areas. Their results indicate that *Dendrocephalus brasiliensis* has higher sensitivity than the temperate organism. Therefore, the authors suggest its use as alternative test species for monitoring contaminants in tropical and subtropical freshwaters. Another example of the toxicity tests realized with the species was conducted by Santos et al. (2019), which tested different surfactants (synthetic and biogenic) using *Daphnia magna*, as a model organism, and *Dendrocephalus brasiliensis* as alternative test species. They intended to find an alternative for monitoring these pollutants in tropical freshwaters. The authors concluded that both species showed a high sensitivity for the anionic compound sodium dodecyl sulphate (SDS) (For the recommended dosage use), as well as higher responsiveness of *Dendrocephalus brasiliensis* for all tested compounds in comparison to *Daphnia magna*, demonstrating its potential for monitoring pollutants in tropical/subtropical environments.

1.4 New Methodologies for Production of Dendrocephalus brasiliensis species

Several studies present, investigate, and create methodologies that can improve the usability of *Dendrocephalus brasiliensis*, since they tackle different problems and solutions aiming to advance the production system, independent of the specimens' phase of development (cyst, nauplii, or adult). One example is Arbeláez-Rojas and da Graça Gama Melão (2022) which investigated, among other things, the effects of the stocking density on biomass and cyst productions of *Dendrocephalus brasiliensis* and concluded that different stocking densities affected the biomass and cyst productions as the survival of *Dendrocephalus brasiliensis*. Thus, for intensive production, the authors suggested rearing the species in a high density of 100 individuals L^{-1} and for higher cyst production 20 pairs/L. Such information presents great value since it can benefit productivity and, consequently, the viability of its use in the future.

Lopes et al. (2007) developed a methodology to produce cysts of *Dendrocephalus brasiliensis* as an alternative to *Artemia sp.* as a food source for shrimp and fingerlings and concluded that the inoculation of cysts in the ponds increases the cyst production. Another author evaluated the influence of food concentration on the growth and reproduction of *Dendrocephalus brasiliensis* at different algal densities and under the conditions studied, concluded that the concentration of $9x10^5$ cells mL^{-1} of *Raphidocelis subcapitata* algae was the best for the species as provided better development (Andrade et al., 2020); all those data enriches the available knowledge about the necessities of *Dendrocephalus brasiliensis*.

Another important aspect that has been studied is the nauplii production, since several authors described low hatching rates, naturally around 7%, according to Lopes et al. (2007). Considering this, Santos et al. (2019) have developed a new process for nauplii production using chitinase and/or chemical treatment, and the results showed that enzyme increased the hatching rate of the *Dendrocephalus brasiliensis*, and the further addition of calcium hydroxide and ascorbic acid increased the hatching efficiency of the nauplii; additionally, the use of enzyme/chemical treatment promotes a reduction of hatching time with more than 80% of total hatching observed on the first day. Such a high rate result is important since increases the applicability of the species but new studies aiming to develop techniques are important, especially using more accessible substances and/or technologies. Therefore, it is necessary to develop hatching techniques that can be easily applied in any situation.

1.4.1 New Experiment Setting Techniques for hatching studies of Dendrocephalus brasiliensis species

The studies for *Dendrocephalus brasiliensis* use, as methodology, a known amount of weight, in grams of cysts (Lopes et al., 2007; Pereira and dos Santos-Neto, 2010; de Vasconcellos, 2010; Santos et al., 2019)) that is believed to contain a certain amount of cysts (an approximation). Some facts are ignored in these studies or considered irrelevant, such as the variation in size of the cysts, and the fact that even cleaned from the substrate the cyst could still carry dirt (such as sand, dehydrated algae, fibers, etc.). Considering the low amount of weight used in studies, and that the impurities present in that weight are an ignored variable, it's acceptable to question the accuracy of the hatching rate currently established as normal for the species.

Therefore, proposing a new experiment setting is important as a form to investigate the species' hatchability. A solution to the questioning is to work with known amounts of cysts in each repetition of the experiment. The cysts can be separated manually using a microscope as a tool. The task however is very laborious and its difficulties bring another challenge to the

research because it's time-consuming. But this challenge itself brings another opportunity as the chance to embrace the use of new technologies, for example, the computational vision that is being used as a tool in several areas of knowledge.

1.4.2 Computer Vision

The use of new technologies can produce solutions to old problems as recognition-related tasks that, in the recent past, depended on human capability only. However, recent technologies are proving that recognition tasks in the future could no longer necessitate direct human labor. Some examples could be the use of computational vision to enhance the recognition of diseases for agriculture (Yuan et al., 2022), people and objects for security purposes (Saad et al., 2019), and environmental conservational efforts (Jalal et al., 2020; Mohamed et al., 2020). As a result, computational vision is an important tool that can be used to automate all kinds of processes, including facilitating the recognition and/or counting of cysts. Therefore, developing software to recognize/count *Dendrocephalus brasiliensis* cysts could be an important contribution to enhancing the species' use viability.

1.4.3 Water Bath

The concept of using a warm water bath was based on the test set of Santos et al. (2019) that were incubated *Dendrocephalus brasiliensis* cysts submerged in water and placed in a warm water bath, at 37°C for 1 hour, later testing the enzymatic activity and filling in the beaker with its necessary volume for the experiment. The author implies to the water bath the possible effect of starting the enzymatic activity. Therefore, it's important to test if this technique presents an influence on the hatching rate or time for the *Dendrocephalus brasiliensis* species.

1.4.4 Sulfuric Acid

The concept of using sulfuric acid as a tool to promote the dormancy break came from the principle that the Anostraca cysts are formed of chitin, and according to Hackman (1962) the chitin undergoes degradation when dissolved in acid. Therefore, it's important to test if this technique presents an influence on the hatching rate or time for the *Dendrocephalus brasiliensis* species.

1.4.5 Aeration

The concept of using aeration to promote cyst dormancy break comes from the principle that the culture medium's constant motion helps to separate the cyst from the substrate, removing the dirt covering the cyst which, in theory, could help the cyst imbibe and hatch. Then, it's important to test if this technique also presents an influence on the hatching rate or time for the *Dendrocephalus brasiliensis* species.

1.4.6 Dimethylsulfoxide and Glycerol

To adopt new test substances, it is important to consider previous knowledge about the characteristics and/or possible effects that they can provoke. Therefore, the concept of using Dimethylsulfoxide and Glycerol was based on Murugan and Dumont (1995) that tested the effects on the hatching rate of other Anostraca cysts, the *Thamnocephalus platyurus* Packard species, and concluded that both substances increased the hatchability significantly. The Dimethylsulfoxide is a clear, hygroscopic, non-volatile liquid; this dipolar organic compound is easily mixed with H_2O in any proportion, acting as an acceptor of hydrogen bonds and making it possible to form complexes with many bonds, serving as an oxidant and reducing agent (Shulyak et al., 2021); All those characteristics give DMSO the capacity to change the membrane permeability, therefore, there is the possibility that its use could increase the hatching rate. Glycerol is a clear, colorless, viscous, water-miscible molecule that enhances protein self-assembly and is used to stabilize enzyme activity and protein structure. Its molecules contain only single bonds making them flexible and able to adapt by optimizing hydrogen bonding with the surrounding water (Abou-Saleh et al., 2019). Glycerol also is present in cysts of brine shrimp (Clegg, 1964), therefore the possibility of its presence in cysts of other Anostraca. All this considered, the result of testing these substances could provide new information about the viability of *Dendrocephalus brasiliensis* use.

1.4.7 pH Range

The pH of the medium is important because can affect the ability of aquatic organisms to regulate the basic life-sustaining processes as exchanges of respiratory gasses and salts with the water, and the failure to regulate these processes can result in sub-lethal effects and mortality in cases when ambient pH exceeds the range physiologically tolerated by the organisms (Robertson-Bryan, 2004). Therefore, studying the pH effects, combined with other factors, could promote new knowledge for the hatching of *Dendrocephalus brasiliensis* species. The ideal pH for *Dendrocephalus*

brasiliensis known in the literature is alkaline (pH 8) (Lopes et al., 2007); but since those are actually the pH measured in natural circumstances it's important to conduct experiments to evaluate the effects different pH ranges cause in the species, especially during the hatching phase.

Chapter 2

Recognizing and counting *Dendrocephalus brasiliensis* (Crustacea: Anostraca) cysts using deep learning

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Abstract: The *Dendrocephalus brasiliensis*, a native species from South America, is a freshwater crustacean well explored in conservational and productive activities. Its main characteristics are its rusticity and resistance cysts production, in which the hatching requires a period of dehydration. Independent of the species utilization nature, it is essential to manipulate its cysts, such as the counting using microscopes. Manually counting is a difficult task, prone to errors, and that also very time-consuming. In this paper, we propose an automatized approach for the detection and counting of *Dendrocephalus brasiliensis* cysts from images captured by a digital microscope. For this purpose, we built the DBrasiliensis dataset, a repository with 246 images

[2]Astolfi ACMN, Astolfi G, Ferreira MGA, Centurião TD, Clemente LZ, de Oliveira BLMC, et al. (2021) Recognizing and counting Dendrocephalus brasiliensis (Crustacea: Anostraca) cysts using deep learning. *PLoS ONE* 16(3): e0248574. https://doi.org/10.1371/journal.pone.0248574

containing 5141 cysts of *Dendrocephalus brasiliensis*. Then, we trained two state-of-the-art object detection methods, YOLOv3 (You Only Look Once) and Faster R-CNN (Region-based Convolutional Neural Networks), on DBrasiliensis dataset in order to compare them under both cyst detection and counting tasks. Experiments showed evidence that YOLOv3 is superior to Faster R-CNN, achieving an accuracy rate of 83,74%, R^2 of 0.88, RMSE (Root Mean Square Error) of 3.49, and MAE (Mean Absolute Error) of 2.24 on cyst detection and counting. Moreover, we showed that is possible to infer the number of cysts of a substrate, with known weight, by performing the automated counting of some of its samples. In conclusion, the proposed approach using YOLOv3 is adequate to detect and count *Dendrocephalus brasiliensis* cysts. The DBrasiliensis dataset can be accessed at: https://doi.org/10.6084/m9.figshare.13073240.

2.1 Introduction

The practice of moving species is common to humans since the agriculture and cattle raising was originated, becoming more intense with the trade expansion across different parts of the world (Macisaac et al., 2010). Once carried, intentionally or not by humans, those species that overcame biogeographic barriers, which another way would not allow their natural dispersal, are defined as alien species (Richardson et al., 2011). From those species, the ones that possess competitive advantages over the native can be the cause of ecological disruption by reducing biodiversity, causing the extinction of native species, being vectors, or spreading diseases (Milardi et al., 2019).

Considering the presented scenario, the choice of native species for conservational efforts or productive activities must prevail when possible. Some species present characteristics that made its use viable in several areas, as such *Dendrocephalus brasiliensis*, which can be used in conservational efforts as a test organism in toxicity (Santos et al., 2018), in the residuary water treatment (Gonçalves, 2001), or in productive activity of aquaculture (Lopes et al., 1998). The utilization of native species, such as, *Dendrocephalus brasiliensis*, avoids the invasive ones that can cause alterations in the environment; but for the successful adoption of a species, independent of its use, the development of technologies aiming to facilitate its management is necessary.

The main characteristics of *Dendrocephalus brasiliensis* species are the rusticity and resistance cysts production that require a period of dehydration to hatch, independent of the conditions. Thus, it is required to manipulate the cysts independent of the species utilization nature. One of the barriers to using the *Dendrocephalus brasiliensis* is the low hatching rate that, according to Lopes et al. (2007), in natural circumstances is around 7%. That way, it is necessary to work

with known amounts of cysts both for experiments or commercialization aiming to achieve better hatching results.

According to Lopes et al. (2007), from 2g of cysts it is possible to produce in 2.000 m^2, 2.075 g/ha/year of cysts and 1g of cyst can generate 380.000 nauplii of *Dendrocephalus brasiliensis*. However, we must consider that the number of cysts present in the substrate may vary according to matrices quality, medium conditions, or any stress that can disrupt the capacity or amount of laying. Besides, working with clean cysts without the substrate is a very laborious task that involves the use of several meshes to help separate cyst and dirt, which is also very time-consuming (Santos et al., 2019). Due to the low hatching rate of the *Dendrocephalus brasiliensis* species, by adopting a gram of the substrate without separating the cysts can create results that are not befitting with reality when used in an experiment. For example, if 1g of substrate that contains almost no cysts is adopted, with the low hatching rate, the results are likely to be different from reality because of data inaccuracy.

There are two options to ensure data accuracy: the manual counting of the cysts present in the substrate or; perform the cleaning and separation of the cysts from the dirt. However, both tasks are very laborious and time-consuming. In this way, alternatives must be developed to improve accuracy and facilitate the cysts counting process. An alternative is to automate the process using domain-specific object detection techniques based on computer vision. These techniques deal with detecting instances of objects of a determined category (such as fruits, fish species, or cysts) in digital images based on features like shape, color, texture, etc (Jiao et al., 2019).

In recent years, several architectures based on computer vision for object detection have emerged and have made significant advances. Among these architectures, YOLO (You Only Look Once) (Redmon et al., 2016) and Faster R-CNN (Region-based Convolutional Neural Networks) (Ren et al., 2017) and their variants stand out due to a wide range in real-world applications. Some researchers have used object-detection based approaches in productive activities such as precision agriculture and also in conservation of species. In the conservation efforts, Jalal et al. (2020) used a unified approach based on YOLO to detect and classify fish in underwater videos, whose fish species classification accuracy varied from 79.8% to 91.64%. Mohamed et al. (2020) also achieved satisfactory results by adopting YOLO to detect and track fish underwater, however, they used images captured from web cameras placed above the pond instead of underwater videos. In the context of precision agriculture, Wang et al. (2020) used several detection methods, including Faster R-CNN and YOLOv3, to detect pests that dominantly attack field crops in order to real-time monitor them. YOLOv3 and Faster R-CNN obtained an average precision in detection

of pests of 63.54% and 51.72%, respectively. Vasconez et al. (2020) achieved video-based fruit counting performances up to 93% on three different fruits using Faster R-CNN. Li et al. (2019) presented an architecture with two-stage to detect aphid, whose detection stage is based on YOLO. The experiments showed that the approach achieved an aphid detection performance of 76.8% average precision. Quan et al. (2019) used an approach based on Faster R-CNN to obtain images from maize seedlings to distinguish maize seedlings and weeds in crops. The approach obtained an average precision in the detection of maize seedlings with respect to soil and weeds of 97.71%. Neupane et al. (2019) used Faster R-CNN to detect and count banana plants on a farm using aerial images collected from a UAV (Unmanned Aerial Vehicle). The approach achieved 97.9%, 91.5%, and 87.2% accuracy on altitudes of 40m, 50m, and 60m, respectively. These are some examples of process automation using domain-specific object detection techniques based on computer vision.

In this paper, we compare state-of-the-art object detection models Faster R-CNN and YOLOv3 in order to propose an automated approach for *Dendrocephalus brasiliensis* cysts detection and counting from images obtained by a digital microscope. Besides, we show that it is possible to infer the number of cysts from a substrate with a known weight. Finally, we introduce the DBrasiliensis dataset, a repository with 246 images containing 5141 cysts of *Dendrocephalus brasiliensis*, a native species from South America. One of the motivations for publishing the DBrasiliensis dataset is related to the importance and potential of this species to productive activities in aquaculture and conservational efforts. A dataset with cysts examples can help to accelerate researches that need *Dendrocephalus brasiliensis* cysts in an automated way using computer vision, as well as new applications for counting and weight inference of cysts.

The contributions of this paper are:

- The publication of a novel annotated *Dendrocephalus brasiliensis* cysts images dataset, called DBrasiliensis, composed of 246 images divided into training and testing. The training set has 111 images containing 3173 annotated cysts. The testing set has 135 images divided into ten subsets, whose labels represent the weight in grams of each one. In all, the testing set has 1968 cyst images. To the best of our knowledge, this is the first *Dendrocephalus brasiliensis* cysts image dataset destined for deep learning. The DBrasiliensis dataset can be accessed at: https://doi.org/10.6084/m9.figshare.13073240.

- Definition of a baseline for detection and counting *Dendrocephalus brasiliensis* cysts using the state-of-the-art YOLOv3 and Faster R-CNN.

- A deep learning-based automatized approach to detect and count *Dendrocephalus Brasilien-*

sis cysts from images obtained by a digital microscope.

The rest of the paper is organized as follows. In the 2.2 Section, we describe the DBrasiliensis dataset, introduce an overview of the YOLOv3 and Faster R-CNN, and also present the experimental setup, followed by the analysis of results in the 2.3 Section and conclusions in 2.4 Section.

2.2 Materials and Methods

2.2.1 DBrasiliensis dataset

The *Dendrocephalus brasiliensis*, whose life stages is presented in Figure 2.1, lays resistance cysts in the bottom of culture medium, such as an aquarium, small lakes, etc. These cysts mix with the substrate present at the bottom of the culture medium which is basically composed of organic and inorganic matter. Figure 2.2 shows a substrate sample, whose cysts are highlighted by a red rectangular bounding box.

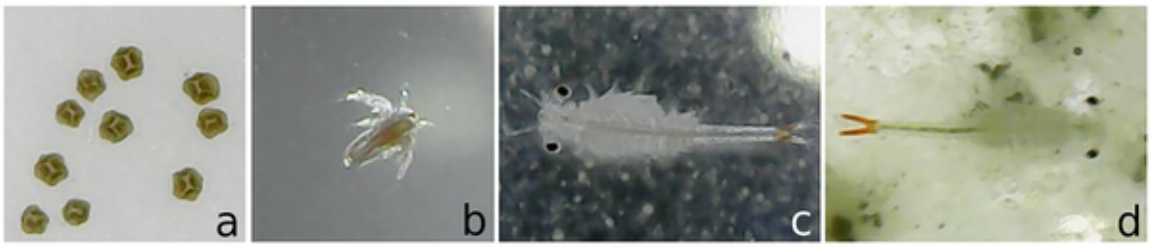

Figure 2.1: Life stages of *Dendrocephalus brasiliensis*: a) cysts, b) nauplius, c) juvenile, and d) adult.

In order to build the DBrasiliensis dataset, we took substrates portions from the bottom of an aquarium that we used as an incubator for the *Dendrocephalus brasiliensis* and split them into two parts: one to capture the training images and the other to capture the test images. Both parts were fixed on white coverslips in order to be observed using a digital microscope. We used an XTRAD USB digital microscope model XT-2036 with 52x magnification to capture the images with resolution of 640×480 pixels.

