Effect of *Helicosporidium* sp. (Chlorophyta; Trebouxiophyceae) infection on *Cyrtobagous salviniae* Calder and Sands (Coleoptera: Curculionidae), a biological control agent for the invasive *Salvinia molesta* D.S. Mitchell (Salviniaceae) in South Africa

by

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A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy at Rhodes University

Makhanda, 2021

RHODES UNIVERSITY

Where leaders learn

Declaration

All the work described in this thesis is my own and has never been submitted for examination with any University. It is being submitted to Rhodes University for the degree of Doctor of Philosophy.

Candidate-Tshililo Emmanuel Mphephu

28 January 2021

Abstract

The effectiveness of established biological control agents depends on biotic and abiotic interactions in the introduced range. The weevil, *Cyrtobagous salviniae* Calder and Sands (Coleoptera: Curculionidae), was released as a biological control against *Salvinia molesta* D.S. Mitchell (Salviniaceae) in South Africa in 1985. This agent has been highly successful against *S. molesta* and has significantly reduced the weed's populations around the country. However, in 2007, the parasitic alga, *Helicosporidium* sp. (an undescribed species), was detected in field-collected *C. salviniae* adults in South Africa. The distribution and impacts of this disease on the weevil and its efficacy as a control agent were not known.

In this thesis, the prevalence, infection load, and impact of *Helicosporidium* sp. on *C. salviniae* was determined. In 2019, adult weevils were collected from 10 sites across the Eastern Cape, KwaZulu-Natal, Limpopo, and Western Cape provinces and screened to determine the occurrence, infection load, and geographic distribution of *Helicosporidium* sp. Transmission mechanisms of this disease in *C. salviniae* were then evaluated. The possible impact of *Helicosporidium* sp. was assessed by comparing the feeding rates and the reproductive output of the diseased and healthy adults of *C. salviniae*. An attempt was then made to eliminate the disease in *C. salviniae* through the application of the antibiotic, ketoconazole. Further, the role of temperature on infection load in *C. salviniae* was also assessed. Finally, recommendations for the long-term biological control programme against *S. molesta* in South Africa were made.

The disease covers the entire distribution range of *C. salviniae* in South Africa, with the disease occurrence rate ranging from 92.15% to 100% insects infected per site. *Helicosporidium* sp. was found to transmit vertically within the populations of *C.*

salviniae. Infection by the *Helicosporidium* sp. disease reduced the reproductive output of *C. salviniae* as well its impact on biomass reduction of *S. molesta* when a diseased culture was compared to a healthy culture from the USA. 98.44 to 98.55% of *Helicosporidium* sp. loads were reduced through multiple applications of ketoconazole concentrations under *in vitro* trials. *In vivo* treatments resulted in 70% control of *Helicosporidium* sp. in the adults of *C. salviniae* that were fed ketoconazole three times over a 21 day period. Adult *C. salviniae* feeding and survival performances were similar when fed fronds of *S. molesta* inoculated with ketoconazole and water. The lowest and highest disease loads of *Helicosporidium* sp. were recorded when the weevils were reared at 30°C and 14°C, respectively. As expected, the highest impact and reproductive output of *C. salviniae* were at 30°C.

The evaluations discussed in this thesis highlight the role of diseases in biological control agents, and gaps in both the pre-release and post-release monitoring that should integrate screening of diseases in these studies. Although the combined application of the antibiotic and temperature will reduce *Helicosporidium* sp. loads and impact, this technology is most likely only applicable where the weevils are reared in small numbers in a rearing facility and not really applicable to the field situation. It is important to release healthy agents that will cause efficient control of the target weed plant species, therefore, when introducing new biological control agents, the health status of such agents needs to be understood. Therefore, long-term field monitoring and assessment of the impact of *C. salviniae* on *S. molesta* should be conducted to track all the changes that may result due to the presence of *Helicosporidium* sp. This long-term monitoring and assessment will give a more informative role of *Helicosporidium* sp. in field populations of *C. salviniae*.

Acknowledgements

This PhD research was funded by the Department of Environment, Forestry and Fisheries: Natural Resources Management Programmes, NRF SARCHI (Insects in sustainable agricultural ecosystems) and Louisiana State University. I thank Rhodes University for its research facilities, and funds for various conferences that I attended.

My special appreciations go to my supervisors, Professors Martin Hill and Julie Coetzee for their mentorship throughout this PhD research study, I could not have made it without you. Most importantly, thank you for realizing my competency to conduct this challenging and interesting PhD research. God bless you!

Dr Grant Martin, thanks for giving me some study sites which I visited for my data collection. Dr Rodrigo Diaz, thank you so much for allowing me to use your research laboratory and equipment, these have helped me to complete major chapters of my PhD thesis. Dr Lenin Chari, thank you for making time to give me insights into R-statistics programme, I could not have made it without the statistical knowledge that you shared with me. Helen Holleman, I deeply thank you for your English corrections that you contributed throughout my PhD study.

Jeanne, thank you so much for always being cheerful, always ready to help and facilitating several purchases for my experiments as well as all the field trips I carried out. You have amazed me with all your kind help when I was outside of South Africa, I will always be grateful. You have made my PhD journey easier than I anticipated or expected. God bless you!

Thanks to Maretha (Waainek Research Station) and Thobeka (South African Sugar Research Institute) for maintaining the insects that you continuously supplied to me throughout my PhD research.

Whenever I felt like I could not carry on anymore, my God carried me through it all, and I give Him the praise.

Also, I thank my previous (MSc research study) supervisors' Drs Terence Olckers and David O. Simelane for introducing me in the biocontrol research fraternity of South Africa. Without the role that both of you played, I wouldn't have embarked on to this exciting PhD research in weed biocontrol.

My wife, Pelewe Itsweni, gave me all the support and encouragements which kept me strong and worked hard to the completion of this PhD research.

My family, father (Thinandavha Council Mphephu), mother (Tendani Mercy Mphephu) and siblings, thank you so much for giving me a place called home. Thank you again for everything that you have done for me. I also thank God for allowing me to achieve this level of education when you are still alive. I am also proud to be the first person to do this PhD research study in our entire royal family.

Most importantly, special thanks to members of the Centre for Biological Control, I cherished all the friendships and interactions we shared.

Lastly, many thanks to Dr. Michael Jukes for helping me with molecular biology experiments when I was doing some revisions of this thesis. I came to your lab with 0% knowledge of molecular tools and procedure and you were always there for me. You have greatly assisted me, and exceeded my expectations from you. Today I can do a lot of molecular work because of you, THANK YOU!.

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CHAPTER 1

General Introduction

1.1. Overview of the study

Several aquatic invasive alien plant species have been introduced into South Africa, during the past century, predominantly through trade as ornamentals (Julien *et al.*, 2009; Coetzee *et al.*, 2011; Hill & Coetzee, 2017). These weeds have proliferated and become widespread across the country's subtropical and more temperate regions (Coetzee *et al.*, 2011). Biological control programmes have been instituted against these aquatic weeds (Julien *et al.*, 2009; Coetzee *et al.*, 2011; Hill & Coetzee, 2017) with the first release of an agent, *Neochetina eichhorniae* Warner (Coleoptera: Curculionidae) on water hyacinth, *Pontederia* (*Eichhornia) crassipes* Mart.(Pontenderiaceae) in 1974, and additional agent releases against this weed between 1987 and 2013 (Hill & Coetzee, 2017). Biological control agents have also been released against *Salvinia molesta* D. S Mitch. (Salviniaceae), *Pistia stratiotes* L. (Araceae), *Myriophyllum aquaticum* (Vell.) Verdc. (Haloragaceae), *Azolla filiculoides* Lam. (Azollaceae) and *Egeria densa* (Hill *et al*., 2020).

Salvinia molesta, the focus of this study, has been successfully controlled by the weevil, *Cyrtobagous salviniae* Calder and Sands (Coleoptera: Curculionidae), across its distribution in South Africa and elsewhere in the world (Julien *et al.*, 2009; Coetzee *et al*., 2011; Sullivan *et al.*, 2011). A long-term quantitative post release evaluation of the effect of *C. salviniae* showed that while the insect has been able to reduce the impact of the weed across the country (Martin *et al*., 2018), the results were less spectacular than elsewhere in the world (Julien *et al.,* 2009). The exact cause for the discrepancy in these results remains unknown, but an infection by *Helicosporidium sp*. (Chlorophyta: Trebouxiophyceae), first suspected in South Africa *C. salviniae* populations by White *et al.* (2007), could be the reason.

This study assessed the described the morphological features of *Helicosporidium* sp.,

identified its taxonomic entity through molecular analysis and measured its effect on *C. salviniae* populations established on the invasive *S. molesta* in South Africa. The outcome of this study could serve as a warning to other countries to ensure that all agents used in biological control programmes against invasive alien plants are healthy.

1.2. Origin and distribution of *Salvinia molesta* in South Africa

Salvinia molesta is a perennial fern native to the south-eastern region of Brazil (Forno & Harley, 1979; Forno *et al.*, 1983), which has established as a weed across Africa, North America, the Caribbean and Oceania, and recently in some countries of Europe (Coetzee & Hill, 2020). In South Africa, *S. molesta* has established in Limpopo, Gauteng, Mpumalanga, Gauteng, KwaZulu-Natal, and the Eastern and Western Cape provinces (Fig.1.1).

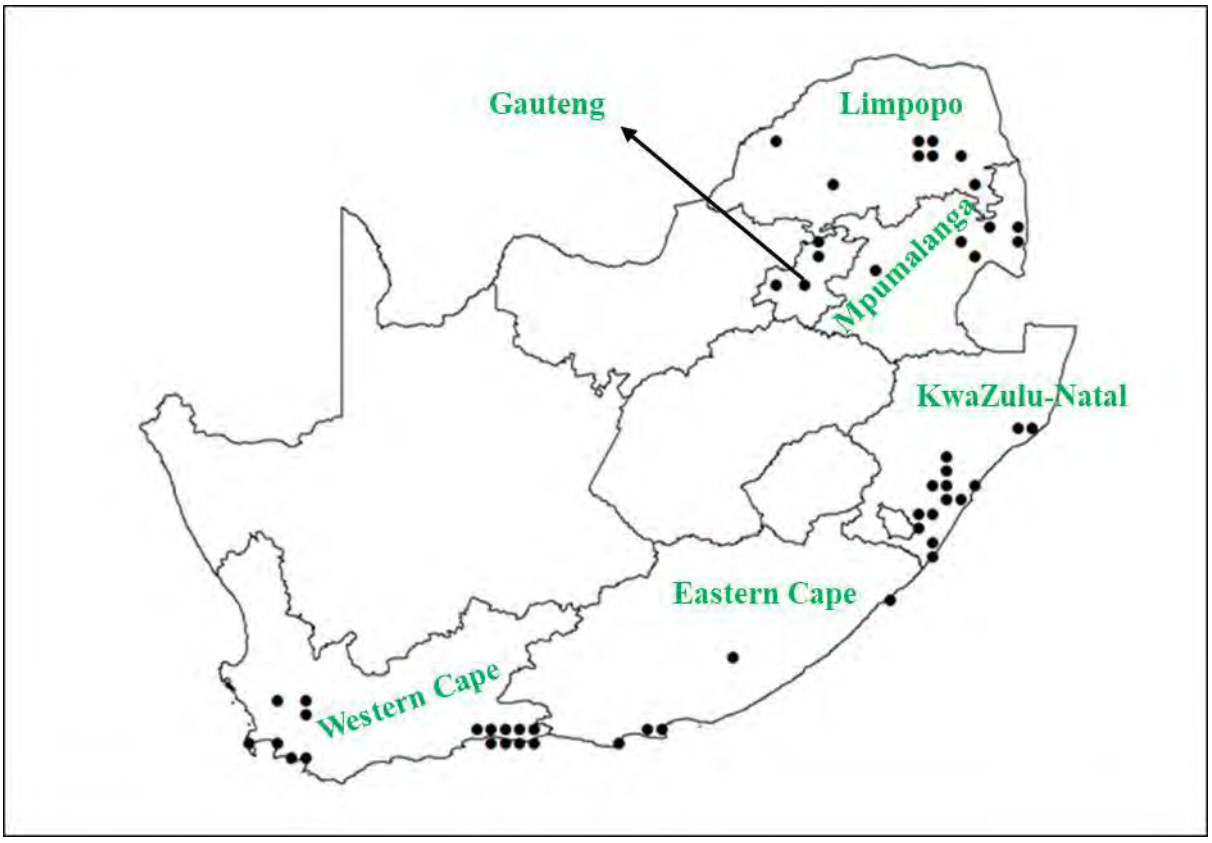

Figure 1. 1. Historical distribution of *Salvinia molesta* in South Africa (Source: Henderson, 2018). Black dots are sites of *Salvinia molesta*.