On the images designated to the training set (see example in Figure 2.3 (a)), we used the LabelImg software to label cysts in both PASCAL VOC (Everingham et al., 2009) and YOLO formats, as shown in Figure 2.3 (b). There are 111 images in all, for training, containing 3173 annotated cysts.

We divided the images designated for the tests into ten small groups. Each small group received a label that indicates the number of cysts in the images group and the weight of the substrate used to capture the images. For building a given group, we split the substrate reserved

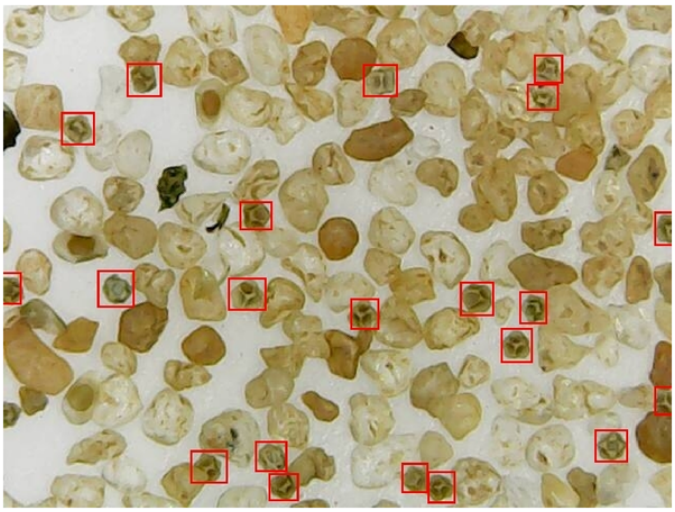

Figure 2.2: Substrate image captured by the XTRAD USB digital microscope model XT-2036 at a 52x magnification. Each red rectangular bounding box displays a cyst.

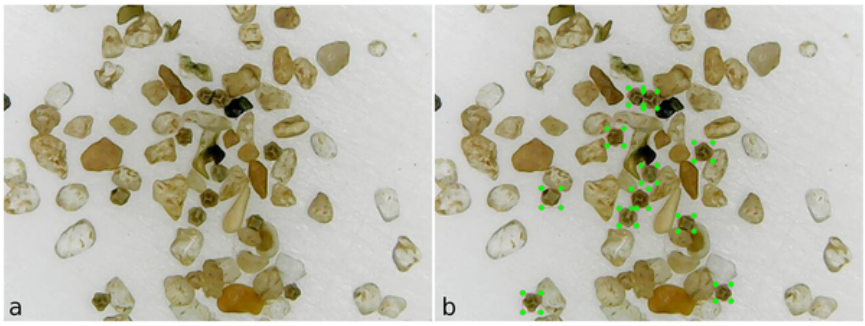

Figure 2.3: The labeling process using the LabelImg software: a) Sample of substrate image captured by the XTRAD USB digital microscope model XT-2036 at a 52x magnification; b) Using the LabelImg software to label samples of cysts. The green rectangles in the image are the labeled cysts.

to it into small portions on a white cover slip and weighed it using a precision scale (see Figure 2.4 (a)). Then, we captured an image of each portion of the substrate using the digital microscope. The captured images were stored in a folder, whose name (label) indicates the amount of cyst and the substrate weight contained in the image group (see Figure 2.4 (b)). Besides, the file name of each image in the folder indicates the image number and amount of cyst contained in it (see Figure 2.4 (c)). The complete testing set has 1968 cysts in 135 images distributed in 10 folders (10 small image groups). The substrate weight used to build the testing set is 4.24 grams. Table 2.1 shows the testing set in detail.

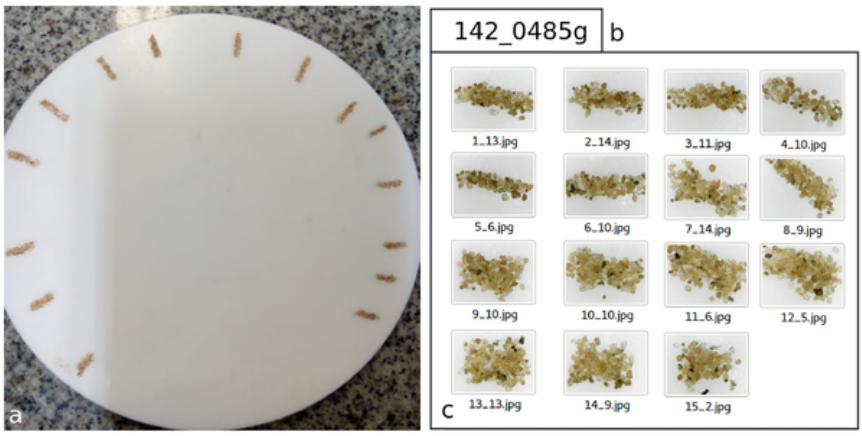

Figure 2.4: The labeling process using the LabelImg software: a) Sample of substrate image captured by the XTRAD USB digital microscope model XT-2036 at a 52x magnification; b) Using the LabelImg software to label samples of cysts. The green rectangles in the image are the labeled cysts.

Table 2.1: The testing set: 10 folders, 135 images, 4.24 grams of substrate, and 1968 cysts.

Folder (label)	Weight (grams)	Number of images	Number of cysts
70_0407g	0.407	14	70
142_0485g	0.485	15	142
149_0420g	0.420	13	149
165_0559g	0.559	16	165
196_0459g	0.459	14	196
213_0223g	0.223	11	213
219_0333g	0.333	14	219
239_0479g	0.479	12	239
256_0419g	0.419	12	256
319_0456g	0.456	14	319

2.2.2 An overview of Faster R-CNN and YOLOv3 architectures

YOLOv3 (Redmon et al., 2016) and Faster R-CNN (Ren et al., 2017) are state-of-the-art object detection architecture and are employed to solve many problems whose aim is to detect and classify objects (Jiao et al., 2019). In this section, we provide an overview of both the architectures.

YOLOv3 Architecture

The YOLOv3 workflow is basically composed of three steps (Redmon et al., 2016). First, it receives an input image and then divides it into a grid. Next, it applies the image classification and localization processes on each grid cell in order to predict class probabilities for objects and their corresponding bounding boxes. For both classification and localization processes, the YOLOv3

uses an open-source CNN (Convolutional Neural Network) called Darknet-53 as backbone, whose 53 first layers are for classification and another 53 additional layers are for detection, resulting in a CNN with a total of 106 layers.

The object detection is done at three different scales in 82nd, 94th, and 106th layers, whose inputs are downsampled by a factor of 32, 16, and 8, respectively. The 82nd layer is responsible for detecting large objects, the 94th layer for medium objects, and the 106th layer for smaller objects. The detection at different layers provides detection of small objects since the upsampled layers are concatenated with the previous layers in order to preserve the object's fine-grained features. During the detection multiple bounding boxes for each object in a grid cell can be predicted. To define the right bounding box for the object, the IoU (Intersection over Union) is calculated between bounding boxes in the grid cell and is selected one with the highest IoU. For those bounding boxes selected, the network calculates conditional class probabilities. Finally, conditional class probabilities and box confidence predictions jointly provide class-specific confidence scores for each bounding box (Redmon et al., 2016).

The Darknet-53 architecture, used by YOLOv3 as a backbone, is mainly composed of successive 3×3 and 1×1 convolutional layers. Each convolution layer is followed by a Batch Normalization layer (Ioffe and Szegedy, 2015) and Dropout operations (Srivastava et al., 2014). At the end of each convolutional block, residual blocks are added in order to perform the identity mapping, whose purpose is to add the output from the previous convolutional layer x to output $F(x)$ of the layer ahead. This allows x and $F(x)$ to be combined as input to the next convolutional layer (He et al., 2016). The final block consists of a Global Average Pooling (Lin et al., 2014a) followed by a fully connected layer and a final layer Softmax (Zoph et al., 2018).

Figure 2.5 shows the general workflow of YOLOv3 applied to cyst detection and counting. After the YOLOv3 was trained using the annotated images of the DBrasiliensis dataset designated for training, a test image captured by the digital microscope is inputted into the model to detect the cysts. Next, the image is divided into several grid cells. For each cell there are predicted several anchor boxes and confidence scores. Then, the boxes with the highest score are selected so that the network calculates conditional class probabilities for each one. For the last step, the conditional class probabilities and box confidence predictions jointly provide cyst class confidence scores for each box, drawing a bounding box around each cyst in the image. We design a post-processing step that counts the cysts detected in the image.

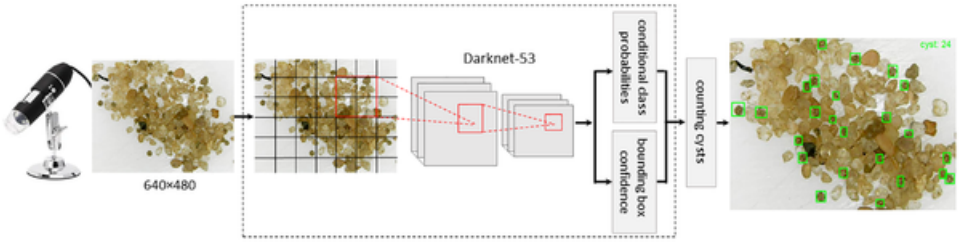

Figure 2.5: Overview of the automatized approach for *Dendrocephalus Brasiliensis* cysts detection and counting using YOLOv3.

Faster R-CNN Architecture

The Faster R-CNN is composed of two modules (Ren et al., 2017): RPN (Region Proposal Network) and Fast R-CNN detector. The RPN receives as input an image that is processed by a CNN in order to obtain features and produce a set of rectangular region proposals with three scales (128×128, 256×256 and 512×512) and three aspect ratios (1:1, 2:1 and 1:2) that possibly have the candidate objects. The Fast R-CNN detector receives input RoIs (Region of Interest) produced from the region proposals generated by RPN. Each RoI is processed by a pooling layer and pooled into a fixed-size feature map that is mapped to a feature vector. This feature vector will be the input for a fully connected layer to classify the RoI. The output is composed of two vectors per RoI: the probabilities and bounding-box for each object class considered. Both RPN and Fast R-CNN detector modules share a common set of convolutional layers which can be provided by a CNN backbone like VGG16 (Simonyan and Zisserman, 2015), ResNet-50 (He et al., 2016), or Inception-v2 (Szegedy et al., 2016). In this paper, we choose the Inception-v2 architecture to act as a backbone for Fast R-CNN.

Inception-v2 architecture (Szegedy et al., 2016) has three initial convolutional layers with 3×3 filters followed by max-pooling. The output of this block is the input for another block with three convolutional layers with 3×3 filters. Next, the architecture has three inception modules in sequence. In the first module, it is performed convolution on an input using filters 1×1 and 3×3, as well as max-pooling. The resulting outputs are concatenated and moved to the next inception module that applies a grid reduction technique to reduce the number of parameters in order to become the model computationally cheaper. The grid reduction consists of 1×n and n×1 convolutions instead of n×n convolutions. Like in the first inception module, the outputs are concatenated and moved to the next inception module. The last inception module is similar to the second, however, it is wider instead of deeper. Finally, before the final layer Softmax, an extra classifier act as a regularizer (Szegedy et al., 2016).

Figure 2.6 shows the general workflow of Faster R-CNN applied to cyst detection and count-

ing. After the training, a test image captured by the digital microscope is inputted into the model to detect the cysts. The image passes through convolutional layers to obtain feature maps, which are inputted into RPN to generate rectangular region proposals. The region proposals are transformed into RoIs and inserted into the Fast R-CNN process that provides cyst class probability and bounding box prediction for each one. Finally, a post-processing step counts the cysts detected in the image.

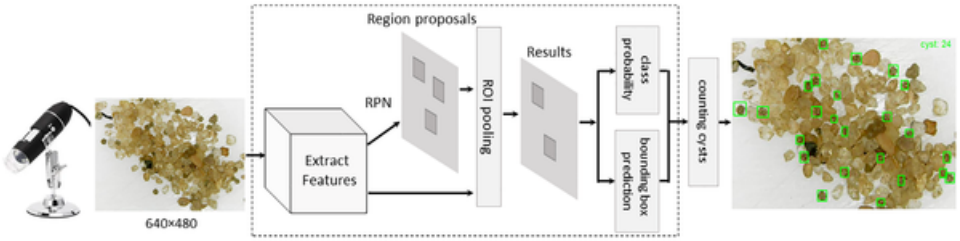

Figure 2.6: Overview of the automatized approach for *Dendrocephalus Brasiliensis* cysts detection and counting using Faster R-CNN architecture.

2.2.3 Experimental setup

Both YOLOv3 and Faster R-CNN architectures were set to use the fine-tuning strategy with all layers initialized with weights from previous training on the MS-COCO (Microsoft Common Objects in COntext) dataset (Lin et al., 2014b). Besides, we set the learning rate at 0.001, the number of iterations at 8,000, and varied the batch size at 2, 4, 8, 16, 32, and 64. We used a small batch size to consume less memory and train the architectures faster since the small batch size allows us to update the network weights more often (Masters and Luschi, 2018). We limited the number of iterations to 8,000, as from that number, the loss rate did not present improvement. We set the learning rate at 0.001 because this value is recommended by (Kingma and Ba, 2015) when used a small number of samples on training. During training, all images in the batch were augmented using random rotation by +30/-30° and exposure between -10% and +10%. Both architectures were trained using a Tesla P100-PCIE-16GB GPU.

We used the DBrasiliensis dataset to train and test both architectures. Thus, 111 images containing 3173 annotated cysts were used in training, i.e., 61.72% of the cysts, and in the test were used 135 images with 1968 cysts, 38.28% of the cysts, arranged into 10 different subsets as presented in Table 2.1. Each built model has been tested ten times using only one test subset at a time. The metric result is an average from the sum of the scores achieved on each one test subset.

We considered a correct detection (true positive) when the predicted cysts have a detection

score of ≥ 0.3, and a wrong detection (false positive) when the detected object isn't a cyst. A false negative is assigned when a cyst is in the image and it isn't detected. The evaluation metrics used were Precision, F1-Score, Accuracy, and Recall. In all formulas below, TP refers to true positives, TN to true negatives, FN to false negatives, and FP to false positives.

$$\text{Accuracy} = \frac{\text{TP} + \text{TN}}{\text{TP} + \text{FP} + \text{FN} + \text{TN}} \tag{2.1}$$

$$\text{Precision} = \frac{\text{TP}}{\text{TP} + \text{FP}} \tag{2.2}$$

$$\text{Recall} = \frac{\text{TP}}{\text{TP} + \text{FN}} \tag{2.3}$$

$$\text{F1-Score} = \frac{2 * (\text{Recall} * \text{Precision})}{(\text{Recall} + \text{Precision})} \tag{2.4}$$

Besides, we evaluated both architectures in terms of MAE (Mean Absolute Error), RMSE (Root Mean Square Error), and R^2. Finally, we applied statistical methods on the Accuracy metric to evaluate the differences among the architectures.

We performed a statistical analysis using the Shapiro-Wilk test (Shapiro and Wilk, 1965) to verify the normality of the data, the one-way Anova hypothesis test, and the Tukey's test (Tukey, 1949) to analyze the difference between the architectures in a pairwise way. We adopted a significance level of 5% for all statistical tests (p-value $< .05$).

2.3 Results and Discussion

The classification results for Precision, F1-Score, Accuracy, and Recall for both architectures are presented in Table 2.2. Table 2.2 shows that the YOLOv3 architecture exhibits higher and more uniform precisions than the Faster R-CNN, indicating that the proportion of true positives concerning the total of predicted positives achieved by it didn't present large distortions. It is important to emphasize that the YOLOv3 achieved the best results with batch size set at 32, except the precision, whose best index was achieved with the batch size set at 4.

Table 2.2: Cyst detection results at average percentage through 10 test subsets on the DBrasiliensis dataset for YOLOv3 and Faster R-CNN. Bold font indicates the best results obtained by each architecture.

Architecture	batch size	Precision	Recall	F1-Score	Accuracy
YOLOv3	2	97.63 ±2.86	77.28 ±5.18	86.11 ±3.06	75.76 ±4.80
	4	**99.54** ±0.76	67.91 ±4.68	80.65 ±3.28	67.69 ±4.56
	8	99.19 ±0.49	68.59 ±7.29	80.89 ±5.08	68.20 ±7.20
	16	99.34 ±0.91	70.39 ±2.55	82.38 ±1.63	70.06 ±2.32
	32	98.24 ±1.31	**85.05** ±5.01	**91.05** ±2.81	**83.73** ±4.76
	64	99.07 ±1.12	84.07 ±3.88	90.89 ±2.23	83.40 ±3.78
Faster R-CNN	2	79.72 ±4.20	**59.66** ±8.24	**67.86** ±5.62	**51.65** ±6.33
	4	**94.44** ±3.64	40.72 ±4.84	56.77 ±5.11	39.82 ±4.95
	8	87.25 ±3.36	47.07 ±5.63	60.93 ±4.86	43.99 ±5.00
	16	88.22 ±5.40	37.86 ±5.47	52.81 ±5.83	36.10 ±5.43
	32	93.88 ±2.03	35.89 ±4.88	51.73 ±5.08	35.05 ±4.65
	64	92.95 ±1.65	35.02 ±4.47	50.70 ±4.67	34.09 ±4.22

The Faster R-CNN achieved 94.44% of precision with batch size set at 4, showing that the observed true positives really were cysts. However, it presented a high false negatives rate that can be observed at the recall of 40.72%. One example of this high false negative rate can be seen in Figure 2.7 (a). From the 20 cysts in the image, the Faster R-CNN detected only 1. On the other hand, the YOLOv3 with batch size also set at 4, in which it achieved the best precision and recall of 67.91%, detected 10 of 20 cysts in the same image (see Figure 2.7 (b)).

We can observe in Table 2.2 that both YOLOv3 and Faster R-CNN with batch size set at 4 achieved better precision. Nevertheless, for other metrics, the YOLOv3 and Faster R-CNN achieved better results with batch size set at 32 and 2, respectively. This high precision with batch size at 4 relates to the low false positive rates achieved by the architectures. YOLOv3 with batch size set at 4 got better precision because it had false positive rates lower than when batch size is 32. Notice in Figure 2.8 that YOLOv3 with batch size set at 4 got only 7 false positives. On the other hand, when the batch size is 32, the number of false positives is 29. The same is true of the Faster R-CNN with batch sizes set at 2 and 4, whose number of false positives were 290 and 42, respectively (see Figure 2.8). However, the high precision of the architectures with batch size at 4 did not translate into detecting more cysts (see Table 2.3).