1.2. Biology of *Salvinia molesta*

1.2.1. Morphological features

Salvinia molesta is a free-floating fern with three static growth phases, *viz.* primary, secondary and tertiary, and at maturity is usually 30 cm in length (Fig. 2A–E) (Julien *et al.*, 2009). During the primary phase (small-leaved phase), *S. molesta* can access both open-water systems and other plant-inhabited water systems (Julien *et al.*, 2009). Eventually, both the secondary and tertiary phases develop and cause long-lived infestations as they grow and reproduce (Harley & Mitchell, 1981). The secondary and tertiary phases can be distinguished by slightly larger fronds (partially folded) and mature folded fronds (densely packed), respectively (Harley & Mitchell, 1981; Julien *et al.*, 2009).

Salvinia molesta fronds are arranged in whorls comprising three layers (of fronds) along a rhizome (Julien *et al.*, 2009). Floating fronds have oblong to obovate or orbicular lamina, a rounded or cordate base and emarginate apex, which can measure approximately 2.5 cm long (Julien *et al.*, 2009). *Salvinia molesta* develops lateral branches in crowded conditions and they become interlocked and form a mat that can measure up to 3–4 cm thick (Harley & Mitchell, 1981).

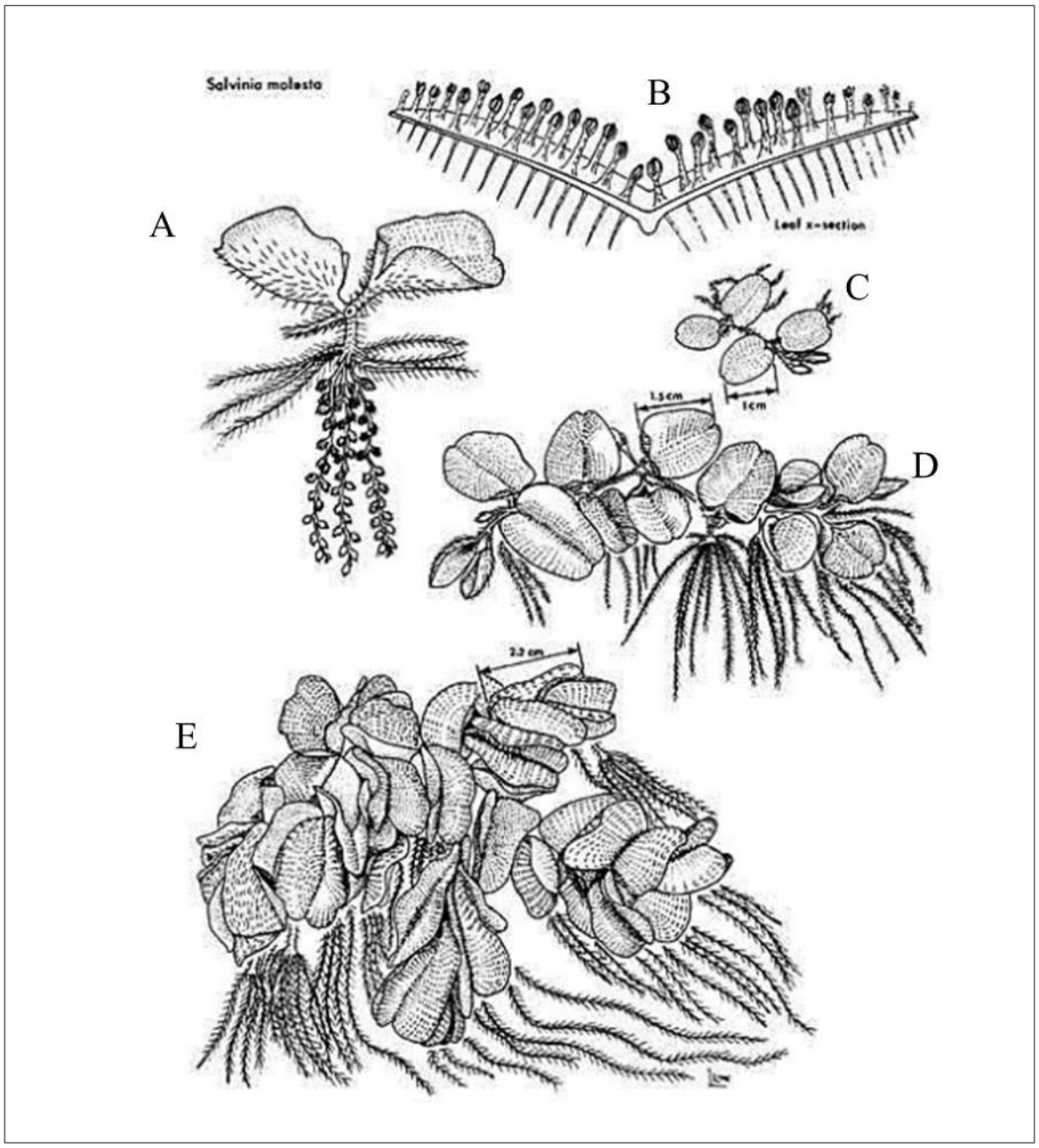

Figure 1. 2. Morphology of *Salvinia molesta*. (A) Individual plant, showing sporocarps, floating fronds (i.e., fronds), and rootlike submerged frond, (B). Floating frond, cross section, (C). Primary form, (D). Secondary form, (E). Tertiary form. (University of Florida, Center for Aquatic and Invasive Plants 2000).

1.2.2. Reproduction

Salvinia molesta is a sterile polyploid (Julien *et al.* 2009) and thus the mode of reproduction is asexual or vegetative. It reproduces solely from branched rhizomes. The ramets (daughter plants) can survive independently, but they are held together in colonies until the rhizomes

break (Room, 1988). When the species is introduced to new habitats, it produces colonizing stage plants which have thin stems and fragment easily, to produce new plants (Room, 1988); this makes the plant highly aggressive and competitive (Kammathy, 1968). Further, Kammathy (1968) argued that the species has the ability to replace water hyacinth and water lettuce, while Owens *et al.* (2005) reported that temperature is probably the greatest factor limiting reproduction of *S. molesta* and affects its growth, survival and spread.

1.2.3. Growth requirements

The growth of *S. molesta* is affected by water salinity, nutrients, and pH (Owens & Smart 2010). The known optimum pH for maximum growth of *S. molesta* ranges from 5.5–7.5 (Owens *et al.*, 2005). Since *S. molesta* acquires nutrients from the water column for its maximum growth, insufficient nutrients with increasing pH greatly reduce the growth rate of *S. molesta*. The nitrogen content of water plays a vital role in the growth and production of new buds of *S. molesta*. However, when nitrogen levels are too low or high, the required content of nitrogen in *S. molesta* buds impedes the productivity of the plant (Owens & Smart, 2010). In spite of the growth requirement parameters, *S. molesta* can grow in waters with a wide range of conductivity.

1.2.4. Survival attributes

In calm water bodies, rhizome fragmentation usually occurs during the senescence stages of *S. molesta* ramets, while harvesting, boating, wind and water currents break down all the plant stages and spread them further (Room, 1983, 1990; Whiteman & Room, 1991). Although fast-moving water carries and disperses *S. molesta* mats quickly infesting other parts of the water body, *S. molesta* also prevails in calm systems (Room, 1983, 1990; Whiteman & Room, 1991).

Salvinia molesta can survive temperatures from freezing to 43˚C and high salinity (Coetzee *et al.*, 2011; Harley & Mitchell, 1981). Further, *S. molesta* thrives in water systems that are polluted with agricultural and industrial run-off, or wastewater (Room, 1983). Dense

mats of *S. molesta* survive on soils that hold a high content of moisture when the water body dries up (Room, 1983). These attributes allow *S. molesta* to colonize various water bodies with different water levels and chemistry (Room, 1983).

1.3. Uses of *Salvinia molesta*

Salvinia molesta has been utilized as manure and as a supplementary fodder for various domesticated animals in some Asian countries (Oliver, 1993a). This species is capable of phytoremediation, and thus treating sewage effluent (Toerien *et al.*, 1983), and is likely to generate biogas (Room & Thomas, 1986; Syaichurrozi, 2017). However, none of these possibilities was developed to large-scale production, probably because of the huge costs associated with the suitable structures and labour (Room & Thomas, 1986; Syaichurrozi, 2017), and none has resulted in the control of the weed.

Salvinia molesta was once widely sold as an ornamental species within the European and Mediterranean Plant Protection Organization (EPPO) region (Harley & Mitchell, 1981; EPPO, 2017). Farmers have also reportedly relied on the dense growth mats of *S. molesta* for removing water pollution (Harley & Mitchell, 1981) because *S. molesta* mats are known to effectively remove excessive nutrients, predominantly nitrogen and phosphorus (Pistori *et al.*, 2010). Nonetheless, this approach has been rarely used due to the various economically detrimental effects associated with *S. molesta* (McFarland *et al*., 2004).

1.4. Ecological and economic impact of *Salvinia molesta*

Mats of *S. molesta* create a barrier to sunlight penetrating to other photosynthesizing plants beneath the water body (Harley & Mitchell, 1981). Further, the flow of water can be reduced, increasing sedimentation, particularly in narrow and shallow water systems (Room, 1983). The presence of the plant mat further reduces water quality by decreasing pH and dissolved oxygen while increasing CO_2 and H_2S concentrations (Mitchell, 1969). The mature, dying *S. molesta* mats create a decomposing bank of litter at the bottom of a water body, which decreases the

oxygen levels that aquatic invertebrates and vertebrates depend on (McFarland *et al*., 2004). Decomposition of *S. molesta* mats occurs at a low rate (Oliver, 1993a), which affects water body nutrient dynamics, and impacts all trophic levels by reducing suitable breeding sites of many organisms (Oliver, 1993b). Bird species that are under conservation are also greatly impacted by *S. molesta* infestations (Oliver, 1993b). For example, several endangered Hawaiian water bird populations have suffered from the destruction of their nesting sites by *S. molesta* mats (Oliver, 1993b).

Major economic impacts of *S. molesta* are its interference with engineering infrastructure, predominantly barrages, flood locks and drains, and in inhibiting livestock from reaching water (Oliver, 1993a). *Salvinia molesta* also affects the value of aquatic ecosystems that form part of ecosystem services to humans by reducing access to water bodies designated for recreational activities (e.g. swimming, fishing, boating or canoeing) (Oliver, 1993a). Further, *S. molesta* mats can encourage the accumulation of vectors that spread various diseases, particularly elephantiasis, encephalitis, malaria and dengue fever, as well as the snail-mediated disease, bilharzia (Oliver, 1993a).

1.5. Management of *Salvinia molesta*

Management of alien invasive weeds should be conducted soon after introduction while the infestations of the weed are still at non-significant levels (Olckers, 2004). Early intervention reduces costs involved in various management measures, particularly manual, chemical and biological control (Olckers, 2004). However, most invasive species are only noticed once they have become problematic throughout a wide range and therefore require wide scale management. When managing weed infestations, three strategies can be considered: eradication, maintenance control, or allowing the species to become integrated within its new range without intervention (Jacono *et al.*, 2001). Manual or chemical eradication can only be achieved through labour-intensive means (Jacono *et al.*, 2001). Consequently, the best

approach is to control and maintain alien weed infestations below environmental injury levels (Julien *et al*., 2002).

1.5.1. Chemical and manual control

Manual control conducted against *S. molesta* has been successful in reducing the plant infestations but requires annual repetition to keep *S. molesta* under control (Oliver, 1993a). Hand removal and giant nets have been successfully used in Australia to get rid of *S. molesta* mats (Oliver, 1993a). Mechanical harvesting is not economically viable compared to chemical control, and the large biomass associated with severe infestations can make the use of both harvesting machines and hand removal impractical (Oliver, 1993a). Removal using booms to accumulate or control the location of mats and machines to collect and remove the weed has been used in many instances, although rarely with great success, and always at great expense (Oliver, 1993a).

In South Africa, aquatic weeds in general have been controlled using mechanical and manual removal, herbicide application and biological control. Although manual removal can be successful, it is always labour intensive. For example, the Working for Water Programme of the Natural Resources Management Programmes of the Department of Environmental Affairs failed to control water weeds through manual removal measures despite investing significant resources (Van Wilgen & Wannenburgh, 2016).