Concerning accuracy, the Faster R-CNN achieved the lowest results at all batch sizes compared to YOLOv3. The best accuracy rate achieved by YOLOv3, 83.73%, is relevant due to the difficulty of detecting cysts in the substrate because, in many instances, only parts of the cyst are visible, or the cysts are glued together, and there is also a considerable quantity of sand

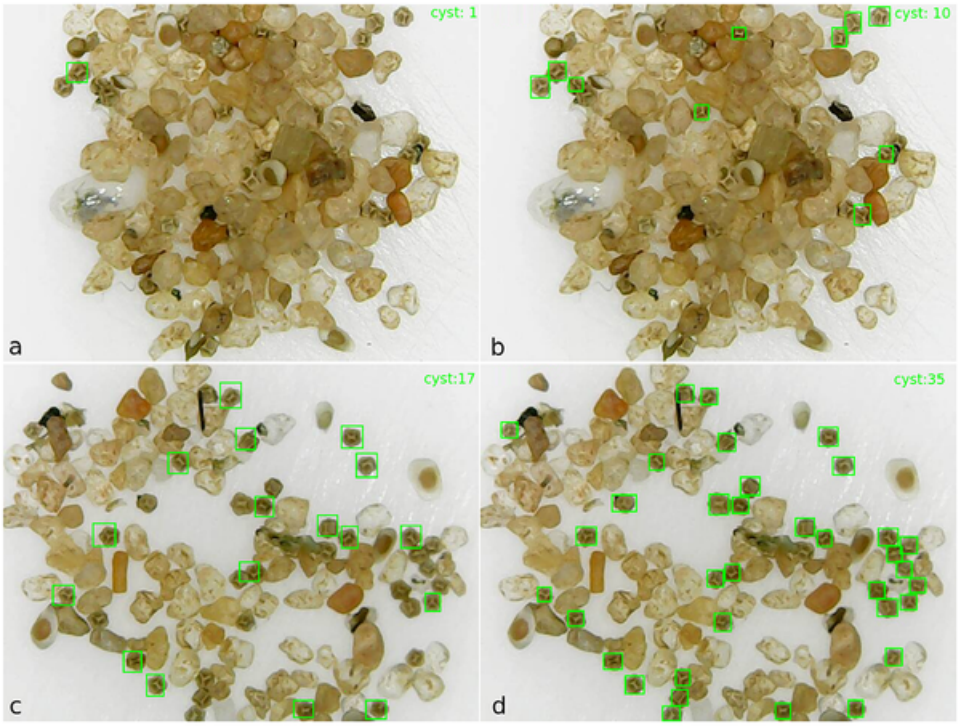

Figure 2.7: Example of detecting and counting cysts of the Faste R-CNN and YOLOv3 architectures: a) The Faster R-CNN detected and counted only 1 of 20 cysts in the image; b) The YOLOv3 detected and counted 10 of 20 cysts in the image; c) The Faster R-CNN detected and counted 17 of 36 cysts in the image; d) The YOLOv3 detected and counted 35 of 36 cysts in the image.

and other residues. Figure 2.7 (c) and (d) show examples of detection of the Faster R-CNN and YOLOv3, respectively, using the same image and batch size set at 2 for Faster R-CNN and 32 for YOLOv3. Although there are no false positives in both images, the Faster R-CNN presented 19 false negatives, by detecting 17 of 36 cysts. On the other hand, the YOLOv3 presented only 1 false negative, i.e., it detected 35 of 36 cysts in image. This difference in accuracy between the architectures was repeated in most of the images of the DBrasiliensis dataset reserved for testing.

We counted the number of false positive detections in different batch sizes (see Figure 2.8). This count confirms the precision achieved by each architecture configurations shown in Table 2.2, i.e., the precision decrease as the number of false positive increases.

We analyzed the false positives and observed that most of them have similar colors to cyst colors and, in some cases, they have parts similar to cyst shapes. Fig 2.9 (a) and (b) show an image in which both architectures, Faster R-CNN and YOLOv3, with batch size set at 2, achieved a high false positives rate. The red rectangular bounding boxes show the false positives, the greens the true positives, and the blues the false negatives.

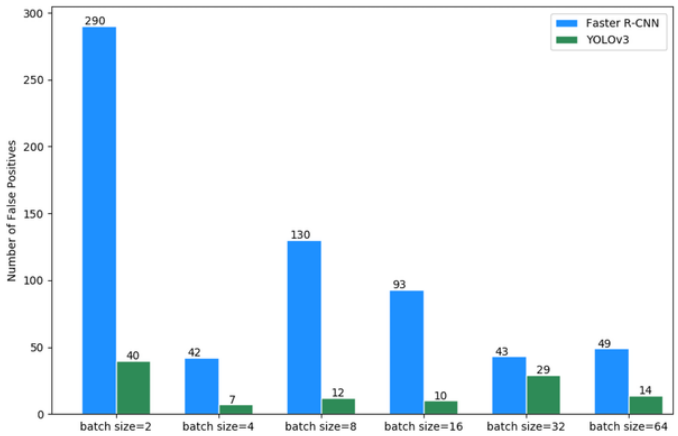

Figure 2.8: Comparison of the number of false positives of the Faster R-CNN and YOLOv3 architectures in different batch sizes.

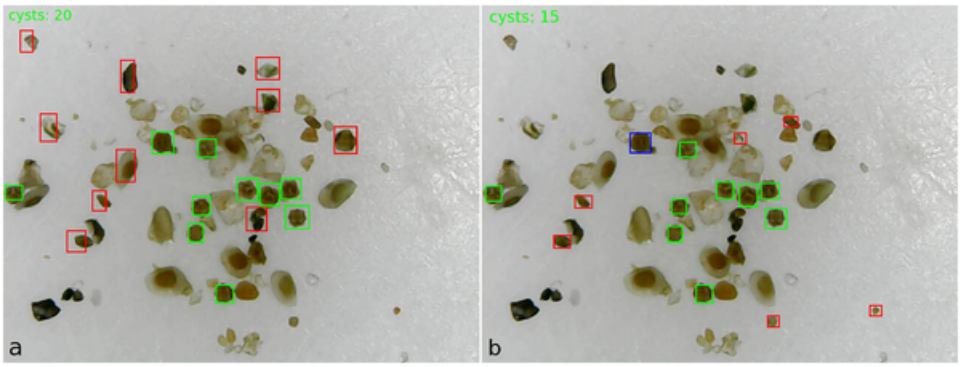

Figure 2.9: Example of detecting and counting cysts of the Faster R-CNN (a) and YOLOv3 (b) architectures, both with batch size set at 2. Green boxes are true positives with score detection ≥ 0.3, red boxes are false positives, and blue boxes are false negatives.

Table 2.3 shows the detection and counting results for all batch sizes on the 10 testing subsets. It can be noted that YOLOv3 outperforms the Faster R-CNN on all testing subsets and has a higher hit rate with batch size set at 32, detecting and counting 1666 of 1968 cysts, a hit percentage of 84.66%. The Faster R-CNN achieved the higher hit percentage with batch size set at 2, detecting and counting 1127 of 1968 cysts, 57.27%.

We carry out an analysis of variance with Anova on a .05 level of significance using the accuracy as metric to determine if there is a difference between the different batch sizes in each architecture, as well as if there is some difference between the average accuracy of both architectures. We adopted the Anova test because, in general, the average accuracy presented normality/homogeneity of variance after we performed the test of normality using the Shapiro-Wilk.

The test between the different batch sizes for Faster R-CNN resulted in a p-value of 0.0001,

Table 2.3: Detection and counting results for all batch sizes on the 10 testing subsets of the DBrasiliensis dataset for YOLOv3 and Faster R-CNN. Bold font indicates the best results obtained by each architecture.

	Yolov3						**Faster-RCNN**					
	Hits in each test set (folder) per batch size						Hits in each test set (folder) per batch size					
cyst in set	2	4	8	16	32	64	2	4	8	16	32	64
70	50	49	45	49	60	59	49	27	32	22	27	26
142	117	98	107	102	123	123	90	59	71	55	53	52
149	114	99	107	104	123	117	107	71	83	67	60	58
165	119	114	103	115	134	135	102	74	74	67	64	62
196	156	127	132	140	174	167	130	91	106	94	88	85
213	169	146	147	146	188	182	121	71	110	76	74	73
219	180	163	163	164	194	190	128	93	99	79	75	73
239	206	180	197	174	224	220	129	102	112	96	78	77
256	181	153	143	166	196	204	118	91	96	81	71	70
319	231	197	201	224	250	256	153	109	125	100	95	94
Total:1968	1523	1326	1345	1384	**1666**	1653	**1127**	788	908	737	685	670

which indicates a statistically significant difference between the different batch sizes average accuracy. The Tukey test showed that the batch size defined in 2 differs from the others.

The test between the different batch sizes for YOLOv3 also resulted in a p-value of 0.0001, indicating a significant difference between the batch sizes average accuracy, except the batch sizes set at 32 and 64 which according to Tukey test didn't present a statistically significant difference between them.

The comparison between YOLOv3 and Faster R-CNN using the accuracy as metric resulted in a p-value $< .05$, indicating a statistically significant difference between the models.

Table 2.4 shows that YOLOv3 reached R^2 of 0.88 for batch sizes 32 and 64. On the other hand, Faster R-CNN achieved the best R^2 using batch size 2 (0.20). These results indicate that YOLOv3 outperforms Faster R-CNN in detecting and counting the cysts since the R^2 metric is a performance indicator, and the higher the result the better the agreement between the resulting count of the architectures and the number of cysts in the DBrasiliensis Dataset.

In terms of RSME and MAE, Table 2.4 shows that YOLOv3 using batch size 32 achieved 3.49 and 2.24, respectively. This result indicates that YOLOv3 has the lowest average standard deviation using batch size 32 between the number of cysts detected and counted and the number of cysts in the DBrasiliensis dataset. From this result, we can tell that YOLOv3 with batch sizes set at 32, and the learning rate at 0.001 is the approach best suited to detect and count cysts, since that configuration achieved the best results for Accuracy, Precision, R^2, RSME e MAE.

We also carry out an analysis of variance with Anova on a .05 level of significance using the accuracy as a metric to determine if the YOLOv3 with batch size set at 32 maintains the

Table 2.4: RSME, MAE, and R^2 through 10 test subsets on the DBrasiliensis dataset for YOLOv3 and Faster R-CNN. Bold font indicates the best results obtained by each architecture.

Architecture	batch size	RMSE	MAE	R^2
YOLOv3	2	4.91	3.30	0.77
	4	6.49	4.76	0.59
	8	6.38	4.61	0.61
	16	6.07	4.33	0.64
	32	**3.49**	**2.24**	**0.88**
	64	3.57	2.33	**0.88**
Faster R-CNN	2	**9.13**	**6.23**	**0.20**
	4	11.64	8.74	-0.31
	8	10.58	7.85	-0.08
	16	12.03	9.12	-0.40
	32	12.39	9.50	-0.48
	64	12.50	9.61	-0.51

average accuracy between the different testing subsets (see Table 2.1). The test resulted in a p-value of 0.1008, therefore, we have no evidence that there is a statistically significant difference in YOLOv3 accuracy on the different testing subsets of the DBrasiliensis dataset.

In that way, taking into account that the testing set of the DBrasiliensis dataset consists of 10 subsets, each of which is associated with the substrate weight used to capture your images (see Section 2.2.1), we can infer the number of cysts for a new portion of substrate with a known weight obtained from the same aquarium where we took the substrate to build the DBrasiliensis dataset. Thus, a producer or researcher associating weights and counts can use the same technique to infer his production.

The inference of the number of cysts from the substrate with a known weight, for both research and cultivation, is a necessary practice because the manual counting of thousands of cysts is not feasible. Thus, we can use YOLOv3, with batch size set at 32, to infer the total number of cysts contained in a substrate, counting a certain number of cysts collected through sampling, with the samples vary according to the need for more/less accuracy of the data. Adopting the inference, we will be able to count cysts with 83.73% of accuracy (Table 2.2). It is up to the producer or scientist to analyze the number of samples (set of images associated with a weight) that best suits their needs.

Although the proposed approach can present disadvantages, such as the work required for annotation of thousands of cysts and the computational cost for training the model, the benefit obtained by it, concerning the accuracy and the counting time, is a factor that supports the adoption of automated cyst counting. For instance, the YOLOv3 takes around 1 minute and 29 seconds to count the cysts of 135 images with 83.73% of accuracy and 98.24% of precision.

We believe that the results obtained by YOLOv3 with batch size set at 32 are enough to build

an automatic detection and counting system of cysts since the visual counting of cysts performed by humans using microscopes is a hard task, prone to errors, and that also very time-consuming. Besides, it is possible to optimize the process of gauging the number of cysts present in a given medium or substrate, inferring the number of cysts without the need for cleaning, drying, and manual counting.

2.4 Conclusion

Due to the potential of *Dendrocephalus brasiliensis* species in the conservational efforts and productive activities, we presented a new technology aimed to improve and facilitate the cysts measurement process. We built a novel annotated images dataset of *Dendrocephalus brasiliensis* cysts called DBrasiliensis and used the YOLOv3 and Faster R-CNN to provide a baseline for detecting and counting cysts. To promote research in the automation of cyst measurements, we also report evidence that the performance of YOLOv3 is superior against Faster R-CNN. Besides, we provided the possibility of inferring the total number of cysts, with an accuracy around 83.73%, from a substrate image set associated with a known weight. The DBrasiliensis dataset can be accessed at: https://doi.org/10.6084/m9.figshare.13073240.

Chapter 3

The key factors for performing hatching tests with *Dendrocephalus brasiliensis* (Crustacea:Anostraca) species

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Abstract: Aquatic invasive species (AIS) are one of the leading causes of the global freshwater biodiversity crisis, and once an AIS is established it is infeasible to be eradicated, with mitigation of its spread as the only solution. Considering the severity of the problems it is crucial to develop technologies that allow the use of native organisms to the detriment of exotic species. Some native species have important characteristics that make their use possible in several areas. *Dendrocephalus brasiliensis* (Crustacea: Anostraca) is an example since it presents characteristics that make it economically and scientifically interesting. However, the species present a low hatching rate, and to overcome such a problem, new studies testing different substances and test conditions (such as experiments with a known amount of cyst,) are necessary. We tested Dimethylsulfoxide (DMSO) and Glycerol because those substances were previously tested in cyst hatchability studies from another Anostraca, and use/not of pH control (MOPS pH ranges of 6.5 and 7.8), and use of saline buffers (Sodium Phosphate and Borate buffer for pH range 7.9 and

8.7, respectively) as well as other mediums commonly used as Natural Water (NW), Reconstituted Water (RW), and different dormancy break methodologies (Cyst Pre-Treatment-CPT) such as a Warm Water Bath (WB) at 37°C for 1 hour, Aired Motion in distilled water cysts for 24 hours, and Sulfuric Acid cyst bath for 5 seconds. The results showed that CPT (precisely, No Pre-Treatment-NPT treatments), followed by pH Control are the variables with more influence over the hatching, and the pre-treated cysts showed the second best results for homogeneity, though not good results for hatching. The pH control didn't affect the rate but promoted better hatch homogeneity. The Glycerol treatments showed, in general, better hatching results than DMSO, but as the CPT variable includes Natural and Reconstituted Water, between the treatments presenting higher hatching rates, then the use of DMSO or Glycerol for *Dendrocephalus brasiliensis* became unjustified. The use of Cyst Sulfuric Acid (H_2SO_4) bath, and saline buffers, resulted in the complete absence of nauplii hatching. Therefore, we recommend the use of Natural or Reconstituted Water as a medium, buffered (only to help with the hatching homogeneity) in the range of 7.3 to 8, and cysts without previous dormancy break attempt (pre-treatment with WB, Aired or Sulfuric Acid). Also, since our results differ from other studies stating the hatching rate of *Dendrocephalus brasiliensis* as 7%, but indicate a variation (in controlled cultures and laboratory environments, with AN and RA) from 9 to >30%. Due to this natural variation, we also recommend that hatching tests be carried out with known amounts of cysts, in order to guarantee results consistent with reality, and that hatching tests for *Dendrocephalus brasiliensis* be performed, at least, in triplicate.

3.1 Introduction

The numbers and distributions of invasive species are increasing in many parts of the world (Seebens et al., 2017). Anthropogenic actions are causing environmental changes and, in the coming decades, will cause biodiversity loss (Sala et al., 2000). Biological invasion is an important threat to biodiversity because alien species alter the composition and functioning of the ecosystems (Vitousek et al., 1997). Aquatic invasive species (AIS) are one of the leading causes of the global freshwater biodiversity crisis (Reid et al., 2019). The situation is worse for lakes and streams that are prone to species loss (Ricciardi and Rasmussen, 1999), caused by primarily land-use changes and exotic invasive species (Sala et al., 2000).

Climate change adds negatively to the situation, introducing new challenges for managing the problem and causing the species' range to shift due to warmer temperatures (Walther et al., 2009). So much so, that according to Dukes and Mooney (1999), climate change is expected to

enable the expansion of some species into regions where they previously could not survive and reproduce. Another aspect of this situation is that once an AIS is established it is infeasible to be eradicated, and the solution is to mitigate it by limiting its spread (Zanden and Olden, 2008). Considering the severity of the problems, it is crucial to develop technologies that allow the use of native organisms to the detriment of exotic species.

Some native species have important characteristics that make their use possible in several areas. Therefore, it is important to develop new methodologies to increase the range of their use. *Dendrocephalus brasiliensis* (Crustacean: Anostracan) is an example of such species since its present characteristics such as high proteic value (da Cruz Daltro et al., 2021), making it economically interesting, and high sensibility to toxic pollutants (Santos et al., 2018), allow its use in ecotoxicological studies, making it scientifically important. However, *Dendrocephalus brasiliensis* presents, according to Lopes et al. (2007), a low hatching rate; and such a characteristic could cause an impediment to its commercial or scientific usability. To overcome such a predicament, new studies testing the use of different substances and conditions are necessary.

Previous knowledge about the characteristics and/or possible effects can be a differential when choosing a substance to be tested. The substances Dimethylsulfoxide (DMSO) and Glycerol, were previously tested in cyst hatchability studies by Murugan and Dumont (1995), for the species *Thamnocephalus platyurus* Packard, and presented positive results such as the increase in the hatchability and absence of toxicity. Therefore, the choice of testing its effects on the hatchability of the Anostraca *Dendrocephalus brasiliensis*, to better evaluate the substances is also important to compare the hatching performance of those substances with different pH ranges, as also with other mediums commonly used as Natural Water (NW) and Reconstituted Water (RW).