In some parts of the world, including South Africa, the use of glyphosate has been shown to significantly control *S. molesta*, but applications should be repeated, otherwise re-infestation of *S. molesta* occurs (Kam-Wing & Furtado, 1977; Divakaran *et al.*, 1980; ; Fairchild *et al.*, 2002). To achieve substantial control with herbicide, skilled operators with the capability of maintaining a long-term follow-up programme to control re-infestation from scattered plants is required. Therefore, any herbicide programme against the weed requires a commitment to an ongoing operation of unlimited duration. It is the lack of a follow-up regime

that has often led to the failure of herbicidal control programmes (Hill & Olckers, 2001). Although herbicide application is often used as part of an integrated management approach (Hill & Coetzee, 2017), Hill *et al.* (2012) showed that several herbicide formulations used in South Africa were toxic to some of the established biological control agents on water hyacinth.

1.5.2. Biological control

Classical biological control programmes are aimed at selecting candidate biological control agents that show a minimal risk of feeding damage on non-target plants species (McFadyen, 1998; Wheeler *et al*., 2017; Mphephu *et al.*, 2017) and introducing them onto their respective target plant species. Because of this approach, biological control agents have been released against their prospective target plant species across the world (Wheeler *et al.*, 2017). In South Africa, this practice has been conducted against many alien invasive weeds for more than 100 years, with a good track record of success (Moran *et al.*, 2013). Therefore, as prolific as *S. molesta* is, with conspicuous adverse ecological impacts, biological control becomes the most viable option against this weed, as it establishes a permanent self-sustaining regulatory system at low cost (Olckers, 2004). *Salvinia molesta* was targeted for biological control in the 1980s in South Africa (Cilliers, 1991).

Most of the known biological control agents with the potential to control *S. molesta* were developed in Australia, not in South Africa (Forno & Bourne, 1985). As part of the development of the classical biological control programme in Australia, four candidate biological control agents were found to be specific and effective, and thus considered for release against *S. molesta* (Forno & Bourne, 1985; Thomas & Room, 1986a). The species collected from their native range in Brazil, included a grasshopper *Paulinia acuminata* De Geer (Orthoptera: Pauliniidae), a pyralid moth *Samea multiplicalis* Guenee (Lepidoptera: Crambidae), and the weevils *Cyrtobagous singularis* Hustache (Coleoptera: Curculionidae), and *Cyrtobagous salviniae* Calder & Sands (Forno & Bourne, 1985; Thomas & Room, 1986b).

Among these biological control agents, *C. salviniae* was the most effective insect agent and successfully controlled *S. molesta* in Australia. Although *C. singularis* was found a promising agent for *S. molesta*, its damage was not effective as that caused by *C. salviniae* on *S. molesta* (Sands & Schotz, 1985). In addition, where *C. singularis* failed to control the plant, *C. salviniae* was found to be very effective (Sands & Schots, 1985). Based on this track record, scientists from several parts of the world (*viz*., Paupa New Guinea, Namibia, Botswana, Zimbabwe) imported weevil colonies and released them on *S. molesta* infestations in their respective countries and achieved substantial control of *S. molesta* (Forno & Bourne, 1985; Room & Thomas, 1986).

The weevil was introduced into South Africa from the Eastern Caprivi, Namibia based on its success there (Cilliers, 1991; Julien *et al*., 2009; Coetzee *et al*., 2011). The decision to import *C. salviniae* and release it into South Africa was also motivated by its high degree of specificity with no record of non-target feeding damage (Thomas & Room, 1986b; Room & Fernando, 1992). The weevil has reduced infestations of *S. molesta* over the past three decades in South Africa, and resulted in substantial control of the plant (Hill & Coetzee, 2017).

1.5.2.1. *Cyrtobagous salviniae*

Cyrtobagous salviniae is a semi-aquatic weevil native to southern Brazil, Bolivia, Paraguay, Uruguay, and Argentina (Madeira *et al.*, 2006), and has been introduced into 16 countries across the world against infestations of *S. molesta* (Julien *et al.*, 2009; Sullivan *et al.*, 2011). Although *C. salviniae* is well known as a biocontrol agent of *S. molesta*, a smaller genotype of the weevil can also develop and complete its life cycle on *S. minima* (Tipping & Center, 2005).

Initially, a population of the weevil occurring on *S. minima* was identified in Florida in 1962 as *Cyrtobagous singularis* Hulst (Kissinger 1966). Calder and Sands (1985) then identified the Florida weevil population as *C. salviniae* during attempts to find a biological control agent and noted a size difference between North and South American populations. The

South American weevils are larger than those of the North American populations (Calder & Sands, 1985).

1.5.2.1.1. Life history of *Cyrtobagous salviniae*

The life cycle of *C. salviniae* was determined by Forno *et al.* (1983) (Fig 1.3A-D). The adult *C. salviniae* is black and can grow up to 2 mm in length, with a long snout which is about 2–3.5 mm long, and has numerous shallow punctures on the elytra. Teneral adults appear brown in colour for the first few days then turn shiny black. Adults feed on and between fronds, within the buds, causing ragged holes in the fronds of the salvinia plant. While underwater, the weevils aquire oxygen from a thin film of air held beneath the body, and they periodically come to the surface to replenish this film of air.

Females are generally larger than males adults, and can live 3–5 months at temperatures between 23°C and 25°C, while cooler weather often extends their longevity (Sands *et al*., 1986). Shallow holes chewed in salvinia buds, fronds, and rhizomes are suitable oviposition sites of adult females. At a constant 25.5 °C, adult female pre-oviposition lasts for about 6–14 days after they emerge as adults (Forno *et al*., 1983). Under favourable conditions, females deposit an average of 208–374 eggs during a lifespan that lasts for 20–36 weeks (Sands *et al*., 1986; Jayanth, 1989; Eisenberg, 2011). Female egg production increases with nitrogen content of the plant (Sands *et al*., 1986). Eggs are shaped like beans and are about 0.5 mm long. Egg incubation period lasts for about 10 days at 25.5 °C (Forno *et al.*, 1983).

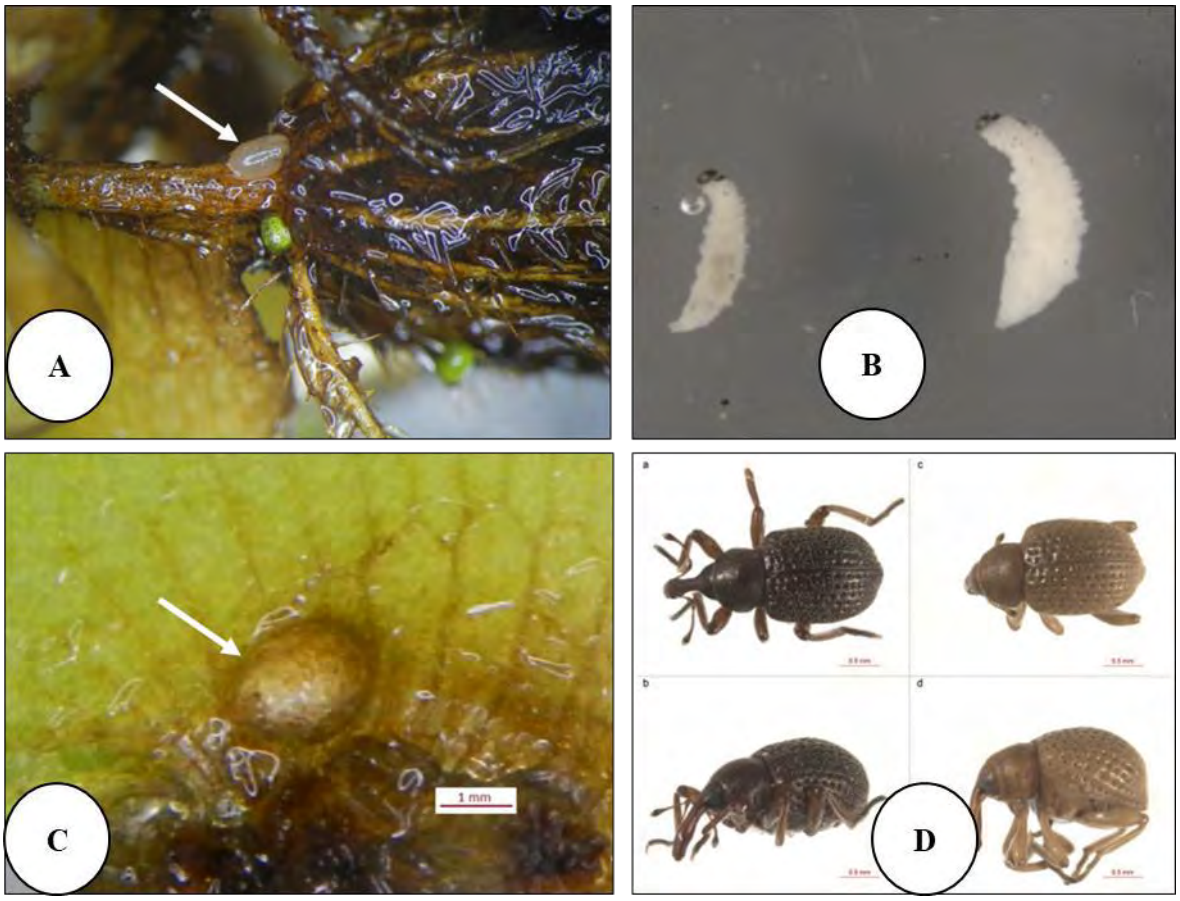

Figure 1. 3. The lifecycle of *Cyrtobagous salviniae*. (A) Egg, (B) larval stages, (C) pupa, and (D) adult (Source: Bug Biz at www.lsuagcenter.com).

Young larvae feed externally on roots and in leaf buds during the first one to two weeks and then tunnel inside the rhizomes or within the base of the leaf and leaf petiole, for further feeding. Internal feeding in the rhizomes is cruicial as these sites constitute higher concentrations of nitrogen, required for larval development (Sands *et al*., 1983). The larval period lasts for about 23 days at 25.5 °C. Full-grown larvae are white with a brown head and are about 2.6 mm long, and often exit the plant and pupate on on the surfaces of rhizomes or root masses. The pupation period lasts 10 to 15 days. Development from egg to adult requires ~ 45 days at 25.5 °C (Forno *et al.*, 1983).

1.5.2.1.2. Distribution and impact of *Cyrtobagous salvinia* on *Salvinia molesta*

The control of *S. molesta* in various tropical and subtropical areas of Africa, and Australia has been accomplished by *C. salviniae* (Room & Thomas, 1986; Thomas & Room, 1986b; Room,

1988). For example, in Australia, infestations of *S. molesta* of about 30 000 tonnes, were cleared within 11 months of initial releases of *C. salviniae*, sourced from Brazil in 1980 (Room, 1990). *Salvinia molesta* infestations have been successfully controlled by this weevil in more than 18 countries (Julien *et al.*, 2009; Winston *et al.*, 2014).

1.5.2.1.3. Impact of *Cyrtobagous salviniae* against *Salvinia molesta* in South Africa

The biological control programme for *S. molesta* in South Africa commenced with the release of *C. salviniae* collected from Eastern Caprivi, Namibia, in 1985 (Cilliers *et al.*, 2003). Despite concerns that the climatic conditions would limit the establishment of *C. salviniae* against *S. molesta*, control of the weed was achieved throughout the Limpopo, KwaZulu-Natal, Mpumalanga and the Eastern and Western Cape provinces (Figure 1.4) (Forno & Bourne, 1985; Coetzee *et al.*, 2011; Hill & Coetzee, 2017). During the late 1990s, biological control of *S. molesta* was regarded as highly successful, and it was concluded that no further control measures were necessary (Cilliers *et al.*, 2003; Coetzee *et al.*, 2011; Hill & Coetzee, 2017). However, further releases of the weevil to newly infested sites were encouraged.

Figure 1. 4. Example of impact of *Cyrtobagous salviniae* on *Salvinia molesta* (source: Grant D. Martin and M. Parkinson, 2009-2011).

1.5.2.1.4. Factors affecting the performance of *Cyrtobagous salviniae*

Both temperature and nutrient levels affect performance of *C. salviniae* against *S. molesta* (Sands & Kassulke, 1984; Julien *et al.*, 2009; Coetzee *et al.*, 2011; Hill & Coetzee, 2017). Feeding damage by *C. salviniae* can be less effective on *S. molesta* fronds when the nitrogen levels in the fronds are either too high or too low (Room & Thomas, 1986; Thomas & Room, 1986b; Room, 1988). Fluctuations of temperature and nitrogen can inhibit oviposition and embryonic development of *C. salviniae* (Forno & Bourne, 1985; Room *et al.*, 1989). This further implies that, in water systems that continuously receive excessive input of nitrogen rich water, *C. salviniae* is unlikely to achieve control of *S. molesta*.

Another concern was the possibility that native parasitoids or predators could influence the efficacy of *C. salviniae* against *S. molesta*. Hill and Hulley (1995) reported that about 40% of established biocontrol agents in South Africa were attacked by parasitoids on their respective alien invasive plant species. High levels of predation by ant species were also expected, as the imported red fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), impacted the effectiveness of *C. salviniae* released on *S. minima* in southern Louisiana (Parys & Johnson, 2012). In South Africa, there have been no known predation impacts by any ant species in the field populations of *C. salviniae*, despite collections of unidentified spiders observed feeding on the weevil cultures at the Centre for Biological Control's Waainek Mass Rearing Facility at Rhodes University (pers. obs.). Although predation of *C. salviniae* by some spider species is known, the prospects of such spider species hampering the efficacy of *C. salviniae* populations against *S. molesta* in South Africa remains extremely low. Further, predation of *C. salviniae* by any other species has never been detected during the post-release assessment of the weevil on *S. molesta* over the past nine years (Martin *et al.*, 2018).

While no predation, or parasitism by insect parasitoids have been recorded, infection by a parasitic green alga *Helicosporidium* sp. infection was detected during screening of *C.*

salviniae populations from South Africa, imported to quarantine in Florida, USA, (White *et al.*, 2007). Nonetheless, it was not confirmed whether the weevils were imported with the infection or they encountered the infection while feeding on the USA plant material (White *et al*., 2007). Although unpublished data by Denton et al. (accessed in 2018) showed the presence of *Helicosporidium* sp. in South African *C. salviniae* populations, further confirmation for the presence of *Helicosporidium* sp. was needed. *Helicosporidia* are unique invertebrate parasites considered to be protists by Keilin (1921), fungi by Weiser (1964) but identified recently as the first invertebrate pathogenic green algae (Tartar *et al*., 2002). Despite annual quantified post-release evaluations on *C. salviniae* since 2008 (Martin *et al.*, 2018), infection levels of *Helicosporidium* sp. or its possible impact on the weevil were not considered. However, Denton et al. (unpublished data, accessed in 2018) investigated the effect of temperature and food quality on *Helicosporidium* sp. infection in mass-reared *C. salviniae* and concluded that the infection posed a threat to the weevil's development, fecundity and longevity, but this was not explicitly tested.

Although *C. salviniae* colonies that were initially released on *S. molesta* in Australia and Namibia were collected from the weevil's native range (Cilliers, 1991), White *et al.* (2007) showed that the *Helicosporidium* sp. infection occurred only in *C. salviniae* collected in South Africa, while weevils from Florida and Australia were free from the infection. These findings support speculation that the *Helicosporidium* sp. infecting *C. salviniae* populations in South Africa could be native to South Africa.

Helicosporidium could well have a negative impact on *C. salviniae*, as other species/isolates of the disease are known to cause increased mortality in beetles. For example, *Helicosporidium* sp. resulted in a high mortality rate in *Rhizophagus grandis* Gyll. (Coleoptera: Rhizophagidae) populations, making them less effective in controlling the weevil, *Dendroctonus micans* (Kugelann) (Coleoptera: Curculionidae) in Turkey (Yaman & Radek,

2007; Yaman, 2008). *Helicosporidium* spp. has been shown to be orally transmitted in four noctuid hosts, *Heliocoverpa zea* (Boddie), *Spodoptera exigua* (Hubner), *Trichoplusia ni* (Hubner) and in *Carpophilus mutilates* (Coleoptera: Nitidulidae) (Bläske & Boucias, 2004).

1.6. *Helicosporidium*

Helcosporidium is a genus of pathogenic green algae which infects various host species, particularly arthropods, insects, mites, crustaceans and trematodes (Keilin, 1921; Sayre & Clark, 1978; Purrini, 1984; Avery & Undeen, 1987; Pekkarinen, 1993), through gut epithelial and hemolymph tissue (Yaman & Radek, 2007).

1.6.1. Description and taxonomic positions of *Helicosporidium* spp.

Helicosporidium was initially detected in the ceratopogonid midge, *Dasyhelea obscura* (Diptera: Ceratopogonidae) and described as *Helicosporidium parasiticum* (Keilin, 1921). More recently, *Helicosporidium* spp. have been isolated from a wide host range, including Coleoptera, Collembola, Diptera, Lepidoptera and mites (Keilin, 1921; Sayre & Clark, 1978; Purrini, 1984; Pekkarinen, 1993; Seif & Rifaat, 2001; Yaman & Radek, 2007). The taxonomy of *Helicosporidium* remains ambiguous, with only *H. parasiticum* recognized (Tartar, 2013).

The first described *H. parasiticum* was classified as a protozoan (Keilin, 1921). Later, Weiser (1970) positioned *H. parasiticum* in the lower fungi class. Most recently, the vegetative development of *Helicosporidium* was matched with the autosporogenic growth of unicellular algae (Boucias *et al.*, 2001). A genetic study examined the genus *Helicosporidium* and classified it as a member of the green algal group, Trebouxiophyceae (Chlorophyta), and further depicted a novel clade of invertebrate pathogens (Boucias *et al.*, 2001, 2002). However, the trebouxiophytes are largely photosynthetic and free-living (Boucias *et al.*, 2001), while their congener *Helicosporidia* (*Prototheca*) are achlorophyllous, non-photosynthetic algae, which specifically infect invertebrate species (Boucias *et al.*, 2001; Tartar, 2013).

To date, in the order Coleoptera, four *Helicosporidium* spp. isolates have been detected

(Conklin *et al*. 2005). Among these isolates, the first one was detected from *Carpophilus mutilatus* Erichson (Coleoptera: Nitidulidae) and was able to infect six nitidulid species, five pyralid lepidopterans, one culicid dipteran and three mite species in Texas (Lindegren & Hoffmann, 1976). A second isolate was identified from adults of the scarabaeid *Oryctes monoceros* (Coleoptera: Scarabaeidea) in Tanzania (Purrini, 1984), and a third isolate was identified from the German bark beetle, *Dendroctonus micans* (Coleoptera: Scolytidae) (Yaman & Radek, 2007).

1.6.2. Life history of other *Helicosporidium* spp.

Helicosporidium parasiticum was studied to understand and represent the life history of other *Helicosporidium* species/isolates. *Helicosporidium* infection appears in its host in three different forms: a mature cyst, a filamentous cell, and vegetative spore stages (Fig. 1.5) (Lindegren & Hoffmann, 1976; Tartar & Boucias, 2004) . Usually, the mature cyst is ingested by a host, and the host's digestive fluid induces the cyst to rupture and release ovoid cells, which differentiate into filamentous cells and pass through the columnar epithelium to establish in the hemocoel (Boucias *et al*., 2001).

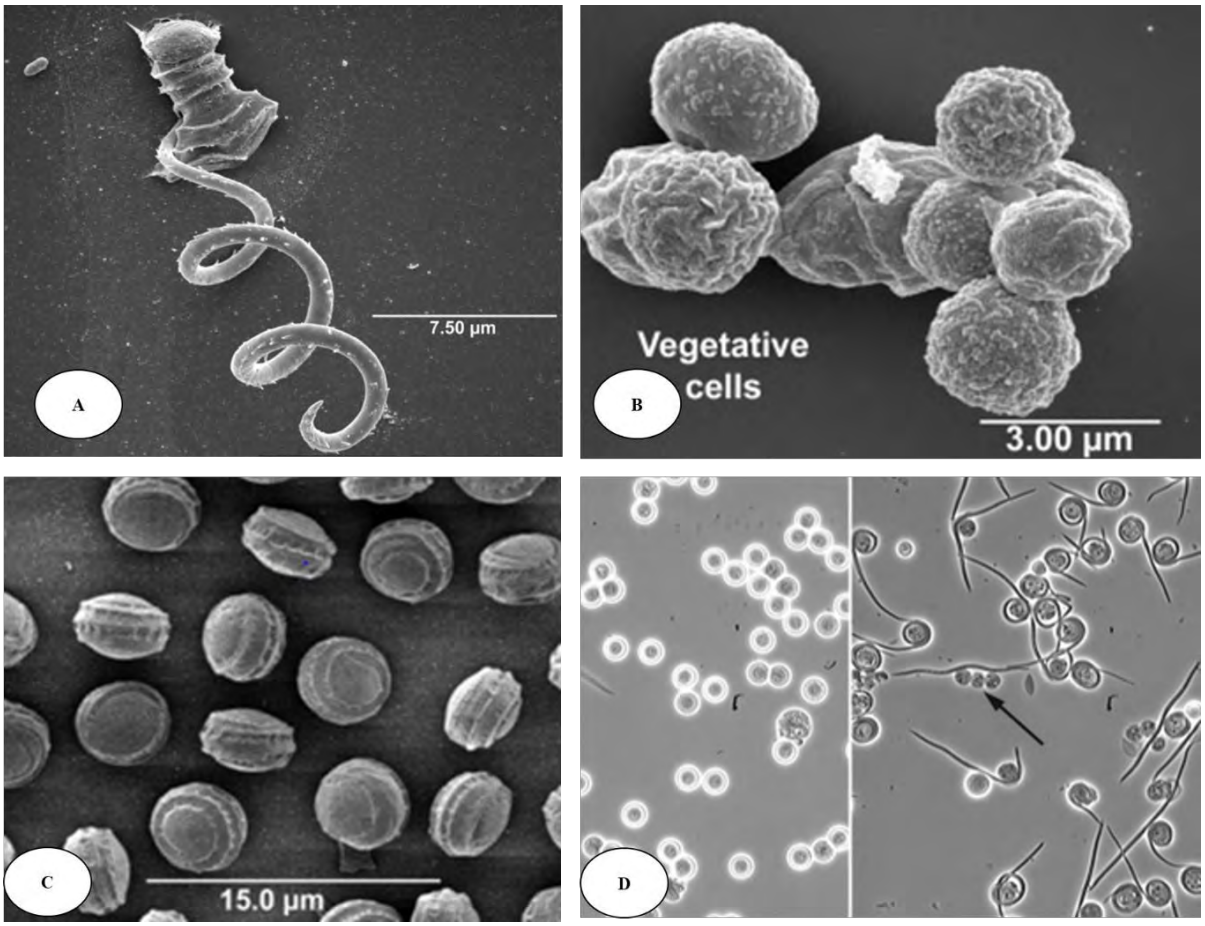

Figure 1. 5. Scanning electron microscopy (SEM) and light microscopy (LM) of life stages of *Helicosporidium* sp. cells. (A) filamentous, (B) vegetative, (C) mature cyst, and (D) mature cyst, captured from light microscope prepared from fresh smears, (source: Boucias *et al*., 2001).

Surfaces of the filamentous cells have conspicuous barbs, which are believed to facilitate mobility through the midgut epithelial layer of a host (Boucias *et al.*, 2001). Eventually, the filamentous cell shatters and produces four to eight daughter cells (Lindegren & Hoffmann, 1976; Boucias *et al.*, 2001), which further differentiate into vegetative spores and infect the entire host (Lindegren & Hoffmann, 1976; Boucias *et al.*, 2001). In the last developmental stage of the infection, the vegetative spores differentiate into the mature cyst and vacate the host insect (Lindegren & Hoffmann, 1976; Boucias *et al.*, 2001).

1.6.3. Transmission of *Helicosporidium* sp.

Helicosporidium spp. cysts are known to be infectious when ingested by the host (Kellen & Lindegren, 1974). This implies that the cysts can persist freely in different environments

outside the susceptible host species, a view supported by Yaman (2008) who reported that the cysts are most commonly contracted through feeding on infected plant material or prey. Once ingested, the cysts rupture and produce three ovoid and one filamentous cell into the midgut of the host (Kellen & Lindegren, 1974). The pH levels of the host are often responsible for triggering cysts to dehisce (Boucias *et al.*, 2001). Further, studies by Boucias et al. (2001) and Bläske-Lietze and Boucias (2005) have showed that it is the filamentous cell which passes through the midgut epithelium and then initiates *Helicosporidium* infection. Once across the midgut, the filamentous cell divides and releases the first vegetative cells of *Helicosporidium* infection (Bläske-Lietze & Boucias, 2005).