A situation observed in several studies (Lopes et al., 2007; Pereira and dos Santos-Neto, 2010; de Vasconcellos, 2010; Santos et al., 2019) with *Dendrocephalus brasiliensis* was that the adopted studies' methodology consisted of using an unknown amount of cysts. In general, the experiments adopted a certain weight (in grams) that was supposed to contain an expected number of cysts, but cysts can vary in size, presence/absence of dirt, presence of already hatched cysts, fibers, etc., and with those variables being ignored, it's acceptable to question the accuracy of the hatching rate currently established as normal for the species. Thus, testing and implementing a different approach for hatching studies that consider a known amount of cysts is important as a form to develop a protocol for the species hatching tests.

Therefore, this study aimed to provide a new approach to the methodology in hatching studies. Also, to increase the hatching rate by studying the responses to Dimethylsulfoxide and Glycerol, in situations with and without pH control. Also, to test the use of cyst pre-treatment techniques

(such as Water bath, sulfuric acid exposure, and aeration) aiming to facilitate the imbibe and, consequently, the embryo metabolism start and posterior hatching. To analyze the responses in hatchability of DMSO and Glycerol when compared to different mediums (Natural, Reconstituted, and Distilled water). The results of these studies are expected to provide information that can be applied to improve the accuracy in hatching studies for *Dendrocephalus brasiliensis* species.

3.2 Methodology

The study was conducted in the laboratory of Limnology (LABLIM) of the Federal University of Mato Grosso do Sul (UFMS).

3.2.1 Dendrocephalus brasiliensis Cyst

Dendrocephalus brasiliensis cysts were acquired from different sources such as aquaculture tanks located in the northeast region of the country, aquarists located in the Midwest and produced in our facilities. The cysts were mixed together aiming to guarantee the genetic viability of the experiment. The cyst production obeyed some criteria as the medium used, photoperiod, maintenance e feeding of the matrices.

Dendrocephalus brasiliensis matrices maintenance

The medium used to produce cysts was Reconstituted Water (RW) and the maintenance was done, according to Santos et al. (2018), by renewing the medium 3 times per week, the cultures used photoperiod of 16/8 (light/dark), the matrices were fed using the algae *Raphidocelis subcapitata*, also cultivated at the laboratory facilities, at a ratio of 10^5 cells per Liter/individual/day.

Cultives of Raphidocelis subcapitata

The culture of *Raphidocelis subcapitata* algae was kept in a growing medium CHU12 (Müller, 1972) and, to obtain the CHU12, it was necessary to prepare a stock solution made as follows:

In 1 liter of distilled water add, in numerical order of 1 to 6, the chemical substances:

1. $Ca(NO_3)_2$ - 4.3 g;
2. K_2HPO_4 - 0.5 g;
3. $MgSO_4.7H_2O$ - 7.5 g;

4. KCl - 0.5 g;

5. Na_2CO_3 - 2.0 g;

6. $FeCl_3.6H_2O$ - 0.05 g.

The stock solution must be kept in an amber glass bottle, refrigerated at a temperature of 4°C, and it is best if used for a period of, maximum, 6 months (Müller, 1972). The algae culture was renewed weekly using a volume of 0.3 L of the stock solution diluted in 1.470 L of distilled water. This solution was kept in an Erlenmeyer, closed with a lid made of hydrophobic cotton and gauze, and autoclaved at 121°C with an atmospheric pressure of 1 atm for 20 minutes.

After the solution cooled down to room temperature the medium was inoculated with an aliquot of 15 ml of *Raphidocelis subcapitata* algae exponentially growing (10^7 to 10^8 cells per ml^{-1}). The algae cultures were kept with constant aeration, at a temperature of 25±2°C, with a photoperiod of 12:12 light/dark and an intensity of luminosity of 1500 lux. Before feeding *Dendrocephalus brasiliensis* matrices aliquots of the algae culture were collected and the number of *Raphidocelis subcapitata* cells was counted using a Neubauer chamber.

3.2.2 Cyst Treatment

Aired Cysts

The cysts were put inside a glass beaker with two liters of distilled water. To aerate and motion the medium, we used an air compressor of 5w potency from the Master® brand, the compressor has two air exits that were coupled with two silicone hoses. To close the beaker without stopping the airflow through the hoses we used cotton balls covered with PVC stretch film. The cysts were kept in motion for 24 hours. After that, we removed the hatched nauplii and, the remaining cysts were dried naturally (sun-exposed) for 7 days.

Water Bath

For that cyst treatment method, we covered the cysts with the respective solutions (pre-heated to 37°C), for 60 minutes. To keep the temperature steady, we accommodated the beakers in a large container containing warm water and a thermostat with a heater. After that period, we completed the beakers with the specific mediums and proceed with the experiment.

Sulfuric Acid

That treatment consisted of covering the cysts with H_2SO_4 for 5 seconds. After that the cysts were abundantly washed with distilled water to eliminate any H_2SO_4 residues and, posteriorly, naturally dehydrated.

3.2.3 Solutions

Reconstituted Water (RW)

The reconstituted water was produced by adding the below substances in distilled water as described by Santos et al. (2018):

- 0.03 g/L of $CaSO_4.2H_2O$;
- 0.61 g/L of $MgSO_4.7H_2O$;
- 0.0048 g/L of $NaHCO_3$;
- 0.01 g KCl.

Natural Water (NW)

Mineral water was acquired and used as a natural water source. That is because after pretests with both spring water collected in the region of Campo Grande (Mato Grosso do Sul state), and commercial mineral water, better results were observed with the commercial mineral water. Since samples for both sources were tested and don't present discrepancies in the results for Dissolved Oxygen, Conductivity, pH, or Hardness, it's possible that the spring water could have some form of contamination that cause the disparity in the hatching results.

Dimethylsulfoxide Solution

There aren't previous studies with the substance Dimethylsulfoxide (DMSO) for the *Dendrocephalus brasiliensis* species. Then, pre-tests were necessary and, to initiate, the concentration that showed the best results in the Murugan and Dumont (1995) studies was adopted as the base. From this initial concentration (0.0375%) others were tested, starting by multiplying from 0.5 times the original concentration, and posterior doubling until four times the original (187.5µl/L, 375 µl/L, 750 µl/L, and 1.5 ml/L), and the best results were shown in the concentration 1.5 ml/L of DMSO (more than 20% of hatching rate). Then, new pre-tests were set up for higher concentrations (3.5 ml, 7.5 ml, and 15 ml), these tests resulted in 0% of hatching. Since the best results

were for the concentration of 1.5ml/L, and amounts over 3.5 ml/L presented no hatching, new pre-tests with the concentrations between 1.5 ml and 3.5 ml were made (with an interval of 0.5 ml). The new set of pre-test (2 to 3.5 ml per liter) resulted in a hatching rate of 14%, inferior to the results previously observed for 15ml/L. Therefore, the concentration of Dimethylsulfoxide adopted in this study was 1.5 ml/L. The solutions were made by dissolving the DMSO in distilled water.

Glycerol solution

For the Glycerol, we used the same principle used for DMSO i.e. with the original concentration that showed better results in the Murugan and Dumont (1995) studies being used as the base for the pre-tests with *Dendrocephalus brasiliensis*. Then, from the initial concentration (0.125%) others were tested, starting by multiplying from 0.5 times the original concentration, and posterior doubling until four times the original (625µl/L, 1.25 ml/L, 2.5 ml/L and 5 ml/L), and the best results were for concentration 1.25 ml/L (approximated to 1.3 ml/L) of Glycerol (more than 20% of hatching rate). The solutions were made by dissolving the Glycerol in distilled water.

3.2.4 pH Control

For the pH control MOPS ($C_7H_{15}NO_4S$) 3-(N-morpholino) propanesulfonic acid was used as a buffer (to pH 6.5 and 7.8), from Sigma-Aldrich, in the respective solutions (DMSO, Glycerol). The mediums under pH control treatment were substituted daily, to guarantee the buffer effect. The mediums' change was carefully made using a Pasteur pipette in order to keep the cysts in the beaker. During those tasks, hatched nauplii could also get counted and removed. Another aspect tested was the effect of saline buffers in the cysts hatching (Sodium Phosphate and Borate buffer).

3.2.5 Physical Chemical Parameters

At the beginning of the tests, the physical-chemical parameters were measured to establish the conditions of the medium. The parameters observed were Dissolved Oxygen (DO), pH, Conductivity, and Hardness. Portable equipment was used to perform those measurements, except for Hardness, which was measured through hardness titration with ethylenediaminetetraacetic acid (EDTA) with Eriochrome Black T as a metal ion indicator. To measure Dissolved Oxygen the equipment used was YR-70 Precision Meter, Conductivity meter AK52 AKSO™, and pH meter AK90 AKSO™.

3.2.6 Experiment Settings

The experiments were composed of 28 groups (Table A1 in A.1); each group was composed of 10 times each treatment, and the treatment was composed of 50 repetitions with 50 cysts, manually separated, per repetition which totalized 1,400 repetitions. The experiment was distributed inside a laboratory incubator, in a completely randomized design, with a temperature of 30±2°C and a photoperiod of 16:8 light/dark.

3.2.7 Statistics

Statistical analysis was conducted in R, using the package party kit and Visreg, to analyze the level of importance of the variables using conditional inference trees. The data also needed to be 'rearranged' to make the analysis possible; the data that were previously arranged in 28 groups were modified to fit 3 columns separated as follows:

- Medium;
- Cyst Pre-Treatment - Aired, Water Bath (sulfuric acid weren't used since resulted in zero hatching);
- pH Control.

Aiming to test if the treatments, composing the variable with the higher hatching rates results, differentiate between themselves was conducted the ANOVA test using the statistical software BioEstat, with the previous normality test of the data was conducted using the Shapiro-Wilk test.

Considering the cyst hatching, was also possible to calculate the following:

- Total Hatched per day (THd)

Σ (x_{n1}, x_{n2}...x_{nx})

'x' being the amount hatched per repetition/per 'n' day, with 'nx' being the final time of the observation.

- Total Hatch (TH)

Σ (THd_1, THd_2...THd_n)

with '$_n$' being the number of days observed.

- Mean Number (MN)

THd/n
with 'n' being the number of repetitions.

- Percentage of Hatching per day (%Hd)

$((MN*100)/t)$
with 't' being the number of cysts used per repetition.

- Relative Frequency (RF)

$((THd*100)/TH)$

- Total.day*d (TDd)

$(D*THd)$
'D' being the time in days (example: day 1, 2... until the day of the last observation).

- Average Final Time (AFT)

$((\Sigma\ TDd\ ) / (\Sigma\ MN))$

- Velocity (V)

$(1/AFT)$

- Accumulated Percentage (AP)

$\Sigma\ (\%Hd)$

3.3 Results and Discussion

The results regarding the physical-chemical tests in the different culture medium used for *Dendrocephalus brasiliensis* hatching tests are presented in the Table 3.1

About the hardness, in cultures of *Dendrocephalus brasiliensis*, Lopes et al. (2008a) describes values between 41 and 63 mg/L of $CaCO_3$, While, in another study, Lopes et al. (2008b), describes the medium as presenting hardness between 7.61 and 16.03 during winter, and 6.73 and 12.02 mg/L of $CaCO_3$ during summer. For another species of freshwater shrimp, the *Macrobrachium*

rosenbergii, the hardness around 13 and 31 mg/L of $CaCO_3$ resulted in survival between 75 and 92%, and concentrations below 53 mg/L resulted in higher growth for the species (Brown et al., 1991). Those values are aligned with the hardness observed in the different culture mediums used in the treatments (minimum of 16.77 and maximum of 43.26 mg/L of $CaCO_3$). Regarding Dissolved Oxygen (DO) the values observed, for all the mediums, surpass 7 mg/L and, according to Sipaúba Tavares (1995), values above 4 mg/L present good conditions for the culture of aquatic organisms.

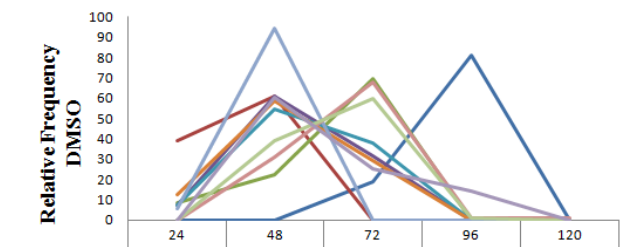
The higher conductivity observed in this study was, as expected, for the medium buffered with sodium phosphate (681 $\mu S/cm$), but Glycerol (156 $\mu S/cm$) with the same buffer presented a conductivity higher than the others, but much lower than Dimethylsulfoxide. Such a result could be due to the characteristics of the substances, as DMSO is the most polar among aprotic solvents, and it's used for solubilization of mineral salts (Verani et al., 1982). Regarding the solubility of NaCl and Na_2SO_4 in glycerol, the reduction of the amount of water in the mixture decreases the solubility of the salt, and the larger solubility values happen at smaller glycerol: water ratios, and such behavior is expected since NaCl solubility is almost four times higher in water than in pure glycerol (Velez et al., 2019). But, we theorize that the effects observed for these two substances, buffered with sodium phosphate, of zero hatching rate were caused by the salinity of the medium. In general, the low conductivity values observed for the majority of the mediums are, probably, the result of distilled water use, and for the natural and reconstituted water, the values are higher since both those mediums present dissolved salts.

The results regarding the relative frequency of the hatching of *Dendrocephalus brasiliensis* in

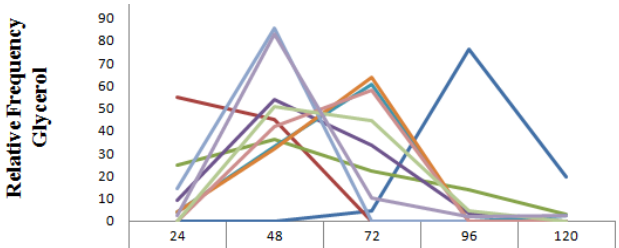
Table 3.1: Results of the physical-chemical tests performed in different culture media (DMSO, Glycerol, Natural Water, Reconstituted Water), with/without buffer (Sodium Sulfate, Sodium Borate, and MOPS) used in the hatching tests for *Dendrocephalus brasiliensis* species.

Treatment/Buffer	pH	Dissolved Oxygen (mg/L)	Conductivity (us/cm)	Hardness (mg $CaCO_3.L^{-}1$)
Reconstituted Water	7.3	7.4	170.6	43.26
Natural Water	7.6	7.9	158	30.39
DMSO (unbuffered)	6.8	13	3	28.82
DMSO (Sodium Phosphate Buffer)	7.9	15	681	28.3
DMSO (Sodium Borate Buffer)	8.7	12	15	27.25
DMSO (MOPS Buffer)	6.5	16	49	16.77
DMSO (MOPS Buffer)	7.8	14.5	20	26.72
Glicerol (unbuffered)	6.9	14.2	0	29.87
Glicerol (Sodium Phosphate Buffer)	7.9	10.1	156	30.39
Glicerol (Sodium Borate Buffer)	8.7	12	42	29.34
Glicerol (MOPS Buffer)	6.5	9.9	42	17.82
Glicerol (MOPS Buffer)	7.8	11.7	30	24.10

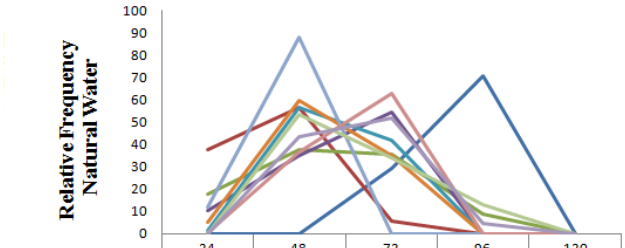
the different culture media and conditions, after the period of assessment of the hatching data, are presented in the Figures 3.1, 3.2, 3.3, and 3.4.



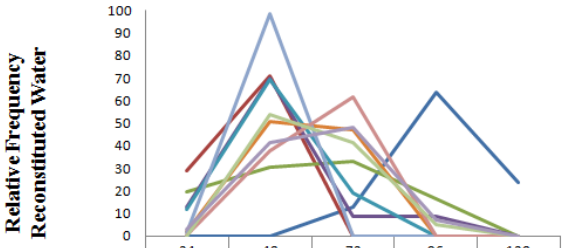
	24	48	72	96	120
DMSO T1	0	0	19.04762	80.95238	0
DMSO T2	39.13043	60.86957	0	0	0
DMSO T3	8.33333	22.22222	69.44444	0	0
DMSO T4	7.317073	60.97561	31.70732	0	0
DMSO T5	7.602339	54.38596	38.0117	0	0
DMSO T6	12.2807	58.77193	28.94737	0	0
DMSO T7	5.714286	94.28571	0	0	0
DMSO T8	0	30.98592	67.60563	0.704225	0.704225
DMSO T9	0	39.32584	59.55056	1.123596	0
DMSO T10	0	60.48387	25	14.51613	0



	24	48	72	96	120
Glycerol T1	0	0	4.347826	76.08696	19.56522
Glycerol T2	54.6875	45.3125	0	0	0
Glycerol T3	25	36.11111	22.22222	13.88889	2.777778
Glycerol T4	8.955224	53.73134	33.58209	3.731343	0
Glycerol T5	3.846154	33.07692	60.76923	0	2.307692
Glycerol T6	4	32	64	0	0
Glycerol T7	14.28571	85.71429	0	0	0
Glycerol T8	0	41.88034	58.11966	0	0
Glycerol T9	0	50.58824	44.70588	4.705882	0
Glycerol T10	2.542373	83.05085	10.16949	1.694915	2.542373



	24	48	72	96	120
Natural Water T1	0	0	29.16667	70.83333	0
Natural Water T2	37.68116	56.52174	5.797101	0	0
Natural Water T3	17.77778	37.77778	35.55556	8.888889	0
Natural Water T4	10.46512	34.88372	54.65116	0	0
Natural Water T5	1.234568	56.79012	41.97531	0	0
Natural Water T6	5.263158	59.64912	35.08772	0	0
Natural Water T7	11.66667	88.33333	0	0	0
Natural Water T8	0.609756	36.58537	62.80488	0	0
Natural Water T9	0	53.19149	34.04255	12.76596	0
Natural Water T10	0	43.42105	51.97368	4.60563	0



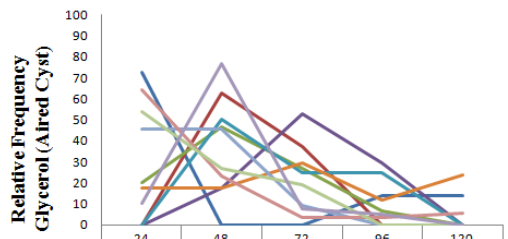
	24	48	72	96	120
Reconstituted Water T1	0	0	12.72727	63.63636	23.63636
Reconstituted Water T2	29.11392	70.88608	0	0	0
Reconstituted Water T3	19.44444	30.55556	33.33333	16.66667	0
Reconstituted Water T4	12.69841	69.84127	8.730159	8.730159	0
Reconstituted Water T5	11.56069	69.36416	19.07514	0	0
Reconstituted Water T6	2.040816	51.02041	46.93878	0	0
Reconstituted Water T7	1.612903	98.3871	0	0	0
Reconstituted Water T8	0.552486	37.56906	61.87845	0	0
Reconstituted Water T9	0	53.78151	41.17647	5.042017	0
Reconstituted Water T10	3.2	41.6	48	7.2	0

Figure 3.1: Relative hatching frequency of organisms of the species *Dendrocephalus brasiliensis*, ten tests each, carried out with Dimethylsulfoxide (DMSO), Glycerol, Natural Water, and Reconstituted Water as culture mediums.