Cysts are released after the death of the host (Bläske & Boucias, 2004). The cyst cells enable horizontal infection from one insect to another in a single population (Denton *et al.*, 2009). *Helicosporidium* cells have not been observed in relation to reproductive organs (De Koning, 2006). Vertical transmission rates are quite low, and it seems that vertical transmission does not play a vital role in the spread of *Helicosporidium* infection (De Koning, 2006). *Helicosporidium* probably relies on the host's death for the cysts to be released and spread into new environments (Blaske-Lietze *et al.*, 2006). The mechanism which encourages cyst dispersal, however, is still unknown (Blaske-Lietze *et al.*, 2006) and is important to ascertain as it influences the spread and multiplication of the infection in an insect species population.

The mode of transmission of *Helicosporidium* sp. involves ingestion and vertical mechanisms (Yaman & Radek, 2007). For example, vertical transmission of *Helicosporidium* sp. infection occurred in an infection isolate transmitted orally from *C. salviniae* to *Helicoverpa zea*, *Spodoptera exigua* and *Trichoplusia ni*, and the infection was subsequently detected in 41.7% of their progeny (Bläske & Boucias, 2004).The parasite can be transmitted from prey to predator; for example, populations of *R. grandis* showed high infection rates after feeding on its infected prey, *D. micans* (Fielding & Evans, 1997). Another possible means of transmission

could be through mating, but this has never been investigated.

1.6.4. Host range of *Helicosporidium* spp.

Helicosporidium spp. has a wide host range within the invertebrate populations and has been detected in the Coleoptera, Diptera, and Lepidoptera (Tartar, 2013). Transmission of *Helicosporidium* sp. isolates from the nitidulid beetle, *Carpophilus mutilates,* into other nine coleopterans, five lepidopterans and one dipteran species showed successful infection, but failed to infect when transferred to orthopteran and hymenopteran insects (Lindegren & Hoffmann, 1976). This suggests that the host range of *Helicosporidium* sp. is likely to be restricted to the three orders: Coleoptera, Lepidoptera and Diptera (Tartar, 2013). Among these orders, a total of 23 insects species in 11 families and 16 genera have been field-detected as hosts susceptible to *Helicosporidium* sp. infection; 13 of them are within Coleoptera (Tartar, 2013) (Table 1.1).

A study aimed at measuring the distribution and abundance of *Helicosporidium* sp. in the coleopteran host beetle, *Dendroctonus micans*, found the infection to be widely distributed, with about 71% rates of infection of the screened beetles across the geographic range of the beetle (Yaman, 2008). Further, the predatory beetle, *Rhizophagus grandis*, of *D. micans* has also shown high rates of *Helicosporidium* sp. infection (Yaman, 2008). These findings have certainly illustrated that, despite the interactions (insect predator-insect prey relationship) between *R. grandis* and *D. micans*, both the beetle species are the hosts most preferred by the infection (Yaman, 2008). Globally, establishing a wide understanding of the distribution, abundance, and impact of *Helicosporidium sp*. would certainly reveal the role of the infection amongst the coleopterans, and other susceptible invertebrate orders (Tartar, 2013).

Table 1. 1. Summary of some of the susceptible insect species (Coleoptera) to *Helicosporidium* sp. infection across the world.

Species name	Family	Location	Field detected host	Laboratory detected host	References
Dendroctonus micans Kug.	Curculionidae	Europe; Asia	1	0	Yaman & Radek, 2007
Rhizophagus grandis Gyll.	Monotomidae	Europe; Asia	1	0	King & Evans, 1984; Gregoire et al., 1985; Fielding et al., 1991; Yuksel, 1996
Carpophilus mutilates (Erichson)	Nitidulidae	Texas	1	0	Kellen & Lindegren 1973; Kellen & Lindegren 1974; Lindegren & Hoffmann, 1976)
Trogoderma variable Ballion	Dermestidae	Texas	0	1	Kellen & Lindegren 1973; Kellen & Lindegren 1974; Lindegren & Hoffmann, 1976)
Oryzaephilus surinamensis (L.)	Silvanidae	Texas	0	1	Kellen & Lindegren 1973; Kellen & Lindegren 1974; Lindegren & Hoffmann, 1976)
Lasioderma serricorne (F.)	Ptinidae	Texas	0	1	Kellen & Lindegren 1973; Kellen & Lindegren 1974; Lindegren & Hoffmann, 1976)
Oryctes Monoceros OL.	Scarabaeoidea	Tanzania	1	0	
Cyrtobagus salviniae Calder & Sands	Erirhinidae	Florida; South Africa	1	0	White *et al*., 2007;
Carpophilus dimidiatus (Fabricius)	Nitidulidae	USA	1	0	Lindegren & Okumura, 1973
Carpophilus freeman (Fabricius)	Nitidulidae	USA	1	0	Lindegren & Okumura, 1973
Carpophilus remipterus (Fabricius)	Nitidulidae	USA	1	0	Lindegren & Okumura, 1973
Carpophilus mutilates (Fabricius)	Nitidulidae	USA, Mexico	1	0	Lindegren & Okumura, 1973; Kellen & Lindegren, 1973
Carpophilus pilosellus (Fabricius)	Nitidulidae	USA	1	0	Lindegren & Okumura, 1973
Conotelus stenoides Erichson	Nitidulidae	USA	1	0	Lindegren & Okumura, 1973
Stelidota geminate Erichson	Nitidulidae	USA	1	0	Lindegren & Okumura, 1973
Urophorus humeralis (Fabricius)	Nitidulidae	Mexico	1	0	Lindegren & Okumura, 1973

0= no; 1= yes.

1.6.5. Attributes and impacts of *Helicosporidium* sp.

The primary sign of *Helicosporidium* sp. infection is a milky white haemolymph (Sayre & Clark, 1978; Boucias *et al.*, 2001), attributed to the reduction of most of the host insect's internal organs (Conklin *et al*. 2005). Usually, an insect infected by *Helicosporidium* sp. moves conspicuously slowly and exhibits severely retarded growth (Keilin, 1921; Hembree, 1981; Conklin *et al*., 2005), malformed adult wings, lower longevity and fecundity (Bläske & Boucias, 2004). This infection poses threats to insects that contribute towards ecosystem services such as decomposition of organic materials, pollination, and biological control. For example, in an experiment conducted to test the effects of *Helicosporidium* sp. infection, reduced fecundity was observed among the infected *S. exigua* adults as well as the transmission of the infection to *S. exigua* progeny (with no evidence of invasion of the reproductive tissue) (Bläske-Lietze & Boucias, 2005). Another study reported a reduction in the body size of mosquitoes and citrus root weevils after exposure to two different isolates of the infection (Conklin *et al*. 2005). Another study reported that the worst effect of *Helicosporidium* sp. was the rupturing of insect body cuticle, chiefly when the insect is heavily infested with the infection (Conklin *et al*. 2005). The rupturing allows the mature cysts of the infection to vacate a host insect into the water while rendering the host insect prone to other diseases and abiotic (e.g., temperature) effects. This suggests that prospects remain high for the newly hatched larvae and adults being prone to the infection through their thin body cuticle.

Similar disease organisms, *Prototheca wickerhamii* K. Tubaki & M. Soneda and *P. zopfii* W. Krüger (Chlorellales: Chlorellaceae) persist as cysts under different environmental conditions (e.g. freezing to hot temperatures; variation of chemical properties and radiations) (Tartar *et al*., 2002, 2003). The prospects for all other stages of *Helicosporidium* sp. infection to respond to the different chemical properties (i.e., antibiotics, aquatic and terrestrial contamination of chemicals) remain high, while the cysts would remain resistant.

The attaributes and impacts of *Helicosporidium* sp. can be measured using different methods to study its germination, development and replication. There are several methods including Koch's postulate (*In vivo*) (Vega & Kaya, 2012) and *In vitro* experiments that have been used successfully to

culture *Helicosporidium* sp. When diagnosing, haemolymph or gut fluids of the infected insects are extracted and propagated in *In vivo* and *In vitro* experiments (Boucias *et al*., 2001). *In vivo* propagations involve the transfer of haemolymph or gut fluids from its host into the larvae of a suceptible insect species for the infection replication (Boucias *et al*., 2001). *In vivo* experiments further help to determine the impact of the infection in the innoculated insect species (Boucias *et al*., 2001). However, *In vitro* propagations are conducted on glass slides, tubes or in petridishes using midgut and haemolyph fluids extracted from the infected insect species (Boucias *et al*., 2001). *In vitro* propagations that uses the purified cells of *Helicosporidium* sp. are often provided with insect cells suplimented with 10% fetal calf serum for an optimum growth medium (Boucias *et al*., 2001; Lynn *et al*., 1982). Other researchers have reported the ability of *Helicosporidium* sp. to develop in various simple salt solutions with pH between 7.0 and 10 (Hembree, 1981; Keilin, 1921). In other studies, culturing of *Helicosporidium* sp. in salt solutions was done to test the adaptabilty of this infection to survive in saline aquatic environments (Hembree, 1981).

Helicosporidium sp. cells are readily detectable from fresh smears prepared from disected or squashed adult, larva or pupa in Ringer's or phosphate buffer solutions mixed with different stains (Yaman, 2008; Keilin, 1921; Kellen & Lindegren, 1974). Lacey and Solter (2012) have reported that, spores of *Helicosporidium* sp. can also be germinated by drying and wetting them in purified water mixed with Giemsa stain to differentiate their developmental pathways. Different types of stains can be used when diagnosing *Helicosporidium* sp. cells, but, filamentous cells of *Helicosporidium* sp. respond differently when exposed to different stains (Keilin, 1921). Further, the nature of *Helicosporidium* sp. mature cyst refractile depends on the screening methods or culturing solutions (Keilin, 1921). Similarly, many different refractile apperences of *Helicosporidium* sp. cysts have been published (Figure 1.6) (Yaman, 2008; Keilin, 1921; Kellen & Lindegren, 1974; Boucias *et al*., 2001; White *et al*., 2007).

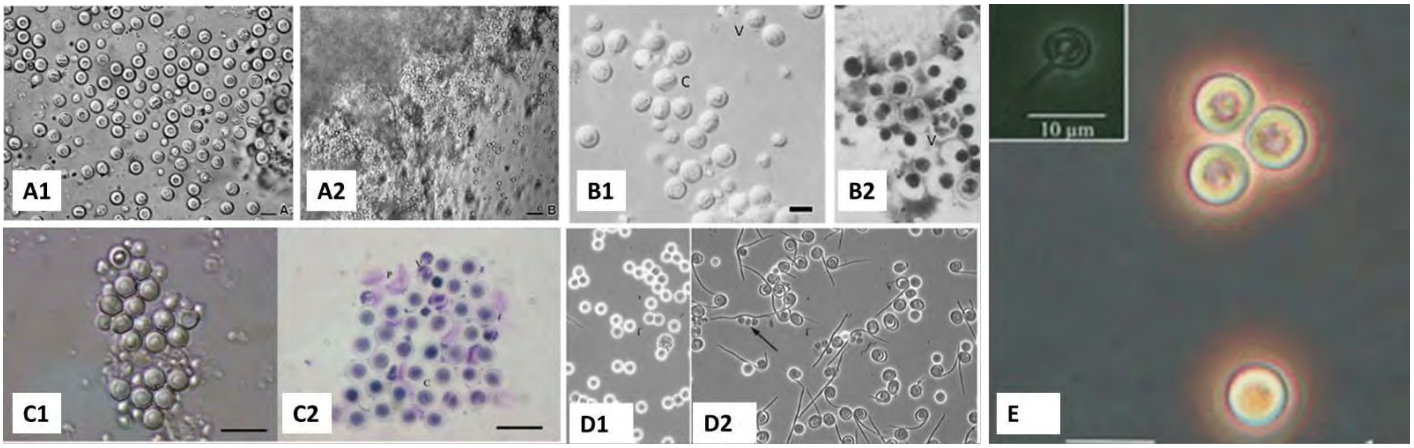

Figure 1. 6. Light micrographs of *Helicosporidium* sp. cyst and filamentous cells (some are Giemsa-stained cells) cells isolated from different host insect species (A) A1 and A2 Cyst cells by Yaman and Radek (2007), (B) B1 and B2 Cyst cells by Yaman et al. (2009), (C) C1, Cyst cells and C2, Giemsa-stained cyst cells by Yaman (2008), (D) D1, Cyst cells and D2, filamentous cells by Boucias et al. (Boucias et al. (2001) and (E) Cyst and filamentous cells by White et al. (2007). Vegetative cell. Scale bars: 10–50 µm.