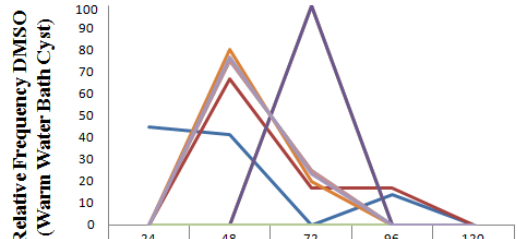
The results of the Inference Tree about all conditions tested for *Dendrocephalus brasiliensis* are presented in Figure 3.5.

The results about the Total Average Time (TAT) and the Velocity of the hatching from *Dendrocephalus brasiliensis* in different culture mediums, after the period of assessment, are presented in the Figure A2 in A.2).

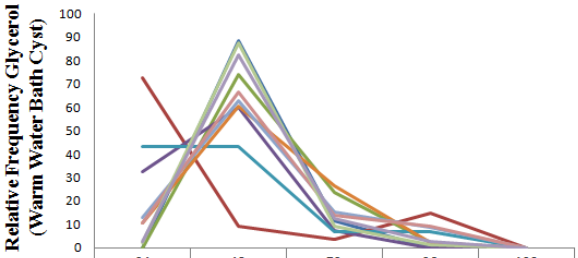
One of the characteristics of the inference tree is that it only presents in its knots the results that are statistically important. The most critical variable is given as Knot one, but the importance of a variable also rests in the number of times it appears in the branches. Therefore,



	24	48	72	96	120
Glycerol (Aired Cyst) T1	72.72727	0	0	13.63636	13.63636
Glycerol (Aired Cyst) T2	0	62.5	37.5	0	0
Glycerol (Aired Cyst) T3	20	46.66667	26.66667	6.666667	0
Glycerol (Aired Cyst) T4	0	17.64706	52.94118	29.41176	0
Glycerol (Aired Cyst) T5	0	50	25	25	0
Glycerol (Aired Cyst) T6	17.64706	17.64706	29.41176	11.76471	23.52941
Glycerol (Aired Cyst) T7	45.45455	45.45455	9.090909	0	0
Glycerol (Aired Cyst) T8	64.28571	23.21429	3.571429	3.571429	5.357143
Glycerol (Aired Cyst) T9	54.05405	27.02703	18.91892	0	0
Glycerol (Aired Cyst) T10	10.25641	76.92308	7.692308	5.128205	0



	24	48	72	96	120
DMSO (Warm Water Bath) T1	44.82759	41.37931	0	13.7931	0
DMSO (Warm Water Bath) T2	0	66.66667	16.66667	16.66667	0
DMSO (Warm Water Bath) T3	0	0	100	0	0
DMSO (Warm Water Bath) T4	0	0	100	0	0
DMSO (Warm Water Bath) T5	0	75	25	0	0
DMSO (Warm Water Bath) T6	0	80	20	0	0
DMSO (Warm Water Bath) T7	0	0	0	0	0
DMSO (Warm Water Bath) T8	0	75	25	0	0
DMSO (Warm Water Bath) T9	0	0	0	0	0
DMSO (Warm Water Bath) T10	0	76.92308	23.07692	0	0

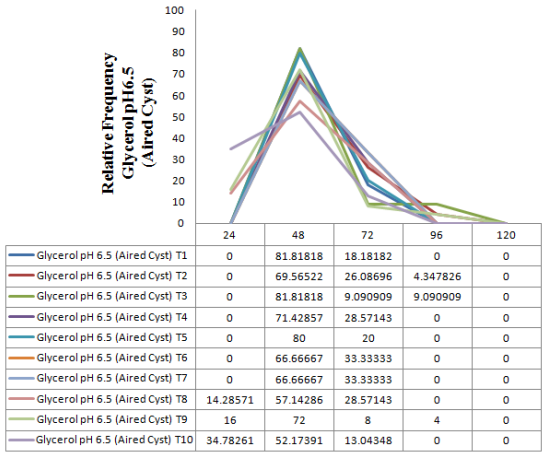


	24	48	72	96	120
Glycerol (Warm Water Bath Cyst) T1	0	88.52459	11.47541	0	0
Glycerol (Warm Water Bath Cyst) T2	72.41379	9.195402	3.448276	14.94253	0
Glycerol (Warm Water Bath Cyst) T3	0	74.1573	23.59551	2.247191	0
Glycerol (Warm Water Bath Cyst) T4	32.65306	60.20408	7.142857	0	0
Glycerol (Warm Water Bath Cyst) T5	43.18182	43.18182	6.818182	6.818182	0
Glycerol (Warm Water Bath Cyst) T6	10.6383	60.6383	26.59574	2.12766	0
Glycerol (Warm Water Bath Cyst) T7	13.04348	63.04348	15.21739	8.695652	0
Glycerol (Warm Water Bath Cyst) T8	10.60606	66.66667	13.6636	9.090909	0
Glycerol (Warm Water Bath Cyst) T9	2.247191	87.64045	8.988764	1.123596	0
Glycerol (Warm Water Bath Cyst) T10	2.739726	82.19178	12.32877	2.739726	0

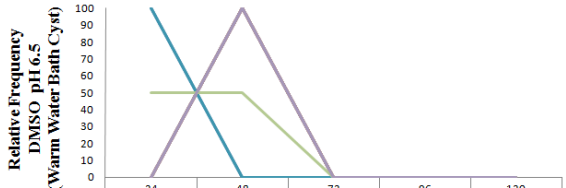
Figure 3.2: Relative hatching frequency of organisms of the species *Dendrocephalus brasiliensis*, ten tests each, carried out with Dimethylsulfoxide (DMSO) with cysts pre-treated with Warm Water Bath, Glycerol with Aired pre-treated cysts, and Glycerol with cysts pre-treated with Warm Water Bath as culture mediums (with the different cysts pre-treatment being the Warm Water Bath at 37°C for 1 hour, and then aired for 24 hours and, posteriorly, dehydrated).

Cyst Pre-Treatment (CPT) and pH Control are the variables that caused more influence over the hatching. The variable resulting in the major hatching rates was CPT, specifically the treatments that used cyst with no pre-treatment (NPT). Among the NPT treatments, we have Natural Water, Reconstituted Water, Glycerol, and DMSO. In the NPT treatments, the hatching average was 38.57%, the highest observed among the experiments.

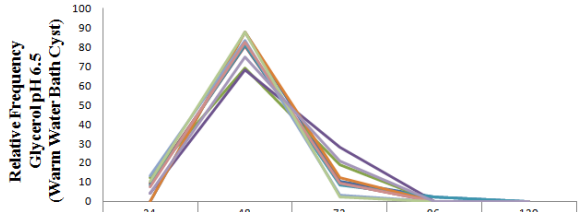
The second most important variable was pH, but in this case, we need to understand that the importance of pH is not when adopted in a specific range, since all positive and negative results presented all three pH forms tested (pH 6.5, 7.8, and without control). Therefore, pH was



	24	48	72	96	120
Glycerol pH 6.5 (Aired Cyst) T1	0	81.81818	18.18182	0	0
Glycerol pH 6.5 (Aired Cyst) T2	0	69.56522	26.08696	4.347826	0
Glycerol pH 6.5 (Aired Cyst) T3	0	81.81818	9.090909	9.090909	0
Glycerol pH 6.5 (Aired Cyst) T4	0	71.42857	28.57143	0	0
Glycerol pH 6.5 (Aired Cyst) T5	0	80	20	0	0
Glycerol pH 6.5 (Aired Cyst) T6	0	66.66667	33.33333	0	0
Glycerol pH 6.5 (Aired Cyst) T7	0	66.66667	33.33333	0	0
Glycerol pH 6.5 (Aired Cyst) T8	14.28571	57.14286	28.57143	0	0
Glycerol pH 6.5 (Aired Cyst) T9	16	72	8	4	0
Glycerol pH 6.5 (Aired Cyst) T10	34.78261	52.17391	13.04348	0	0



	24	48	72	96	120
DMSO pH6.5 (Warm Water Bath Cyst) T1	100	0	0	0	0
DMSO pH6.5 (Warm Water Bath Cyst) T2	0	100	0	0	0
DMSO pH6.5 (Warm Water Bath Cyst) T3	0	100	0	0	0
DMSO pH6.5 (Warm Water Bath Cyst) T4	0	100	0	0	0
DMSO pH6.5 (Warm Water Bath Cyst) T5	100	0	0	0	0
DMSO pH6.5 (Warm Water Bath Cyst) T6	0	100	0	0	0
DMSO pH6.5 (Warm Water Bath Cyst) T7	0	100	0	0	0
DMSO pH6.5 (Warm Water Bath Cyst) T8	0	100	0	0	0
DMSO pH6.5 (Warm Water Bath Cyst) T9	50	50	0	0	0
DMSO pH6.5 (Warm Water Bath Cyst) T10	0	100	0	0	0

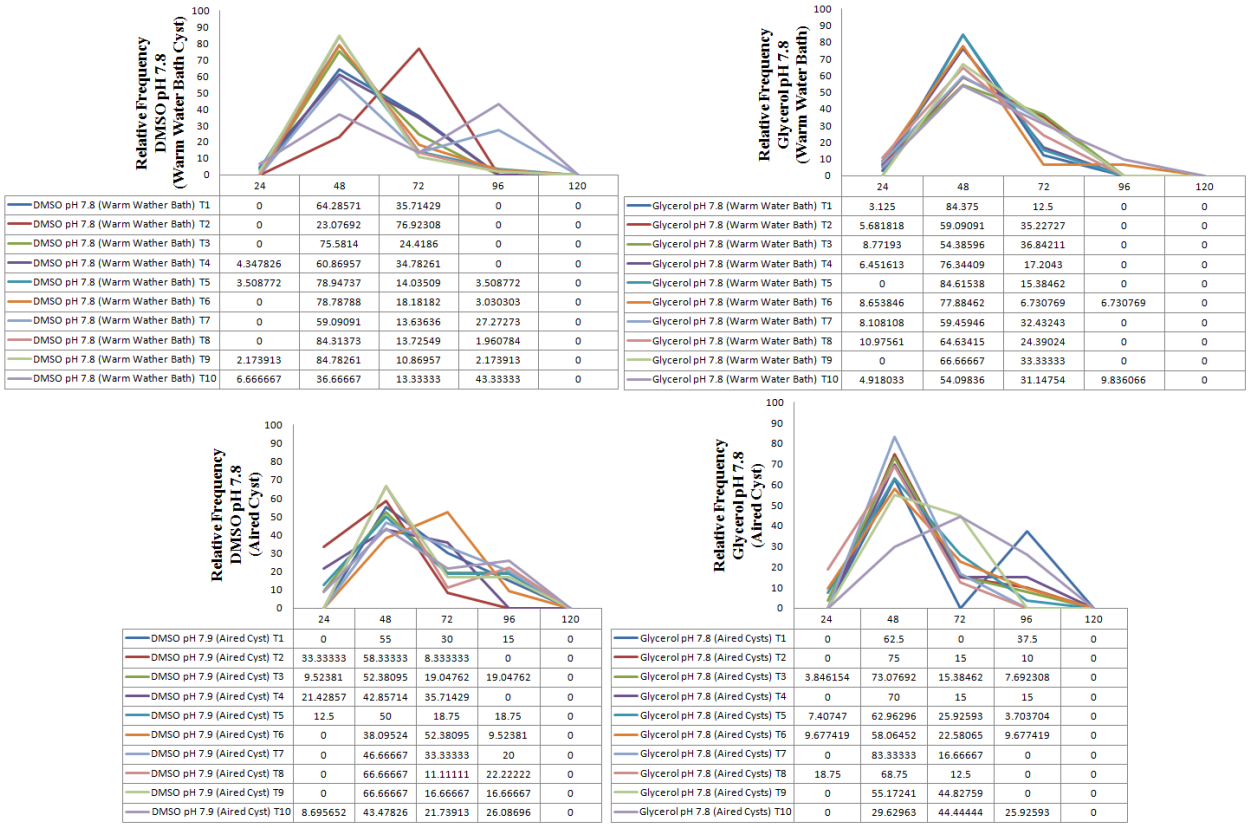


	24	48	72	96	120
Glycerol pH 6.5 (Warm Water Bath Cyst) T1	0	87.5	10.41667	2.083333	0
Glycerol pH 6.5 (Warm Water Bath Cyst) T2	9.615385	80.76923	9.615385	0	0
Glycerol pH 6.5 (Warm Water Bath Cyst) T3	12.06897	68.96552	18.96552	0	0
Glycerol pH 6.5 (Warm Water Bath Cyst) T4	4	68	28	0	0
Glycerol pH 6.5 (Warm Water Bath Cyst) T5	8.333333	81.25	8.333333	2.083333	0
Glycerol pH 6.5 (Warm Water Bath Cyst) T6	0	87.5	12.5	0	0
Glycerol pH 6.5 (Warm Water Bath Cyst) T7	13.33333	83.33333	3.333333	0	0
Glycerol pH 6.5 (Warm Water Bath Cyst) T8	7.692308	82.69231	9.615385	0	0
Glycerol pH 6.5 (Warm Water Bath Cyst) T9	10.20408	87.7551	2.040816	0	0
Glycerol pH 6.5 (Warm Water Bath Cyst) T10	4.166667	75	20.83333	0	0

Figure 3.3: Relative hatching frequency of organisms of the species *Dendrocephalus brasiliensis*, ten tests each, carried out with Dimethylsulfoxide (DMSO) with cysts pre-treated with Warm Water Bath and pH 6.5, Glycerol with Aired pre-treated cysts and pH 6.5, and Glycerol with cysts pre-treated with Warm Water Bath and pH 6.5 as culture mediums (with the different cysts' pre-treatment being the Warm Water Bath at 37°C for 1 hour, and then Aired for 24 hours and, posteriorly, dehydrated).

considered an important variable because it was a repetitive factor appearing several times, between the best and worst treatments, as shown in the inference tree results. The pH range of the mediums, even without buffer (Natural and Reconstituted Waters) presented pH in the range of 7.3 to 7.8, similar to the 7.8 in the pH-controlled medium, except for the lowest pH of 6.5. This range was the same found in other studies with *Dendrocephalus brasiliensis*, such as Lopes et al. (1998), which described the species as non-demanding since it survived in conditions such as 2.78 mg/L of DO, temperature of 25.5°C, and pH of 7.3. Another study made by Lopes et al. (2007), described the natural pH of cultivating tanks ranging from 8 to 8.5.

Therefore, the mediums with a pH range above 7 were expected to be a positive variable for the hatchability, but the results showed that the pH itself didn't result in any enhancement



	24	48	72	96	120
DMSO pH 7.8 (Warm Wather Bath) T1	0	64.28571	35.71429	0	0
DMSO pH 7.8 (Warm Wather Bath) T2	0	23.07692	76.92308	0	0
DMSO pH 7.8 (Warm Wather Bath) T3	0	75.5814	24.4186	0	0
DMSO pH 7.8 (Warm Wather Bath) T4	4.347826	60.86957	34.78261	0	0
DMSO pH 7.8 (Warm Wather Bath) T5	3.508772	78.94737	14.03509	3.508772	0
DMSO pH 7.8 (Warm Wather Bath) T6	0	78.78788	18.18182	3.030303	0
DMSO pH 7.8 (Warm Wather Bath) T7	0	59.09091	13.63636	27.27273	0
DMSO pH 7.8 (Warm Wather Bath) T8	0	84.31373	13.72549	1.960784	0
DMSO pH 7.8 (Warm Wather Bath) T9	2.173913	84.78261	10.86957	2.173913	0
DMSO pH 7.8 (Warm Wather Bath) T10	6.666667	36.66667	13.33333	43.33333	0

	24	48	72	96	120
Glycerol pH 7.8 (Warm Water Bath) T1	3.125	84.375	12.5	0	0
Glycerol pH 7.8 (Warm Water Bath) T2	5.681818	59.09091	35.22727	0	0
Glycerol pH 7.8 (Warm Water Bath) T3	8.77193	54.38596	36.84211	0	0
Glycerol pH 7.8 (Warm Water Bath) T4	6.451613	76.34409	17.2043	0	0
Glycerol pH 7.8 (Warm Water Bath) T5	0	84.61538	15.38462	0	0
Glycerol pH 7.8 (Warm Water Bath) T6	8.653846	77.88462	6.730769	6.730769	0
Glycerol pH 7.8 (Warm Water Bath) T7	8.108108	59.45946	32.43243	0	0
Glycerol pH 7.8 (Warm Water Bath) T8	10.97561	64.63415	24.39024	0	0
Glycerol pH 7.8 (Warm Water Bath) T9	0	66.66667	33.33333	0	0
Glycerol pH 7.8 (Warm Water Bath) T10	4.918033	54.09836	31.14754	9.836066	0

	24	48	72	96	120
DMSO pH 7.9 (Aired Cyst) T1	0	55	30	15	0
DMSO pH 7.9 (Aired Cyst) T2	33.33333	58.33333	8.333333	0	0
DMSO pH 7.9 (Aired Cyst) T3	9.52381	52.38095	19.04762	19.04762	0
DMSO pH 7.9 (Aired Cyst) T4	21.42857	42.85714	35.71429	0	0
DMSO pH 7.9 (Aired Cyst) T5	12.5	50	18.75	18.75	0
DMSO pH 7.9 (Aired Cyst) T6	0	38.09524	52.38095	9.52381	0
DMSO pH 7.9 (Aired Cyst) T7	0	46.66667	33.33333	20	0
DMSO pH 7.9 (Aired Cyst) T8	0	66.66667	11.11111	22.22222	0
DMSO pH 7.9 (Aired Cyst) T9	0	66.66667	16.66667	16.66667	0
DMSO pH 7.9 (Aired Cyst) T10	8.695652	43.47826	21.73913	26.08696	0