Most importantly, either the freshly isolated or cultured cells of *Helicosporidium* sp. could be subjected to further molecular studies. The use of molecular tools to characterize cells of *Helicosporidium* sp. provides the more precise identification of this infection. Several studies have used different and similar molecular methods to detect and study the cells of *Helicosporidium* spp. (Mancera *et al*., 2012; Ueno *et al*., 2005; Roesler *et al*., 2006; Tartar, 2002). These methods could be integrated in studying the attributes of *Helicosporidium* sp. in the present study.

1.6.6. Management of *Helicosporidium* spp.

There have been no attempts to manage or control *Helicosporidium* spp. infections in insects. Although there are no available control options for *Helicosporidium* sp., there are other effective mechanisms that have been used against other diseases that infect insects (Bassi, 1835, 1836; Montllor *et al.*, 2002; Russell & Moran, 2006). These options include the use of antibiotics or heat treatments (Bassi, 1835, 1836; Montllor *et al.*, 2002; Russell & Moran, 2006). However, the lack of a single successful treatment of *Helicosporidium* sp. suggests that, this infection could not have drastic impacts on the susceptible insect species.

1.7. Aims and rationale of this study

The aim of this study is to determine the role of *Helicosporidum* sp. infection on populations of *C.*

salviniae in South Africa. Studies conducted by White *et al.* (2007) and Denton *et al.* (unpublished data, accessed in 2018) on *Helicosporidium* sp. isolated from *C. salviniae* supported the premise that the infection has the ability to influence the efficacy of *C. salviniae* against *S. molesta*. Although the presence, occurrence, distribution, and impacts of *Helicosporidium* sp. are widely known, the spread of this infection and its effects on *C. salviniae* remain unknown. Thus, the presence of *Helicosporidium* sp. was determined in Chapter 2 through describing morphological features and the molecular characterization of this infection. The distribution and infection load of *Helicosporidium* sp. in the populations of *C. salviniae* in South Africa, was determined in Chapter 3. Understanding the distribution and loads of *Helicosporidium* sp. infection is important in justifying interventions and developing appropriate control strategies. Therefore, it was important to study the transmission mechanisms of *Helicosporidium sp*. at different life stages of *C. salviniae*, with the purpose of identifying the transmittable cell of *Helicosporidium* sp. and its replication in *C. salviniae*; this investigation is described in Chapter 4.

Cyrtobagous salvinae was not screened for *Helicosporidium* sp. prior to its release into South Africa and its potential impact was unknown. Although the performance of *C. salviniae* might be reduced, *S. molesta* is still under excellent biological control. Thus, the experiments in chapter 5 quantify the effect of *Helicosporidium* sp. infection on *C. salviniae*. Understanding the effect of *Helicosporidium* sp. on *C. salviniae* populations would enable the study to predict whether *S. molesta* would likely escape control by *C. salviniae*. However, it is crucial to ascertain the mechanisms in which *Helicosporidium sp*. is likely to transmit and thrive within *C. salviniae* populations.

Finally, and most importantly for the programme, the effect of antibiotics and heat treatments on *Helicosporidium* sp. in *C. salviniae* colonies were tested (Chapter 6 and 7, respectively).

Suggestions on how to advance the control of *Helicosporidium* sp. in *C. salviniae* populations in South Africa and the direction of future research, as well as the implications of this study for other biological control programmes, are discussed in the general discussion chapter (Chapter 8).

CHAPTER 2

The morphological and molecular characterization of *Helicosporidium* sp. isolated from *Cyrtobagous salvinae*

2.1. INTRODUCTION

White et al. (2007) isolated *Helicosporidium* sp. in the USA from *Cyrtobagous salvinae* collected in South Africa. However, it was not clear from that study if *Helicosporidium* sp. had originated in South Africa, or the insect had possibly become contaminated in the USA. Therefore, the focus of the present chapter was to use gross morphological features and molecular techniques to confirm the presence of *Helicosporidium* sp. in *C. salviniae* collected in South Africa.

The taxonomy of *Helicosporidium* sp. is not complete and thus makes it difficult to understand the relationships between known species of this group and other similar genera (Tartar *et al*., 2002; Bläske-Lietze, 2006, 2005; Keilin, 1921). Morphological features of *Helicosporidium parasiticum* have guided many studies in identifying the cells of *Helicosporidium* spp. in various invertebrate hosts (Tartar *et al*., 2002; Bläske-Lietze, 2006, 2005; Keilin, 1921), and recently, rDNA or genomic DNA (molecular) extraction and analysis procedures have also been established to detect the presence of *Helicosporidium* sp. (Mancera *et al*., 2012; Tartar, 2002). These molecular procedures help place *Helicosporidium* spp. with other closely related infectious species.

Several studies have reported monophyletic and paraphyletic relationships of *Helicosporidium* sp. with other algae mostly, *Prototheca* spp., and *Chlorella* spp. (Mancera *et al*., 2012; Tartar, 2002). Genetic analyses at the 18S region showed that *Helicosporidium* and *Prototheca* arise from a common non-photosynthetic ancestor (Mancera *et al*., 2012; Ueno *et al*., 2005; Roesler *et al*., 2006). However, Mancera et al. (2012) concluded that, more genomic DNA of *Helicosporidium* spp. extractions and analysis were required to establish a well-supported phylogenetic tree of the genus. Although the present chapter has only aimed at confirming the presence of *Helicosporidium* sp. in *C. saliviniae* in South Africa,

the results of this study could contribute to the knowledge required in phylogenetic reconstructions and refining the positions of many *Helicosporidium* spp. isolates with other algae species.

The present chapter aimed to use both morphological description and genomic DNA analysis to confirm the presence of *Helicosporidium* sp. in colonies of *C. salviniae* in South Africa. It was expected that isolates of *Helicosporidium* sp. ex *C. salviniae* would associate with other isolates of *Helicosporidium* spp. and *Prototheca* spp. in the generated phylogenetic tree.

2.2. MATERIALS AND METHODS

2.2.1. Collection of insects

To test for the presence of *Helicosporidium* sp. in *C. salviniae*, two groups of 20 adults (*viz.* total of 40 adults) were collected from two mass-rearing facilities (the Waainek Mass-Rearing facility in Makhanda, Eastern Cape Province and the South African Sugarcane Research Institute mass-rearing facility in Mt Edgecomb, KwaZulu-Natal Province with approximately 1000km separating the two facilities) and processed separately for the light microscopy. These facilities were targeted because they have been the only sources of *C. salviniae* for field releases in South Africa. This implies that infectious species that are present in these facilities could also be in the field populations of *C. salviniae*. All morphological and molecular data on *Helicosporidium* sp. generated from these facilities were targeted to be used as a reference for future relevant studies.

2.2.2. Preparation of insect material for light microscopy

The presence of *Helicosporidium* sp. could be confirmed based on the occurrence of common cells, the vegetative, filamentous and the mature cysts (White *et al*., 2007; Yaman & Radek, 2007; Yaman, 2008; Keilin, 1921; Kellen & Lindegren, 1974; Boucias *et al*., 2001; White *et al*., 2007).

The adult weevils were homogenized (*viz*. ground) separately in a series dilution of 2000 µl, 1600 µl and 400 µl of distilled water (ddH_2O), phosphate buffer (pH 7.4) and Giemsa stain solution (Yaman & Radek, 2007), respectively, in 1.5 ml tubes, using a glass rod. The localization of *Helicosporidium* sp. colonies in *C. salviniae* was determined through screening haemolymph fluids extracted from the legs and guts of the adults of *C. salviniae*. Legs of *C. salviniae* were preferred to ensure that the insects were infected by the disease and not simple passing *Helicosporidium* particles in the gut. Legs of *C. salviniae* were pulled off their bodies using twizers. Guts were extracted by dissecting adults of *C. salviniae* with surgical blades. Collected leg and gut organs were homogenized and processed separately.

The homogenization of the adults of *C. salviniae* allowed the collection of haemolymph cells of this weevil for *in vitro* culturing medium of *Helicosporidium* sp. at room temperature (Figure 2.1). The

in vitro culturing method was adopted and modified from the methods used by Boucias et al. (2001) and, Kellen and Lindegren (1974) in culturing and maintenance of *Helicosporidium* sp. under laboratory conditions. The haemolymph of insects contains water, inorganic salts, proteins, lipids, and carbohydrates (Sutcliffe, 1963) and these contents are also available in the artificial serum that has been successfully used to culture *Helicosporidium* sp. in tubes and discs (Bläske-lietze *et al*., 2006; Boucias *et al*., 2001). 50 µl wet smears were separately mounted on glass slides (*n*=20 slides), covered with coverslips and observed under a compound microscope (Olympus, CX21) at 400x. When the common cells of *Helicosporidium* sp. were observed, many glass slides were prepared and incubated at room temperature for 14 days under a 12-hour light/dark cycle. The incubation period followed the developmental period of *H. parasiticum* described by Keilin (1921). To determine the effectiveness of this culturing method and the infectivity of *Helicosporidium* sp., the number of *Helicosporidium* sp. cells were counted using a hemocytometer before and after the incubation period. Phosphate buffer was added to preserve water from the haemolymph cells and boost the salinity of the growth medium for *Helicosporidium* sp. Giemsa stain was added to distinguish different cells of *Helicosporidium* sp. (Laguerre *et al*., 2015; Yaman & Radek, 2007; Yaman, 2008; Keilin, 1921). The use of Giemsa stain in culturing *Helicosporidium* sp. was learned from a study that focused on developing a simple and efficient methods for preparing cell slides and staining techniques without using cytocentrifuge and cytoclips (Laguerre *et al*., 2015). To monitor the developmental stages of *Helicosporidium* sp., some glass slides were examined and photographed at seven- and 14-days of the incubation period under a compound microscope (Olympus, CX21) at 400x.

Figure 2. 1. Wet solution of the homogenized adults of *C. salviniae* before Giemsa stain was added.

2.2.3. Preparation of insect material for Scanning Electron Microscopy

When the presence of *Helicosporidium* sp. was confirmed under the light microscope, the medium was prepared for scanning electron microscopy (SEM) using the methods developed by Boucias et al. (2001) (slightly modified). All the glass slides with many and well-developed cells of *Helicosporidium* sp. were gently washed off into a sterile petri dish with a minimal amount (*viz*. between 50µl and 100µl) of phosphate buffer. This solution was then transferred into five 1.5 ml centrifuge tubes and centrifuged at a low speed of 5000 rcf for 10 minutes to harvest a pellet with *Helicosporidium* sp. cells. The pellets were resuspended in 200 µl of ddH_2O and re-centrifuged at the same speed and time three times to purify pelleted cells. Purified pellets were resuspended into 100 µl of sterile water to prepare the final solution for SEM. Then, an amount of 50 µl from each tube was separately mounted on thin slides coated with buffered 2.5% glutaraldehyde and air-dried at room temperature. The samples were resuspended in the buffered 2.5% glutaraldehyde, followed by fixing in 1% Osmium tetroxide for five minutes in each. Fixed samples were then dehydrated in an ethanol series and stored in absolute ethanol (99.99%) for 12 hours for critical drying. Samples were air-dried and rinsed with Hexamethylldisilazane (HDML) and then air-dried again for 30 minutes. Samples were then coated with gold and examined with a VEGA TESCAN scanning electron microscope operating at 20 kV. Digital images were captured, processed, and measured with TESCAN software.

2.2.4. Genetic identification and analysis

2.2.4.1. DNA extraction and sequencing

The molecular analysis relied on the amplification of *Helicosporidium* sp. 18S rDNA using genus-specific primers adopted from other studies that have successfully detected *Helicosporidium* spp. from various host species (Mancera *et al*., 2012; Countway & Caron, 2006; Audemard *et al*., 2004; Tartar *et al*., 2002; Boucias *et al*., 2001). The remaining amounts of the purified pellets with *Helicosporidium* sp. cells were used for the genomic DNA extraction. Some cells of *Helicosporidium* sp. were collected

directly from the larvae and adults of *C. salviniae*. The larvae and adults were squashed in the petri dish to release their haemolymph for the extraction of the genomic DNA of *Helicosporidium* sp. Also, water samples were collected from pools that maintained cultures of *C. salviniae* and tested for the presence of *Helicosporidium* sp. A total amount of 1.5 ml was collected from 15 different locations (*viz*. 100µl collected from each location) in each of three pools and processed. This approach was done to check if *Helicosporidium* sp. (if present) in *C. salviniae* can persist in the water, following experiments conducted by Mancera et al. (2012) to test for the survival of *Helicosporidium* sp. isolates in different water pools. The cultured and uncultured haemolymph, and pool water samples were processed for the genomic DNA extraction following YeaStar Genomic DNA Extraction Kit (Zymo Research, USA) protocol. The presence of the genomic DNA in the processed samples were visualized on a 1% agarose gel electrophoresed at 80V for 30 min, photographed and analysed with ChemiDoc™ XRS+ (Bio-Rad,
USA) system.

Following confirmation of genomic DNA bands, these prepared DNA samples were used as templates mixed with specific 18S primers MGF and MGR in Polymerase Chain Reactions (PCR) (Mancera et al., 2012). The primer sequences were MGF (5'-AGGATAGAGGCCTACCATGGTTTCAA-3') and MGR (5'-CCCCGACTCCCTCTCCAT-3') (Boucias *et al*., 2001). PCR reactions comprised 12.5 µl 2× Ampliqon Taq DNA Polymerase (Ampliqon, Denmark), 1 µl each of the forward and reverse primers (10 mM), 2 µl DNA template, with the reaction made up to 25 µl with ddH_2O. The 18S primers were used to amplify the 18S rDNA region of the genomic DNA of *Helicosporidium* sp. The 18S rDNA region was targeted because it has been successfully used to identify protist species including, *Helicosporidium* spp. and *Prototheca* spp. (Boucias *et al*., 2001). The PCR cycle conditions were modified from Mancera et al. (2012), with an initial cycle at 95 °C for 3 minutes, followed by 30 cycles at 95°C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 3 minutes, with a final elongation step of 72 °C for 3 minutes. Following PCR, the resulting amplicons were resolved on a 1% agarose gel by electrophoresis and imaged using a ChemiDoc™ XRS+ to determine if the

amplification of the 18S rDNA region of the processed genomic DNA of *Helicosporidium* sp. was successful or not. The GeneRuler 1kb DNA ladder (Thermo Scientific, USA) was run alongside samples on each agarose gel as a standard. Successful amplicons were sent to the Inqaba Biotechnical Industries (Pty) Ltd (South Africa) for sequencing.

2.2.4.2. Sequence alignment and phylogenetic analyses

The resulting sequences were aligned with the sequences of the closely related algal species downloaded from the NCBI GenBank, guided by the phylogenetic tree reported by Mancera et al. (2012). The sequences were downloaded with Geneious R11 version 11.1.5 (BioMatters, New Zealand). A multiple nucleotide alignment of all the sequences was performed using Muscle in MEGA XI (Tamura et al. 2021) with default settings.

The complete 18S rDNA alignment consisted of a dataset that contained 40 taxa. MEGA XI was further used to identify the most appropriate maximum likelihood (ML) base substitution model for this dataset. The Jukes-Cantor model was selected by finding the best-fit substitution model in MEGA XI based on the lowest Bayesian Information Criterion (BIC) score generated during this analysis. The ML analyses that incorporated the model and parameters calculated by MEGA XI. ML bootstrap analyses were conducted using MEGA XI in 1000 replicates. The phylogenetic tree corresponding to the ML analyses was produced and edited with MEGA XI.

Relatedness percentage of the sequence of *Helicosporidium* sp. ex *Cytobagous salviniae* of the present study to other sequences extracted from the same or related species. This percentage relatedness was generated in Geneious R11 version 11.1.5 (BioMatters, New Zealand).

2.3. RESULTS

2.3.1. *Helicosporidium* sp. determination using light microscopy

The common cells of *Helicosporidium* sp., the vegetative, filamentous cells and mature cyst cells were observed from the prepared uncultured and cultured mediums from both the mass rearing facilities (Figure 2.2). Mature cyst cells were uniform in sizes in both the uncultured and cultured mediums. The number of *Helicosporidium* sp. cells increased from an average of 24.99% uncultured to an average of 53.94% cultured cells of this infection (*n*= 20 slides).

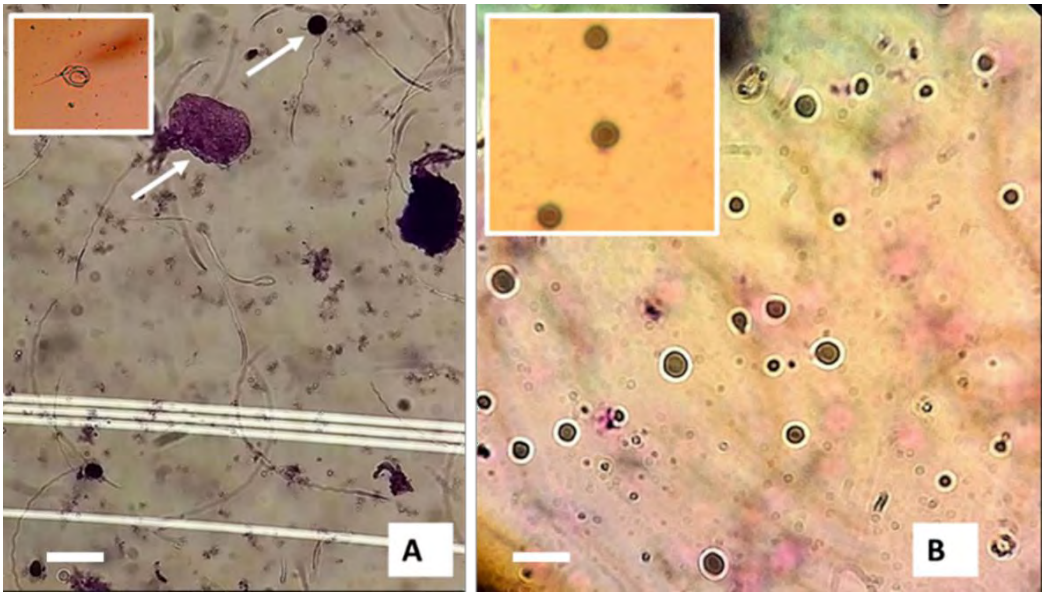

Figure 2. 2. Light micrographs of *Helicosporidium* sp. (Giemsa-stained cells) cells isolated from adults of *Cyrtobagous salviniae*. (A) Non incubated solution and (B) Incubated solution. Arrows are distinguishing vegetative spores or cells of *Helicosporidium* sp. Scale bars: 50µm.

2.3.2. *Helicosporidium* sp. determination using Scanning Electron Microscopy

All the common cells, the filamentous, vegetative, and mature cyst of *Helicosporidium* sp. were shown under the scanning electron microscope. The cyst and vegetative cells (Figure 2.3 A & B) sizes ranged between 3.2–6.2 µm and 3.6–5.4 µm (*n*= 100, for each cell), respectively. Filamentous cells were long and characterized by barb cells (Figure 2.4 A & B). Enclosed cyst and vegetative cells in pellicles were observed. Some pellicles were empty indicating the release of either the mature cyst or vegetative cells (Figure 2.4 A & B).

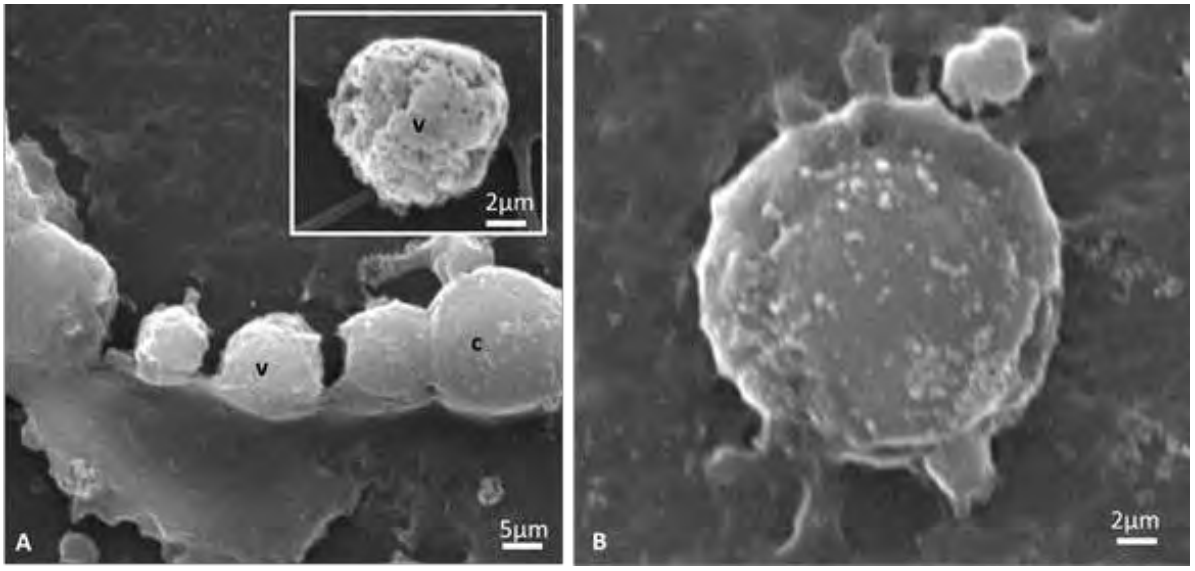

Figure 2. 3. Scanning electron micrographs of *Helicosporidium* sp. cells. (A) Vegetative and cyst cells and (B) Mature cysts. Abbreviations: V (vegetative cell) and C (Cyst).

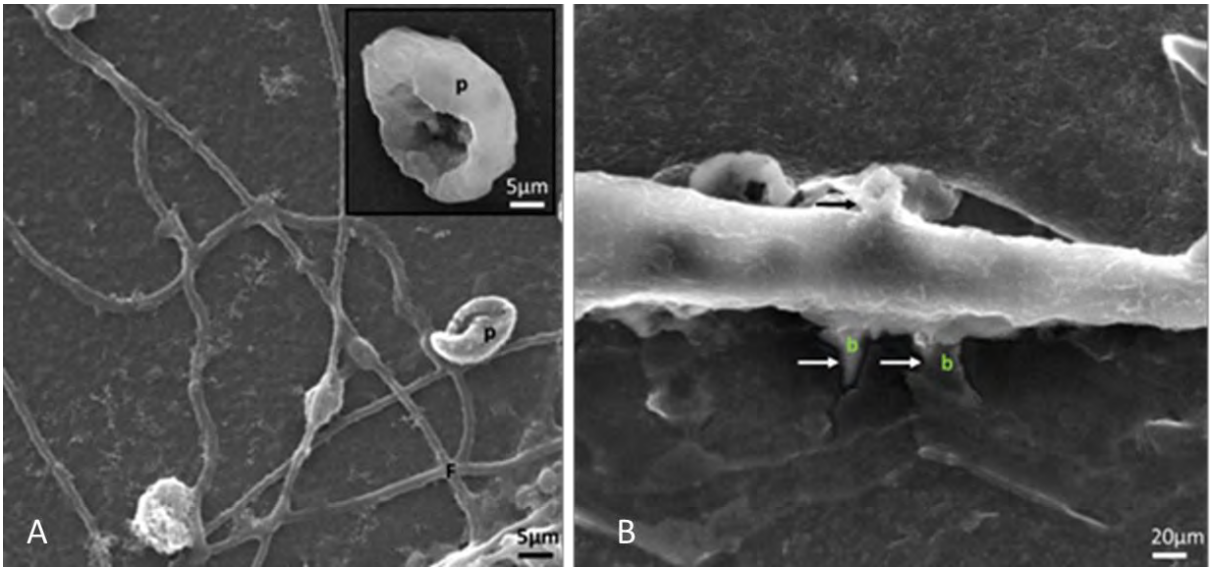

Figure 2. 4. Scanning electron micrographs of filamentous cells of *Helicosporidium* sp. cells. Abbreviations: F (filament), P (pellicle) and B (barb).

2.3.3. Localization of *Helicosporidium* sp. in *Cyrtobagous salviniae*

Cells of *Helicosporidium* sp. were present in both the gut and haemolymph fluids extracted from the adults of *C. salviniae*. More mature cysts were located from the gut than haemolymph fluids of *C. salviniae* (Figure 2.5). The sizes of the mature cysts extracted from these two fluids were slightly uniform.