	24	48	72	96	120
Glycerol pH 7.8 (Aired Cysts) T1	0	62.5	0	37.5	0
Glycerol pH 7.8 (Aired Cysts) T2	0	75	15	10	0
Glycerol pH 7.8 (Aired Cysts) T3	3.846154	73.07692	15.38462	7.692308	0
Glycerol pH 7.8 (Aired Cysts) T4	0	70	15	15	0
Glycerol pH 7.8 (Aired Cysts) T5	7.40747	62.96296	25.92593	3.703704	0
Glycerol pH 7.8 (Aired Cysts) T6	9.677419	58.06452	22.58065	9.677419	0
Glycerol pH 7.8 (Aired Cysts) T7	0	83.33333	16.66667	0	0
Glycerol pH 7.8 (Aired Cysts) T8	18.75	68.75	12.5	0	0
Glycerol pH 7.8 (Aired Cysts) T9	0	55.17241	44.82759	0	0
Glycerol pH 7.8 (Aired Cysts) T10	0	29.62963	44.44444	25.92593	0

Figure 3.4: Relative hatching frequency of organisms of the species *Dendrocephalus brasiliensis*, ten tests each, carried out with Dimethylsulfoxide (DMSO) with cysts pre-treated with Warm Water Bath and pH 7.8, Dimethylsulfoxide (DMSO) with Aired pre-treated cysts and pH 7.8, Glycerol with Aired pre-treated cysts and pH 7.8, and Glycerol with cysts pre-treated with Warm Water Bath and pH 7.8 as culture mediums (with the different cysts' pre-treatment being the Warm Water Bath at 37°C for 1 hour, and then Aired for 24 hours and, posteriorly, dehydrated).

of hatchability for *Dendrocephalus brasiliensis*. However, the lowest pH of 6.5, in addition to the medium DMSO and aired cysts, presented the lower hatching results. We speculate that the addition of Dimethylsulfoxide, with pre-treated cysts, could have caused an interrelated negative reaction. According to Galvao et al. (2013), despite its aprotic and amphipathic characteristics, and apparent low toxicity at concentrations <10% (we adopted the concentration of 0.15%), the indiscriminate use and widespread application of DMSO may be neglecting important toxic effects at the cellular level. Another author, Mukerjee and Ostrow (1998), affirms that DMSO can strongly affect the pH values of acetate and phosphate buffers and also change the pKa in typical carboxylic/acetic acids. These studies show that DMSO has the potential to cause negative effects and could corroborate our results that present the lowest hatching rate in the cultures with Dimethylsulfoxide, indicating that the combination of the substance with acidic or non-buffered mediums (susceptible to variations) and pre-treated cysts, can affect negatively the

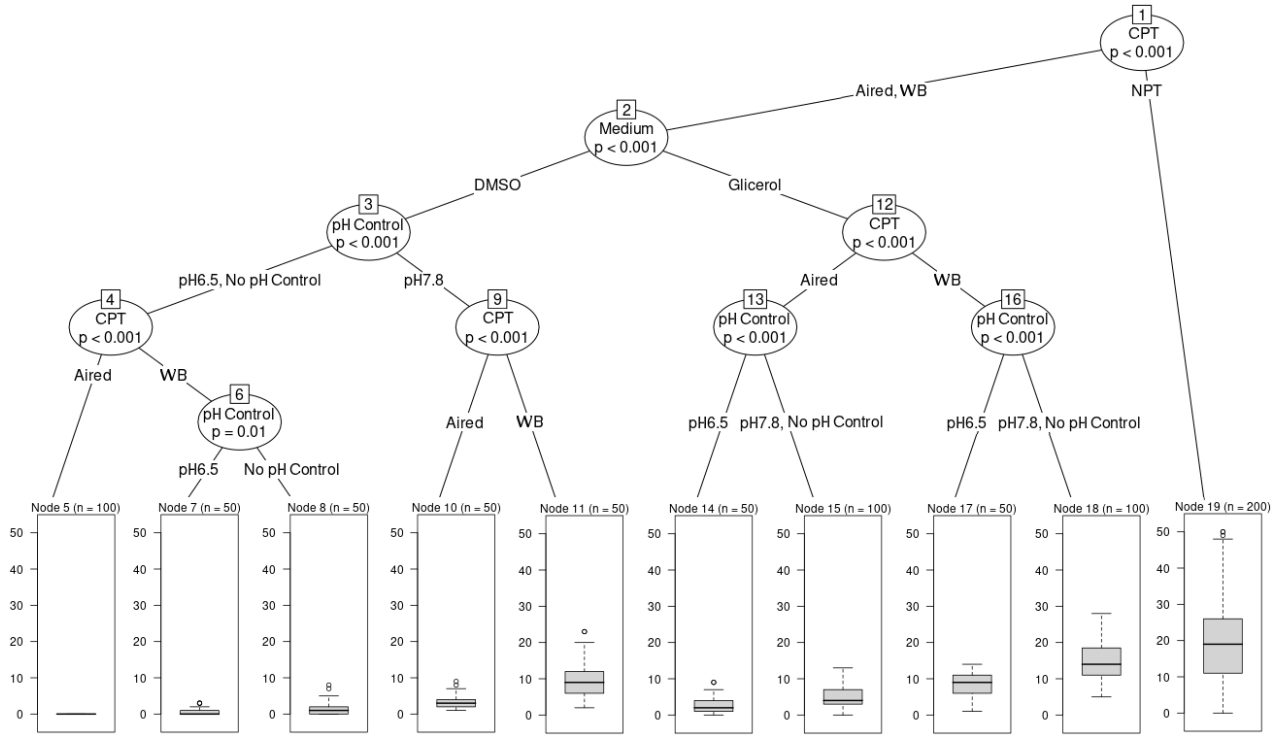


Figure 3.5: Inference Tree showing the statistically significant variables responsible for the results in the hatching of *Dendrocephalus brasiliensis* species, in different culture mediums and conditions tested (DMSO, Glycerol, Natural Water, and Reconstituted Water), pH (without control, pH 6.5 and 7.8) and cyst pre-treatment (kept in Warm Water Bath (WB) at 37°C for 1 hour, or Aired for 24 hours and subsequent dehydration).

hatching for *Dendrocephalus brasiliensis* species.

The treatments with Glycerol presented better hatching rates than the results of DMSO mediums. Glycerol mediums, in all tested pH's (6.5, 7.8, and without buffer) and using cysts pre-treated with Warm Water Bath resulted in the second and third best results. With the lack of previous studies describing the particularities of *Dendrocephalus brasiliensis* cysts, it is necessary to speculate about the possible similarities with other Anostracas. Several studies about the dormancy and diapause break are from the species *Branchipus stagnalis*. Therefore, considering the studies of resistance cyst from brine shrimp, some facts are known, according to Clegg (1964), such as the presence of Glycerol in two locations in the cyst: within the embryo, and between the embryo and cyst shell, and that the Glycerol in the latter location is released into the medium during hatching.

Clegg (1964), also proposed that the accumulation of free glycerol produces a corresponding increase in the internal osmotic pressure in the cyst, which results in an increased ability of water imbibe and continuation of the development. An osmotic rupturing of the shell would then

occur if the extent of this increase in the internal osmotic pressure eventually exceeded the environment pressure. Therefore, we speculated that, with the possibility of glycerol presence within the *Dendrocephalus brasiliensis* cyst, the addition of the glycerol in the medium could also have promoted the break of quiescence and diapause, and consequently increase the hatching rate. Though, the data analysis with the inference tree showed higher hatching rates weren't for the Medium, nor specifically for the Glycerol, they were observed for the variable CPT. The variable CPT relates to all treatments where the cysts received pre-treatment, and within the variable CPT, there is the sub-variable NPT, which represents all the treatments where the cysts didn't receive pre-treatment.

The sub-variable NPT is composed of the treatments with Glycerol and DMSO (without pre-treatment or pH control) and the mediums Natural Water and Reconstituted Water. Such a result indicates that the pre-treatment adopted in this study, WB and Aeration, not only didn't increase the hatching but resulted in lower hatching rates. Also, since the hatching rate was higher for NPT mediums, it means that DMSO and Glycerol, in the conditions tested, didn't promote significant increase in the hatching rate that justify its use. To test the hypothesis that the treatments NPT didn't differentiate, we conducted an ANOVA test with only the NPT mediums, and the test showed no statistical difference ($p = 0.3320$). This result indicates that Natural Water (NW) and Reconstituted Water (RW) produce results as positive as the one observed with DMSO or Glycerol. Also, our results differ from other studies' results stating the hatching rate of *Dendrocephalus brasiliensis* as 7%, but indicates a variation (in controlled cultures and laboratory environments, with NW and RW) from 9 to >30%.

About the use of pH buffer, we observed a peculiarity in the results, notwithstanding the lack of increase in hatchability, the buffered mediums presented a hatching Relative Frequency (Figuras 3.1, 3.2, 3.3, 3.4.) which was more homogeneous, while in mediums without the buffer, the cysts hatched more sparsely through the time. There is the possibility of the unbuffered mediums presenting natural pH fluctuations. It could have happened because, while there were daily changes to guarantee a proper buffering effect in the treatments with pH control, and this renewing offered the opportunity to remove the hatched organisms, the same didn't happen in the mediums without buffer. According to Wurts (2003), CO_2 concentrations and pH are affected by respiration and photosynthesis, since carbon dioxide is released during respiration and consumed for photosynthesis resulting in pH variations throughout the day. Then, in the unbuffered mediums, the biological degradation of dead nauplii or its metabolic activities and natural exchanges with the environment could cause pH fluctuations. This result is important because it could facilitate the species' usability in situations where the hatching uniformity is

necessary, such as toxicological tests for example.

Another form to evaluate the uniformity in which the hatching occurred is to calculate the Total Average Time (TAT) and the Velocity. Such data is important because as the higher the velocity and lower the average time it takes for the nauplii to hatch, the more cohesive will be the organisms' hatching. Therefore, the number of nauplii in similar developmental stages in the medium will be as high as the uniformity of the hatching. Then, we observed that the lower results for TAT and higher Velocity were shown for pH and CPT, respectively (Figure A2). Such a result indicates that, even if not necessary/increasing for the hatching, the pH control promoted, in this study, more uniformity in the hatching. According to Robertson-Bryan (2004), about the common sense in the literature on the effects of rapid pH reductions on benthic macro-invertebrates, the rapid pH reductions (one unit or more) with pH kept between 6.5 and 8.5, would not cause chronic or adverse effects on individuals because the effects of rapid pH changes are insignificant when pH is maintained within the acceptable ambient range.

Such affirmation can be corroborated also for the Anostraca *Dendrocephalus brasiliensis*, once the pH doesn't affect the embryo survival, a fact further confirmed by the nauplii hatch. But, the species *Dendrocephalus brasiliensis* hatches from resistance cysts, and the "Why" and the "How" the pH affected positively the velocity, and consequently the uniformity, of the hatching is unknown. Could the pH variation disrupt/retard the processes needed to initiate the physiological signaling that initiates the hatch? Since CPT, the cysts pre-treated specifically, also resulted in higher velocity, could it be related to a faster imbibed cyst? Those are questions that remain open and require further investigation with a new approach of studies that focus on the pH and pre-treatment effect on the velocity. According to Hand et al. (2016), a single suite of mechanisms did not apply to all cases with the diapause termination through different species; so further investigations are needed specifically for *Dendrocephalus brasiliensis*.

Dendrocephalus brasiliensis is a freshwater species, and the current methodology adopted for nauplii production involves salinity of the medium of 0.0 PSU (LOPES, 2007; Yaflaar and Oliveira, 2003). There isn't published data, until our study, about the effects of saline substances on the hatching of the *Dendrocephalus brasiliensis*. Other *Dendrocephalus* species were tested for saline tolerance, *Dendrocephalus geayi*, and *Dendrocephalus spartaenovae*, and the results showed that 1% of Sodium chloride (NaCl) completely inhibits the cyst hatching (Garcia et al. 2000). For *Dendrocephalus brasiliensis* we tested saline buffers (Sodium Phosphate buffer for pH 7.9, and sodium borate 8.7) aiming to analyze the response to the salt and to create a protocol for use of saline buffers in hatching studies. From the 16 treatments, with 10 tests realized per treatment (80 tests for each buffer), there wasn't hatching observed. This indicates the complete

hatching inhibition by use of those saline buffers for *Dendrocephalus brasiliensis* species. Further investigations are needed to evaluate if the exposure to the saline medium makes the posterior cyst use unfeasible.

About the dormancy break, we also tested the effect of sulfuric acid (H_2SO_4) as a tool to facilitate cyst hatching. The Anostraca cysts are formed of chitin, more exactly as chitinous walls present in cysts. According to Hackman (1962), the chitin undergoes degradation when dissolved in acid. Therefore, we consider if the sulfuric acid exposure could degrade the cyst tegument promoting the imbibe and, consequently, facilitating the hatch. However, there was no hatching observed in the 6 treatments with H_2SO_4 pre-treated cysts. Hackman (1962) affirms that acids (including sulfuric acid) degrade the chitin in a few minutes dissolving it and forming oligosaccharides (deacetylated and N·Acetyl-D.glucosamine). According to Zhang et al. (2021), the chitin biosynthesis pathway of crustaceans should be similar to that of insects and, in insects, it involves eight important enzymes which begin with trehalase followed by hexokinase, glucose-6-phosphate isomerase, glutamine: fructose6-phosphate aminotransferase, and glucosamine-6-phosphate N-acetyltransferase, 6-phosphate acetylglucosamine mutase and UDP-N-acetylglucosamine pyrophosphorylase, and finally chitin synthase. However, Hackman (1962) also affirms that after the chitin acid degradation the glucosamine wasn't found, except in the smallest traces. We speculate if, since the glucosamine is part of hexokinase pathways, and since the sulfuric acid disrupts the chitin synthase, this could have caused damage and prevented the cyst hatching. Those theories demand further investigation.

3.4 Conclusion

Through the data results analysis, we concluded that the Cyst Pre-Treatment (CPT), precisely cysts without treatment (NPT), followed by pH Control are the variables with more influence over the hatching rate for *Dendrocephalus brasiliensis* species. The pH control, or lack of it, didn't affect the hatching rate but promoted the best results for hatch homogeneity. About the CPT, pre-treated cysts showed the second best results for homogeneity, though not the best hatching rate. The results in the hatching, for the CPT (specifically the pre-treated cysts), could be related not only to the cyst treatment procedure, but the interrelation between the treated cyst and tested substances, specifically DMSO, and the lower pH of 6.5, that presented the lowest hatching rates. In general, Glycerol mediums showed better hatching rates results than DMSO, but since Natural and Reconstituted Water are between the treatments presenting higher hatching rates, then it's unjustified to use DMSO or Glycerol for *Dendrocephalus brasiliensis* cultures. Regarding

the cyst sulfuric acid (H_2SO_4) bath, it resulted in the complete absence of nauplii hatching. As for the use of saline buffers, for medium pH control for *Dendrocephalus brasiliensis*, it also resulted in the complete absence of nauplii hatching. Considering the current literature, our results differ from other authors' studies that state the hatching rate of *Dendrocephalus brasiliensis* as 7% but indicates a variation (in controlled cultures and laboratory environments, with NW and RW) from 9 to >30%.

3.4.1 Considerations

Therefore, we recommend the use of Natural or Reconstituted Water as a medium, buffered in a range above 6.5, specifically from 7.3 to 8 (8 being the recommended in the literature for the species) being the buffer not necessary to increase the rate, but to provide homogeneity in the hatching; we also recommend, the use of cysts without previous dormancy break (pre-treatment), especially in the conditions tested (Water Bath (WB), Sulfuric Acid Bath, and Aired motioned bathing for 24 hours). We precisely condemn the use of Sulfuric Acid (H_2SO_4) bath, as in the conditions tested in this study, for dormancy break since it resulted in zero nauplii presence, which indicates negative effects over the hatching for *Dendrocephalus brasiliensis* species. To control the pH during tests, or in laboratory cultivates, we indicate the use of non-saline buffers, with special avoidance for Sodium Phosphate and Borate buffers as directly tested in this study, since these resulted in the complete absence of nauplii hatching. And, since our results differ from other studies stating the hatching rate of *Dendrocephalus brasiliensis* as 7%, but indicates a variation (in controlled cultures and laboratory environments, with NW and RW) from 9 to >30%. Due to this natural variation, we also recommend that hatching tests be carried out with known amounts of cysts, in order to guarantee results consistent with reality, and that hatching tests for *Dendrocephalus brasiliensis* be performed, at least, in triplicate.

Chapter 4

Conclusions

4.1 Considerations

The objective of this thesis was to provide a new approach to how to perform hatching studies for *Dendrocephalus brasiliensis* species, evaluate the effects of using different mediums such as Dimethylsulfoxide, Glycerol, Reconstituted, and Natural water and their effects on the hatching rate, the effects of controlled/not the mediums pH, as the effect of saline buffers use over the cyst hatch. This thesis also aimed to automatize and facilitate cyst processing for *Dendrocephalus brasiliensis* using computer vision as a tool. The stated results and considerations provided by this study, are important information that can be applied to improve *Dendrocephalus brasiliensis* studies and production. In Chapter 2: The experiments were designed to evaluate the viability of automating cyst recognizing and counting using domain-specific object detection techniques based on computer vision. Then, we trained two state-of-the-art object detection methods, YOLOv3 (You Only Look Once) and Faster R-CNN (Region-based Convolutional Neural Networks), on DBrasiliensis dataset in order to compare them under both cyst detection and counting tasks. To promote research in the automation of cyst measurements, we also reported evidence that the performance of YOLOv3 is superior to Faster R-CNN. Additionally, we provided the possibility of inferring the total number of cysts, with an accuracy around 83.73%, from a substrate image set associated with a known weight. In conclusion, the proposed approach using YOLOv3 is adequate to detect and count *Dendrocephalus brasiliensis* cysts. The DBrasiliensis

dataset, created in this study, can be accessed at: https://doi.org/10.6084/m9.figshare.13073240.

In Chapter 3: The experiments were designed to differ from the traditional methodology using known amounts of *Dendrocephalus brasiliensis* cysts in all experiments and not quantified per gram, also analyzes the effects of the substances Dimethylsulfoxide and Glycerol, compared with Natural and Reconstituted Water, with variations in other conditions as presence/absence pH control, use of saline buffers, cyst dormancy break (cyst pre-treatment in a warm water bath, aeration, and sulfuric acid bath). The results indicate the Natural and/or Reconstituted Water as a preferential medium to be used, buffered (to help with hatching homogeneity) in a range of 7.3 to 8 (being 8 the recommended in the literature for the species), using cysts without previous dormancy break attempt (pre-treatment).

4.2 Future Work

In this work we tested the viability of automating cyst recognizing and counting using domain-specific object detection techniques, and obtained positive results (>80% accuracy). Therefore, a suggestion for future research includes the creation and implementation of a online system (site and/or applicative) to identify and count *Dendrocephalus brasiliensis* trough image upload.

We also tested different substances (DMSO, Glycerol, Natural and Reconstituted Water) aiming to evaluate its effects on the hatching rate for *Dendrocephalus brasiliensis* species. The results showed the lower hatching rates for the substance DMSO, especially with lower pH mediums and pre-treated cysts. Then, a suggestion for future research is further investigation of the DMSO effects on *Dendrocephalus brasiliensis* and its possible causes, also the interrelation between the substance effects in acidic mediums (pH < 7).

We tested mediums with and without pH control, so could the pH variation. Then, other suggestion for future research is to test if pH variation could disrupt/retard the processes needed to initiate the physiological signaling that initiates the hatch.

Finally, we tested different cyst dormancy break techniques, and the results indicates that not only the treatments didn't increase the hatching but caused negative effects. Therefore, a suggestion for future research is the analyzes of the effects of such treatments on the *Dendrocephalus brasiliensis* species cyst walls and embryo, especially the effects of sulfuric acid, in different concentrations, since our results showed zero hatching rate for the cysts pre-treated with that substance.

4.3 Recommendations

Therefore, after extensive result analysis, we recommend the use of Natural or Reconstituted Water as a medium, buffered in a range above 6.5, specifically from 7.3 to 8 (8 being the recommended in the literature for the species), being the buffer not necessary to increase the rate, but to provide homogeneity in the hatching; we also recommend the use of cysts without previous dormancy break (pre-treatment), especially in the conditions tested (Warm Water Bath (WB), Sulfuric Acid Bath, and Aired motioned bathing for 24 hours). We precisely condemn the use of Sulfuric Acid (H_2SO_4) bath, as in the conditions tested in this study, for dormancy break since it resulted in zero nauplii presence, which demonstrates probable negative effects over the hatching for *Dendrocephalus brasiliensis* species. To control the pH during tests, or in laboratory cultivates, we indicate the use of non-saline buffers, with special avoidance for Sodium Phosphate and Borate buffers as directly tested in this study, since they resulted in the complete absence of nauplii hatching. And, since our results differ from other studies stating the hatching rate of *Dendrocephalus brasiliensis* as 7%, but indicates a variation (in controlled cultures and laboratory environments, with NW and RW) from 9 to >30%. Due to this natural variation, we also recommend that hatching tests be carried out with known amounts of cysts, in order to guarantee results consistent with reality, and that hatching tests for *Dendrocephalus brasiliensis* be performed, at least, in triplicate.

Bibliography

Abou-Saleh, R. H., McLaughlan, J. R., Bushby, R. J., Johnson, B. R., Freear, S., Evans, S. D., and Thomson, N. H. (2019). Molecular effects of glycerol on lipid monolayers at the gas–liquid interface: Impact on microbubble physical and mechanical properties. *Langmuir*, 35(31):10097–10105. 12

Albinati, R., Carneiro, R., Silva, J., Socorro, E., and Neves, A. (2005). Uso do microcrustáceo branchoneta (dendrocephalus brasiliensis) na ração para tucunaré. *Revista Brasileira de Saúde e Produção Animal*, 5. 8

Andrade, L. S., Macedo, C. F., and de Freitas, M. C. (2020). CONCENTRAÇÃO DA DIETA NA BIOLOGIA POPULACIONAL DE DENDROCEPHALUS BRASILIENSIS PESTA 1921, EM ESTUFA AGRíCOLA. In *Ciência e Tecnologia do Pescado: Uma Análise Pluralista*, pages 184–197. Editora Científica Digital. 10

Arbeláez-Rojas, G. A. and da Graça Gama Melão, M. (2022). Production performance and nutritional quality of the fairy shrimp *Dendrocephalus brasiliensis* Pesta, 1921 (Crustacea, Anostraca) cultured with fish effluent in recirculation system. *Aquaculture*, 548:737692. 9

BELK, D. and COLE., G. (1975). Adaptational biology of desert temporary-pond inhabitants. In *Environmental Physiology of Desert Organisms.*, pages 207–226. Dowden, Hutchinson and Ross ; distributed by Halsted Press Stroudsburg, Pa. : [New York], Cambridge, MA, USA. 4

Brown, J. H., Wickins, J. F., and MacLean, M. H. (1991). The effect of water hardness on growth and carapace mineralization of juvenile freshwater prawns, macrobrachium rosenbergii de man. *Aquaculture*, 95(3):329–345. 40

Brtek, J. (2002). Taxonomical survey of the anostraca, notostraca cyclestherida, spinicaudata and laevicaudata. *Acta Rerum Naturualium Musei Nationalis Slovaci*, 48:49–59. 4

César, I. I. (1989). Comparative study on the resting eggs of several anostracans (crustacea). key for the determination of the species based upon the egg structure and diameter. *Studies on Neotropical Fauna and Environment*, 24(4):169–181. 5

Chaves, T. P., Lacau, S., and Rabet, N. (2011). Illustrated key to the brazilian dendrocephalus (crustacea: Anostraca: Thamnocephalidae). *Nauplius*, 19:1–5. viii, 4, 6, 7

Clegg, J. S. (1964). The Control of Emergence and Metabolism by External Osmotic Pressure and the Role of Free Glycerol in Developing Cysts of Artemia Salina. *Journal of Experimental Biology*, 41(4):879–892. 12, 45

César, I. I., ARMENDÁRIZ, L. C., BECERRA, R. V., and LIBERTO, R. (2004). Biodiversidad de crustácea (anostraca, notostraca, spinicaudata, laevicaudata, ostracoda, amphipoda y brachyura trichodactylidae) de la mesopotamia argentina. *Insugeo, Miscelánea*, 12:247 – 252. 5

da Cruz Daltro, A., Macedo, C. F., Serafim-Junior, M., Brandão, L. L. L., and Gauthier, N. B. (2021). The effect of fairy shrimp "branchoneta" (dendrocephalus brasiliensis) as the initial diet of tambaqui postlarvae. *Acta Scientiarum. Animal Sciences*, 43:e53196. 1, 8, 33

da Silva, S. M. B. C., dos Santos, L. B. G., Lopes, J. P., Gálvez, A. O., and Coimbra, M. R. M. (2013). Sexual reproduction in freshwater fairy shrimp, dendrocephalus brasiliensis (pesta, 1921) inferred by amplified fragment length polymorphism markers. *Ciência Rural*, 43(6):1076–1081. 6

de Paiva Barros-Alves, S., Alves, D. F. R., Jr, E. A. B., Rabet, N., and Hirose, G. L. (2016). Morphological review of the freshwater fairy shrimp dendrocephalus brasiliensis pesta, 1921 (anostraca: Thamnocephalidae). *Nauplius*, 24(0). viii, 5, 7

de Vasconcellos, M. G. (2010). Características populacionais, desenvolvimento e produção de dendrocephalus brasiliensis pesta, 1921 sob as condições climáticas da região sudeste do país. *Revista Brasileira de Zoociências*, 12(2). 10, 33

Dukes, J. S. and Mooney, H. A. (1999). Does global change increase the success of biological invaders? *Trends in Ecology Evolution*, 14(4):135–139. 32

Everingham, M., Gool, L. V., Williams, C. K. I., Winn, J., and Zisserman, A. (2009). The pascal visual object classes (VOC) challenge. *International Journal of Computer Vision*, 88(2):303–338. 18

Freita, F. R. V., de Lucena, I. C., Alencar, D. R., Santos, I. J., and Pinheiro, A. P. (2017). Occurrence of dendrocephalus brasiliensis pesta, 1921 (crustacea, anostraca) in the caras river, southern ceara, brazil. *Anais da Academia Brasileira de Ciências*, 89(2):1047–1049. viii, 5, 6

Galvao, J., Davis, B., Tilley, M., Normando, E., Duchen, M., and Cordeiro, M. F. (2013). Unexpected low-dose toxicity of the universal solvent dmso. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 28. 44

Gonçalves, J. L. (2001). Algae removal via feeding by microcrustacean *Dendrocephalus brasiliensis* (Crustacea: Anostraca). Master's thesis, Federal University of Mato Grosso do Sul, Campo Grande, MS, Brazil. 15

Hackman, R. H. (1962). Studies on chitin v. the action of mineral acids on chitin. *Australian Journal of Biological Sciences*, 15:526–537. 11, 48

Hand, S. C., Denlinger, D. L., Podrabsky, J. E., and Roy, R. (2016). Mechanisms of animal diapause: recent developments from nematodes, crustaceans, insects, and fish. *American journal of physiology. Regulatory, integrative and comparative physiology*, 310(11):1193–1211. 47

He, K., Zhang, X., Ren, S., and Sun, J. (2016). Deep residual learning for image recognition. In *2016 IEEE Conference on Computer Vision and Pattern Recognition (CVPR)*, pages 770–778. 21, 22

Ioffe, S. and Szegedy, C. (2015). Batch normalization: Accelerating deep network training by reducing internal covariate shift. In *Proceedings of the 32nd International Conference on International Conference on Machine Learning - Volume 37*, ICML'15, page 448–456. JMLR.org. 21

Jalal, A., Salman, A., Mian, A., Shortis, M., and Shafait, F. (2020). Fish detection and species classification in underwater environments using deep learning with temporal information. *Ecological Informatics*, 57:101088. 2, 11, 16

Jiao, L., Zhang, F., Liu, F., Yang, S., Li, L., Feng, Z., and Qu, R. (2019). A survey of deep learning-based object detection. *IEEE Access*, 7:128837–128868. 16, 20

Kingma, D. P. and Ba, J. (2015). Adam: A method for stochastic optimization. In *3rd International Conference on Learning Representations, ICLR 2015, San Diego, CA, USA, May 7-9, 2015, Conference Track Proceedings*. 23

Leprieur, F., Beauchard, O., Blanchet, S., Oberdorff, T., and Brosse, S. (2008). Fish invasions in the world's river systems: When natural processes are blurred by human activities. *PLoS Biology*, 6(2):e28. 1

Li, R., Wang, R., Xie, C., Liu, L., Zhang, J., Wang, F., and Liu, W. (2019). A coarse-to-fine network for aphid recognition and detection in the field. *Biosystems Engineering*, 187:39–52. 17

Lin, M., Chen, Q., and Yan, S. (2014a). Network in network. In Bengio, Y. and LeCun, Y., editors, *2nd International Conference on Learning Representations, ICLR 2014*, pages 1–10, Banff, AB, Canada. 21

Lin, T.-Y., Maire, M., Belongie, S., Hays, J., Perona, P., Ramanan, D., Dollár, P., and Zitnick, C. L. (2014b). Microsoft COCO: Common objects in context. In *Computer Vision – ECCV 2014*, pages 740–755. Springer International Publishing. 23

Linder, F. (1941). Contributions to the morphology and the taxonomy of the branchiopoda anostraca. *Zoologiska Bidrag från Uppsala*, 20:101–303. 5

LOPES, J. P. (2007). *Dinâmica Reprodutiva de Branchoneta Dendorcephalus brasiliensis Pesta, 1921 Como Incremento na Produção de Alimento Vivo para Peixes Ornamentais*. PhD thesis, Universidade Federal do Rio Grande do Norte. 47

Lopes, J. P., da Silva, A. L., Santos, A. J. G., and Tenório, R. A. (1998). Branchoneta: A notable contribution to larviculture and hatching of freshwater carnivorous fish. *Panorama da aquicultura*, 8(50):31– 34. 15, 43

Lopes, J. P., de Castro Bezerra Gurgel, H., Araujo, A., and Pontes, C. S. (2008a). Influência da inoculação de cistos na produção de biomassa de "branchoneta" dendrocephalus brasiliensis pesta, 1921 (crustacea: Anostraca). *Ciência e Agrotecnologia*, 32(2):598–603. 8, 39

Lopes, J. P., de Castro Gurgel, H., Galvez, A. O., and Pontes, C. (2007). Production of cysts of "branchoneta" dendrocephalus brasiliensis (crustacean: Anostracan). *Biotemas*, 20(2):33–39. 2, 8, 9, 10, 13, 15, 16, 33, 43

Lopes, J. P., Gurgel, H. D. C. B., and Pontes, C. S. (2011). COMPORTAMENTO REPRODUTIVO DE dendrocephalus brasiliensis, pesta 1921 (CRUSTACEA: ANOSTRACA). *Ciência Animal Brasileira*, 12(4). 6

Lopes, J. P., Pontes, C. S., and ARAÚJO, A. (2008b). Fatores bióticos e abióticos que influenciam o desenvolvimento de branconeta (crustacea: Anostraca). *Revista Brasileira de Engenharia de Pesca*, 3(1):76–90. 39

Lutz, A. (1929). Dous phyllopodes, observados no rio grande do norte. *Mem Del Inst Oswaldo Cruz*, 5:3–9. 5

Macisaac, H. J., Tedla, R. A., and Ricciardi, A. (2010). Patterns and rate of growth of studies in invasion ecology. In *Fifty Years of Invasion Ecology*, pages 49–60. Wiley-Blackwell. 1, 15

Mai, M. G., Silva, T. A. S., Almeida, V. L. S., and de Leão Serafini, R. (2008). First record of the invasion of dendrocephalus brasiliensis pesta, 1921 (crustacea: Anostraca: Thamnocephalidae) in são paulo state, brazil. *Pan-American Journal of Aquatic Sciences*, 3(3):269–274. viii, 5, 6

Masters, D. and Luschi, C. (2018). Revisiting small batch training for deep neural networks. *CoRR*, abs/1804.07612. 23

Milardi, M., Gavioli, A., Soininen, J., and Castaldelli, G. (2019). Exotic species invasions undermine regional functional diversity of freshwater fish. *Scientific Reports*, 9(1). 15

Mohamed, H. E.-D., Fadl, A., Anas, O., Wageeh, Y., ElMasry, N., Nabil, A., and Atia, A. (2020). MSR-YOLO: Method to enhance fish detection and tracking in fish farms. *Procedia Computer Science*, 170:539–546. 2, 11, 16

Mukerjee, P. and Ostrow, J. (1998). Effects of added dimethylsulfoxide on pka values of uncharged organic acids and ph values of aqueous buffers. *Tetrahedron Letters*, 39(5):423–426. 44

Murugan, G. and Dumont, H. (1995). Influence of light, dmso and glycerol on the hatchability of *Thamnocephalus platyurus* Packard cysts. In *Studies on Large Branchiopod Biology and Aquaculture II*, pages 175–178. Springer. 3, 12, 33, 36, 37

Müller, H. (1972). Wachstum und phosphatbedarf von *Nitzschia actinastroides* (lemn.) v. Goor in statischer und homokontiuierliecher kultur unter phosphatlimitierung. *Arch. Hydrobiol*, 38:399–484. 34, 35

Neupane, B., Horanont, T., and Hung, N. D. (2019). Deep learning based banana plant detection and counting using high-resolution red-green-blue (RGB) images collected from unmanned aerial vehicle (UAV). *PLOS ONE*, 14(10):e0223906. 17

Pereira, D. C. and dos Santos-Neto, M. A. (2010). InfluÊncia do Ácido ascÓrbico e dos Íons ca+2 na eclosÃo de cistos do anostraca branconeta (dendrocephalus brasiliensis, pesta, 1921). *Revista Brasileira de Engenharia de Pesca*, 5:28–41. 10, 33

Pyšek, P. and Richardson, D. M. (2006). The biogeography of naturalization in alien plants. *Journal of Biogeography*, 33(12):2040–2050. 1

Quan, L., Feng, H., Lv, Y., Wang, Q., Zhang, C., Liu, J., and Yuan, Z. (2019). Maize seedling detection under different growth stages and complex field environments based on an improved faster r–CNN. *Biosystems Engineering*, 184:1–23. 17

Rabet, N. and Thiéry, A. (1996). The neotropical genus dendrocephalus (crustacea: Anostraca: Thamnocephalidae) in brazil (south america), with a description of two new species. *Journal of Natural History*, 30(4):479–503. 5, 6

Redmon, J., Divvala, S., Girshick, R., and Farhadi, A. (2016). You only look once: Unified, real-time object detection. In *2016 IEEE Conference on Computer Vision and Pattern Recognition (CVPR)*, pages 779–788, Las Vegas, NV, USA. IEEE. 16, 20, 21

Reid, A. J., Carlson, A. K., Creed, I. F., Eliason, E. J., Gell, P. A., Johnson, P. T. J., Kidd, K. A., MacCormack, T. J., Olden, J. D., Ormerod, S. J., Smol, J. P., Taylor, W. W., Tockner, K., Vermaire, J. C., Dudgeon, D., and Cooke, S. J. (2019). Emerging threats and persistent conservation challenges for freshwater biodiversity. *Biological Reviews*, 94(3):849–873. 32

Ren, S., He, K., Girshick, R., and Sun, J. (2017). Faster r-CNN: Towards real-time object detection with region proposal networks. *IEEE Transactions on Pattern Analysis and Machine Intelligence*, 39(6):1137–1149. 16, 20, 22

Ricciardi, A. and Rasmussen, J. B. (1999). Extinction rates of north american freshwater fauna. *Conservation Biology*, 13(5):1220–1222. 32

Richardson, D. M., Carruthers, J., Hui, C., Impson, F. A. C., Miller, J. T., Robertson, M. P., Rouget, M., Roux, J. J. L., and Wilson, J. R. U. (2011). Human-mediated introductions of australian acacias - a global experiment in biogeography. *Diversity and Distributions*, 17(5):771–787. 15

Robertson-Bryan, I. (2004). Technical memorandum: ph requirements of freshwater aquatic life. Technical report, California's Central Valley Water Quality Control Board. 12, 47

Rogers, D. C. (2013). Anostraca catalogus (crustacea: Branchiopoda). *THE RAFFLES BULLETIN OF ZOOLOGY*, 61:525–546. 4

Saad, F. S. A., Lee, M. V., and Basah, S. (2019). Face recognition using machine vision for security system. *IOP Conference Series: Materials Science and Engineering*, 705(1):012005. 11

Sala, O. E., Chapin, F. S., III, Armesto, J. J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L. F., Jackson, R. B., Kinzig, A., Leemans, R., Lodge, D. M., Mooney, H. A., Oesterheld, M., Poff, N. L., Sykes, M. T., Walker, B. H., Walker, M., and Wall, D. H. (2000). Global biodiversity scenarios for the year 2100. *Science*, 287(5459):1770–1774. 32

Santos, V. S. V., Campos, C. F., de Campos Júnior, E. O., and Pereira, B. B. (2018). Acute ecotoxicity bioassay using *Dendrocephalus brasiliensis*: alternative test species for monitoring of contaminants in tropical and subtropical freshwaters. *Ecotoxicology*, 27(6):635–640. 2, 9, 15, 33, 34, 36

Santos, V. S. V., Silveira, E., and Pereira, B. B. (2019). Ecotoxicological assessment of synthetic and biogenic surfactants using freshwater cladoceran species. *Chemosphere*, 221:519–525. 9, 10, 11, 16, 33

Seebens, H., Blackburn, T. M., Dyer, E. E., Genovesi, P., Hulme, P. E., Jeschke, J. M., Pagad, S., Pyšek, P., Winter, M., Arianoutsou, M., Bacher, S., Blasius, B., Brundu, G., Capinha, C., Celesti-Grapow, L., Dawson, W., Dullinger, S., Fuentes, N., Jäger, H., Kartesz, J., Kenis, M., Kreft, H., Kühn, I., Lenzner, B., Liebhold, A., Mosena, A., Moser, D., Nishino, M., Pearman, D., Pergl, J., Rabitsch, W., Rojas-Sandoval, J., Roques, A., Rorke, S., Rossinelli, S., Roy, H. E., Scalera, R., Schindler, S., Štajerová, K., Tokarska-Guzik, B., van Kleunen, M., Walker, K., Weigelt, P., Yamanaka, T., and Essl, F. (2017). No saturation in the accumulation of alien species worldwide. *Nature Communications*, 8(1). 32

Shapiro, S. S. and Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika*, 52(3-4):591–611. 24

Shulyak, A., Goydyk, V., Gusakovsky, S., Grygorenko, V., Mytsyk, Y., and Badiuk, N. (2021). Modern aspects of the use of dimethyl sulfoxide (dmso). *Pharmacology online*, 1:82–89. 12

Simonyan, K. and Zisserman, A. (2015). Very deep convolutional networks for large-scale image recognition. In *3rd International Conference on Learning Representations, ICLR 2015*, pages 1–14, San Diego, CA, USA. ICLR. 22

Sipaúba Tavares, L. H. (1995). *Limnologia aplicada à aqüicultura*. Unesp. 40

Srivastava, N., Hinton, G., Krizhevsky, A., Sutskever, I., and Salakhutdinov, R. (2014). Dropout: A simple way to prevent neural networks from overfitting. *J. Mach. Learn. Res.*, 15(1):1929–1958. 21

Szegedy, C., Vanhoucke, V., Ioffe, S., Shlens, J., and Wojna, Z. (2016). Rethinking the inception architecture for computer vision. In *2016 IEEE Conference on Computer Vision and Pattern Recognition (CVPR)*, pages 2818–2826, Las Vegas, NV. IEEE. 22

Tukey, J. W. (1949). Comparing individual means in the analysis of variance. *Biometrics*, 5(2):99. 24

Vasconez, J., Delpiano, J., Vougioukas, S., and Cheein, F. A. (2020). Comparison of convolutional neural networks in fruit detection and counting: A comprehensive evaluation. *Computers and Electronics in Agriculture*, 173:105348. 17

Velez, A., Mufari, J. R., and Rovetto, L. J. (2019). Sodium salts solubility in ternary glycerol+water+alcohol mixtures present in purification process of crude glycerol from the biodiesel industry. *Fluid Phase Equilibria*. 40

Verani, L. H. W. et al. (1982). Formação de micelas de lauril sulfato de sódio em soluções aquosas de dimetilsulfóxido n, n-dimetilformamida en, n-dimetilacetamida. 40

Vitousek, P. M., Mooney, H. A., Lubchenco, J., and Melillo, J. M. (1997). Human domination of earth's ecosystems. *Science*, 277(5325):494–499. 1, 32

Walther, G.-R., Roques, A., Hulme, P. E., Sykes, M. T., Pyšek, P., Kühn, I., Zobel, M., Bacher, S., Botta-Dukát, Z., and Bugmann, H. (2009). Alien species in a warmer world: risks and opportunities. *Trends in Ecology Evolution*, 24(12):686–693. 32

Wang, Q.-J., Zhang, S.-Y., Dong, S.-F., Zhang, G.-C., Yang, J., Li, R., and Wang, H.-Q. (2020). Pest24: A large-scale very small object data set of agricultural pests for multi-target detection. *Computers and Electronics in Agriculture*, 175:105585. 16

Wardle, D. (2005). Effects of biodiversity on ecosystem functioning: A consensus of current knowledge. *Ecological monographs.*, 75(1). 1

Wurts, W. A. (2003). Daily ph cycle and ammonia toxicity. *World Aquaculture*, 34(2):20–21. 46

Yaflaar, B. Z. and Oliveira, A. (2003). Utilização de náuplios de “branchoneta” dendrocephalus brasiliensis (pesta, 1921) na alimentação de larvas do "camarão cinza" litopenaeus vannamei (boone, 1931). *Acta Scientiarum. Biological Sciences*, 25(2). 8, 47

Yuan, Y., Chen, L., Wu, H., and Li, L. (2022). Advanced agricultural disease image recognition technologies: A review. *Information Processing in Agriculture*, 9(1):48–59. 11

Zanden, M. J. V. and Olden, J. D. (2008). A management framework for preventing the secondary spread of aquatic invasive species. *Canadian Journal of Fisheries and Aquatic Sciences*, 65(7):1512–1522. 33

Zhang, X., Li, J. Y. F., and Xiang, J. (2021). Chitin synthesis and degradation in crustaceans: A genomic view and application. *Marine Drugs*, 153(19):1–23. 48

Zoph, B., Vasudevan, V., Shlens, J., and Le, Q. V. (2018). Learning transferable architectures for scalable image recognition. In *2018 IEEE/CVF Conference on Computer Vision and Pattern Recognition*, pages 8697–8710, Salt Lake City, UT, USA. IEEE. 21

Appendices

A Tables and Figures

A.1 Table describing all the treatment groups tested in this study

Table A1: Description of the treatment groups adopted in this study.

Group	Amount of tests	Description
Group Natural Water (NW)	10 tests	Cysts with no scarification in a natural water medium
Group Reconstituted Water (RW)	10 tests	Cysts with no scarification in a reconstituted water medium
Group DMSOZ0	10 tests	Cysts with no scarification in a DMSO solution medium
Group GlycerolZ0	10 tests	Cysts with no scarification in a Glycerol solution medium
Group DMSOZ1	10 tests	Cysts with no scarification in a DMSO solution medium + Controlled pH 6.5
Group DMSOZ2	10 tests	Cysts with no scarification in a DMSO solution medium + Controlled pH 7.9
Group DMSOZ3	10 tests	Cysts with no scarification in a DMSO solution medium + Controlled pH 8.7
Group GlycerolZ1	10 tests	Cysts with no scarification in a Glycerol solution medium + Controlled pH 6.5
Group GlycerolZ2	10 tests	Cysts with no scarification in a Glycerol solution medium + Controlled pH 7.9
Group GlycerolZ3	10 tests	Cysts with no scarification in a Glycerol solution medium + Controlled pH 8.7
Group DMSOE1	10 tests	Cysts with warm water scarification in a DMSO solution medium + Controlled pH 6.5
Group DMSOE2	10 tests	Aired Cysts scarification in a DMSO solution medium + Controlled pH 6.5
Group DMSOE3	10 tests	Cysts with sulfuric acid scarification in a DMSO solution medium + Controlled pH 6.5
Group GlycE1	10 tests	Cysts with warm water scarification in a Glycerol solution medium + Controlled pH 6.5
Group GlycE2	10 tests	Aired cysts scarification in a Glycerol solution medium + Controlled pH 6.5
Group GlycE3	10 tests	Cysts with sulfuric acid scarification in a Glycerol solution medium + Controlled pH 6.5
Group DMSOE4	10 tests	Cysts with warm water scarification in a DMSO solution medium + Controlled pH 7.9
Group DMSOE5	10 tests	Aired Cysts scarification in a DMSO solution medium + Controlled pH 7.9
Group DMSOE6	10 tests	Cysts with sulfuric acid scarification in a DMSO solution medium + Controlled pH 7.9
Group GlycE4	10 tests	Cysts with warm water scarification in a Glycerol solution medium + Controlled pH 7.9
Group GlycE5	10 tests	Aired cysts scarification in a Glycerol solution medium + Controlled pH 7.9
Group GlycE6	10 tests	Cysts with sulfuric acid scarification in a Glycerol solution medium + Controlled pH 7.9
Group DMSOE7	10 tests	Cysts with warm water scarification in a DMSO solution medium + Controlled pH 8.7
Group DMSOE8	10 tests	Aired Cysts scarification in a DMSO solution medium + Controlled pH 8.7
Group DMSOE9	10 tests	Cysts with sulfuric acid scarification in a DMSO solution medium + Controlled pH 8.7
Group GlycE5	10 tests	Cysts with warm water scarification in a Glycerol solution medium + Controlled pH 8.7
Group GlycE6	10 tests	Aired cysts scarification in a Glycerol solution medium + Controlled pH 8.7
Group GlycE7	10 tests	Cysts with sulfuric acid scarification in a Glycerol solution medium + Controlled pH 8.7

A.2 Figure presenting the Total Average Time and Velocity of Dendrocephalus brasiliensis hatching in different culture media conditions

Test	Treatment	Total Average Time	Velocity
1	DMSO	3.810	0.263
2	DMSO	1.609	0.622
3	DMSO	2.611	0.383
4	DMSO	2.244	0.446
5	DMSO	2.304	0.434
6	DMSO	2.167	0.462
7	DMSO	1.943	0.515
8	DMSO	2.711	0.369
9	DMSO	2.618	0.382
10	DMSO	2.540	0.394
	Mean	2.456	0.414
1	DMSO (Warm Bath)	1.828	0.547
2	DMSO (Warm Bath)	2.500	0.400
3	DMSO (Warm Bath)	3.000	0.333
4	DMSO (Warm Bath)	3.000	0.333
5	DMSO (Warm Bath)	2.250	0.444
6	DMSO (Warm Bath)	2.200	0.455
7	DMSO (Warm Bath)		
8	DMSO (Warm Bath)	2.250	0.444
9	DMSO (Warm Bath)		
10	DMSO (Warm Bath)	2.231	0.448
	Mean	2.407	0.444
1	DMSO 6.5 (Warm Bath)	1.000	1.000
2	DMSO 6.5 (Warm Bath)	2.000	0.500
3	DMSO 6.5 (Warm Bath)	2.000	0.500
4	DMSO 6.5 (Warm Bath)	2.000	0.500
5	DMSO 6.5 (Warm Bath)	1.000	1.000
6	DMSO 6.5 (Warm Bath)	2.000	0.500
7	DMSO 6.5 (Warm Bath)	2.000	0.500
8	DMSO 6.5 (Warm Bath)	2.000	0.500
9	DMSO 6.5 (Warm Bath)	1.500	0.667
10	DMSO 6.5 (Warm Bath)	2.000	0.500
	Mean	2.000	0.500
1	DMSO 7.8 (Aired Cyst)	2.600	0.385
2	DMSO 7.8 (Aired Cyst)	1.750	0.571
3	DMSO 7.8 (Aired Cyst)	2.476	0.404
4	DMSO 7.8 (Aired Cyst)	2.143	0.467
5	DMSO 7.8 (Aired Cyst)	2.438	0.410
6	DMSO 7.8 (Aired Cyst)	2.714	0.368
7	DMSO 7.8 (Aired Cyst)	2.733	0.366
8	DMSO 7.8 (Aired Cyst)	2.556	0.391
9	DMSO 7.8 (Aired Cyst)	2.500	0.400
10	DMSO 7.8 (Aired Cyst)	2.652	0.377
	Mean	2.528	0.396
1	DMSO 7.8 (Warm Bath)	2.357	0.424
2	DMSO 7.8 (Warm Bath)	2.769	0.361
3	DMSO 7.8 (Warm Bath)	2.244	0.446
4	DMSO 7.8 (Warm Bath)	2.304	0.434
5	DMSO 7.8 (Warm Bath)	2.175	0.460
6	DMSO 7.8 (Warm Bath)	2.242	0.446
7	DMSO 7.8 (Warm Bath)	2.682	0.373
8	DMSO 7.8 (Warm Bath)	2.176	0.459
9	DMSO 7.8 (Warm Bath)	2.130	0.469
10	DMSO 7.8 (Warm Bath)	2.933	0.341
	Mean	2.274	0.440
Test	Treatment	Total Mean Time	Velocity
1	Natural Water	3.708	0.270
2	Natural Water	1.681	0.595
3	Natural Water	2.356	0.425
4	Natural Water	2.442	0.410
5	Natural Water	2.407	0.415
6	Natural Water	2.298	0.435
7	Natural Water	1.883	0.531
8	Natural Water	2.622	0.381
9	Natural Water	2.596	0.385
10	Natural Water	2.612	0.383
	Mean	2.461	0.412
1	Reconstituted Water	4.109	0.243
2	Reconstituted Water	1.709	0.585
3	Reconstituted Water	2.472	0.404
4	Reconstituted Water	2.135	0.468
5	Reconstituted Water	2.075	0.482
6	Reconstituted Water	2.449	0.408
7	Reconstituted Water	1.984	0.504
8	Reconstituted Water	2.613	0.383
9	Reconstituted Water	2.513	0.398
10	Reconstituted Water	2.592	0.386
	Mean	2.465	0.406

Test	Treatment	Total Average Time	Velocity
1	Glycerol	4.152	0.241
2	Glycerol	1.453	0.688
3	Glycerol	2.333	0.429
4	Glycerol	2.321	0.431
5	Glycerol	2.638	0.379
6	Glycerol	2.600	0.385
7	Glycerol	1.857	0.538
8	Glycerol	2.581	0.387
9	Glycerol	2.541	0.394
10	Glycerol	2.186	0.457
	Mean	2.466	0.411
1	Glycerol (Aired Cyst)	1.955	0.512
2	Glycerol (Aired Cyst)	2.375	0.421
3	Glycerol (Aired Cyst)	2.200	0.455
4	Glycerol (Aired Cyst)	3.118	0.321
5	Glycerol (Aired Cyst)	2.750	0.364
6	Glycerol (Aired Cyst)	3.059	0.327
7	Glycerol (Aired Cyst)	1.636	0.611
8	Glycerol (Aired Cyst)	1.625	0.615
9	Glycerol (Aired Cyst)	1.649	0.607
10	Glycerol (Aired Cyst)	2.077	0.481
	Mean	2.244	0.468
1	Glycerol (Warm Bath)	2.115	0.473
2	Glycerol (Warm Bath)	1.609	0.621
3	Glycerol (Warm Bath)	2.281	0.438
4	Glycerol (Warm Bath)	1.745	0.573
5	Glycerol (Warm Bath)	1.773	0.564
6	Glycerol (Warm Bath)	2.202	0.454
7	Glycerol (Warm Bath)	2.196	0.455
8	Glycerol (Warm Bath)	2.212	0.452
9	Glycerol (Warm Bath)	2.090	0.478
10	Glycerol (Warm Bath)	2.151	0.465
	Mean	2.133	0.469
1	Glycerol 6.5 (Aired Cyst)	2.182	0.458
2	Glycerol 6.5 (Aired Cyst)	2.348	0.426
3	Glycerol 6.5 (Aired Cyst)	2.273	0.440
4	Glycerol 6.5 (Aired Cyst)	2.286	0.438
5	Glycerol 6.5 (Aired Cyst)	2.200	0.455
6	Glycerol 6.5 (Aired Cyst)	2.333	0.429
7	Glycerol 6.5 (Aired Cyst)	2.333	0.429
8	Glycerol 6.5 (Aired Cyst)	2.143	0.467
9	Glycerol 6.5 (Aired Cyst)	2.000	0.500
10	Glycerol 6.5 (Aired Cyst)	1.783	0.561
	Mean	2.236	0.447
1	Glycerol 6.5 (Warm Bath)	2.146	0.466
2	Glycerol 6.5 (Warm Bath)	2.000	0.500
3	Glycerol 6.5 (Warm Bath)	2.069	0.483
4	Glycerol 6.5 (Warm Bath)	2.240	0.446
5	Glycerol 6.5 (Warm Bath)	2.042	0.490
6	Glycerol 6.5 (Warm Bath)	2.125	0.471
7	Glycerol 6.5 (Warm Bath)	1.900	0.526
8	Glycerol 6.5 (Warm Bath)	2.019	0.495
9	Glycerol 6.5 (Warm Bath)	1.918	0.521
10	Glycerol 6.5 (Warm Bath)	2.167	0.462
	Mean	2.055	0.487
1	Glycerol 7.8 (Aired Cyst)	2.750	0.364
2	Glycerol 7.8 (Aired Cyst)	2.350	0.426
3	Glycerol 7.8 (Aired Cyst)	2.269	0.441
4	Glycerol 7.8 (Aired Cyst)	2.450	0.408
5	Glycerol 7.8 (Aired Cyst)	2.259	0.443
6	Glycerol 7.8 (Aired Cyst)	2.323	0.431
7	Glycerol 7.8 (Aired Cyst)	2.167	0.462
8	Glycerol 7.8 (Aired Cyst)	1.938	0.516
9	Glycerol 7.8 (Aired Cyst)	2.448	0.408
10	Glycerol 7.8 (Aired Cyst)	2.963	0.338
	Mean	2.392	0.428
1	Glycerol 7.8 (Warm Bath)	2.094	0.478
2	Glycerol 7.8 (Warm Bath)	2.295	0.436
3	Glycerol 7.8 (Warm Bath)	2.281	0.438
4	Glycerol 7.8 (Warm Bath)	2.108	0.474
5	Glycerol 7.8 (Warm Bath)	2.154	0.464
6	Glycerol 7.8 (Warm Bath)	2.115	0.473
7	Glycerol 7.8 (Warm Bath)	2.243	0.446
8	Glycerol 7.8 (Warm Bath)	2.134	0.469
9	Glycerol 7.8 (Warm Bath)	2.333	0.429
10	Glycerol 7.8 (Warm Bath)	2.459	0.407
	Mean	2.222	0.455

Figure A2: Total Average Time and Velocity of *Dendrocephalus brasiliensis* in different culture media conditions (DMSO, Glycerol, Natural Water, and Reconstituted Water), pH (without control, pH 6.5 and 7.8) and pre-treatment of cysts (kept in Warm Water Bath at 37°C for 1hour, or Aired for 24 hours and subsequent dehydration).