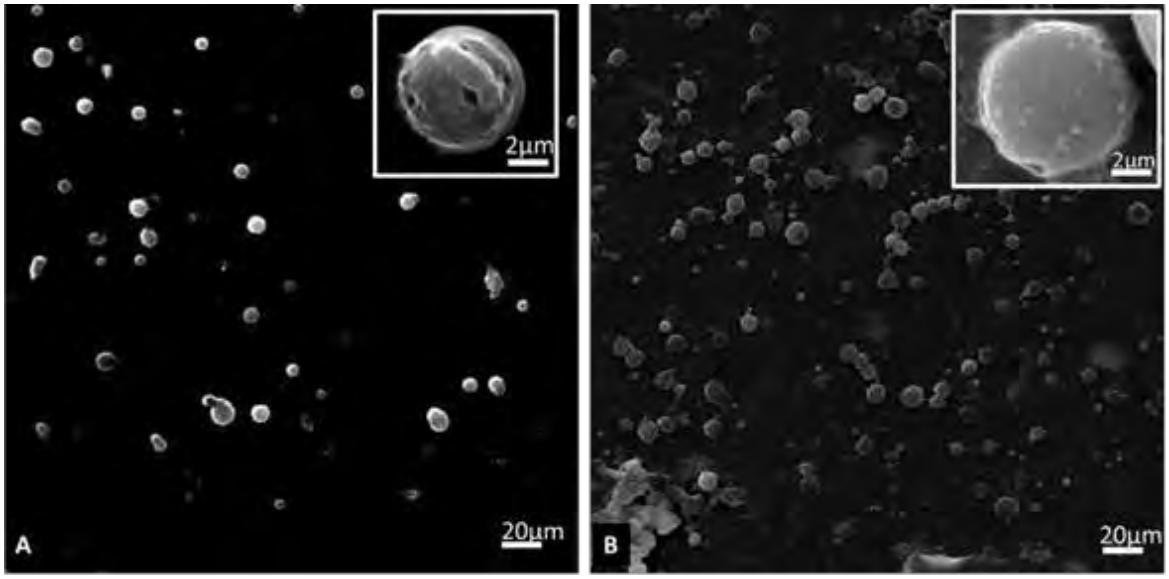

Figure 2. 5. SEM micrographs of *Helicosporidium* sp. cyst cells isolated from different parts of *C. salviniae*. (A) Cyst cells from haemolymph fluid from the legs of *C. salviniae* and (B) Cyst cells from the gut fluid of *C. salviniae*.

2.3.4. *Helicosporidium* sp. DNA extraction and analysis

The genomic DNA of *Helicosporidium* sp. was successfully extracted (Figure 2.6), and the 18S rDNA region of the genomic DNA of *Helicosporidium* sp. was successfully amplified (Figure 2.7). An attempt to extract *Helicosporidium* sp. genomic DNA from the pool water that supports the culture of *C. salviniae* was not successful. Genomic DNA was visible for all larval and adult samples but not in the pool water sample (Figure 2.6). Similarly, amplicons were generated for all larval and adult genomic DNA samples while no amplicon was observed for the water sample. (Figure 2.7).

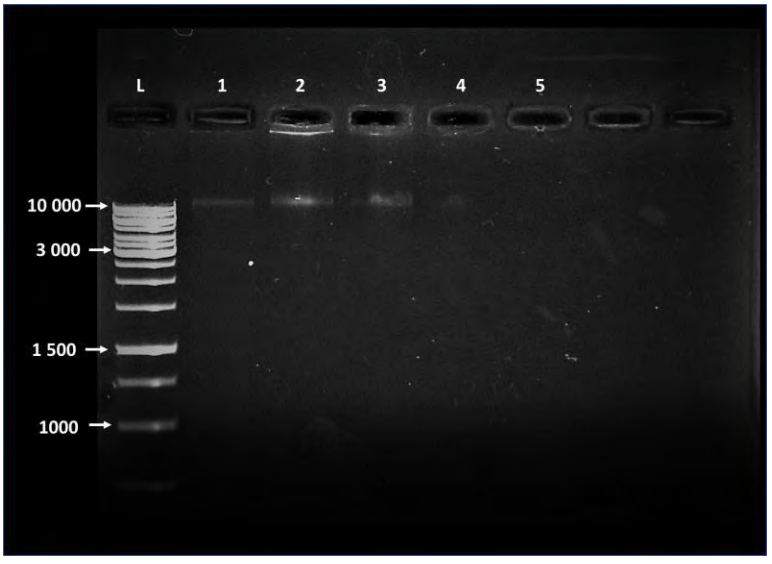

Figure 2. 6. Images of genomic DNA extracts from *Helicosporidium* sp. resolved on a 1% agarose gel by electrophoresis. Abbreviations: (L) GeneRuler 1 kb DNA Ladder, (1) *C. salviniae* larval sample, (2) *C. salviniae* adult sample (uncultured cells), (3) *C. salviniae* adult sample (cultured cells), (4) Mixed *C. salviniae* adult, larval sample (cultured cells) and (5) Pond water sample containing *C. salviniae* cultures.

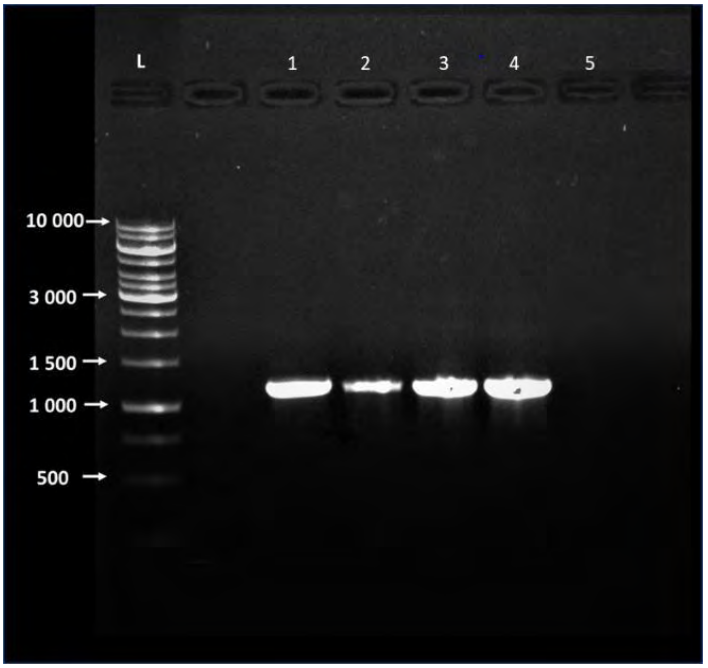

Figure 2.7. Image of *Helicosporidium*-specific 18S PCR amplicons resolved on a 1% agarose gel by electrophoresis (18S rDNA PCR amplicons at the expected region of approximately 1100 bp). Abbreviations: (L) GeneRuler 1 kb DNA Ladder, (1) Pond water sample from *C. salviniae* cultures, (2) *C. salviniae* larval sample, (3) *C. salviniae* adult sample (uncultured cells), (4) *C. salviniae* adult sample (cultured cells), (5) Mixed *C. salviniae* adult and larval sample (cultured cells).

The PCR amplicons generated from both cultured and uncultured *Helicosporidium* sp. ex. *C. salviniae* were sequenced. The phylogenetic tree was inferred from the prepared sequences aligned with 40 nucleotide sequences (*viz*. of different or similar species) downloaded from NCBI GenBank. The isolate (*viz*. *Helicosporidium* sp.) ex. *C. salviniae* was classified as a member of the green algae, and this placement was supported by a significant bootstrap value (85%) (Figure 2.8). The tree was rooted in Chlorophyceae with *Chlorella reinhardtii* as an outgroup. The 18S rDNA nucleotide sequences of *Helicosporidium* sp. have formed a polyphyly relationship with other *Helicosporidium* spp. Sequences obtained from the NCBI GenBank. These sequences have also shown a paraphyletic relationship with both *Helicosporidium* spp. and *Prototheca* sp. isolates from the GenBank database. The relationship of

these isolate sequences was supported (high bootstrap value) with the ancestral root of the phylogenetic tree, *Chlorophyta* species (Figure 2.8).

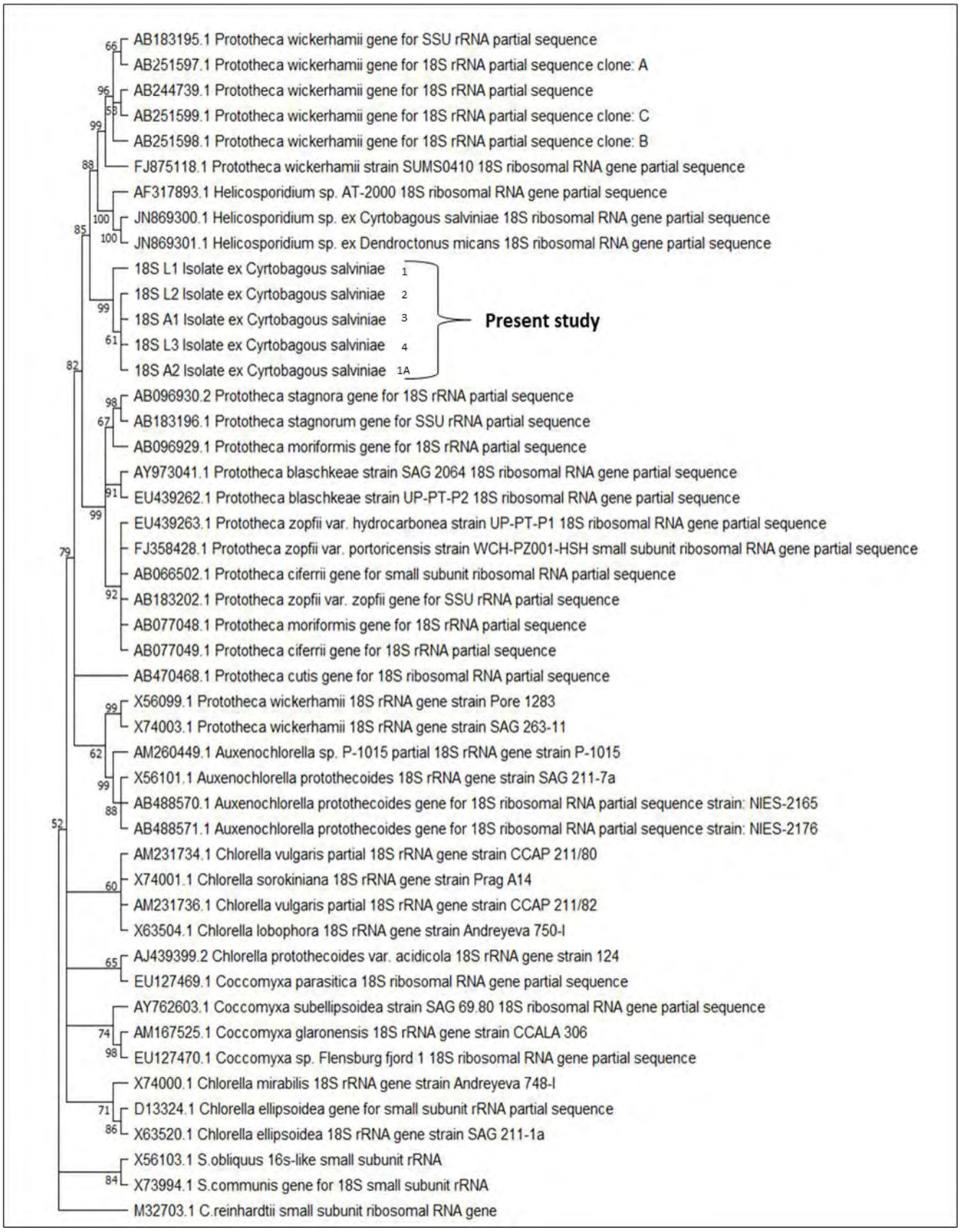

Figure 2. 8. Maximum Likelihood (ML) phylogram constructed from 18S rDNA gene sequences from Trebouxiophyceae and incorporating isolates made from *Cyrtobagous salviniae* sequences. Sequences generated throughout this study are categorised as the present study. Comparative sequences were downloaded from GenBank (accession or reference numbers are presented). The tree is rooted in Chlorophyceae. ML bootstrap values >50 (1000 replicates) are indicated by numbers at the nodes, whereas nodes that were not supported by bootstrap analysis (value <50) are excluded from the tree nodes. Isolates (1) *C. salviniae* larval sample, (2) *C. salviniae* adult sample (uncultured cells), (3) *C. salviniae* adult sample (cultured cells), (4) Mixed *C. salviniae* adult, larval sample (cultured cells). Isolate 1A gel was not presented.

More than 50% similarities between the isolate *Helicosporidium* sp. ex *Cyrtobagous salvinae* of the present study were established when compared to other sequences of *Helicosporidium* sp. isolated from either the same or different insect species (Table 2.1).

Table 2.1. Percentage identity among the aligned sequences of *Helicosporidium* sp. and other related sequences obtained from different species.

	18S L1 (1)	**18S L2 (2)**	**18S A1 (3)**	**18S L3 (4)**	**18S A2 (1A)**	**AF317893.1**	**JN869300.1**	**JN869301.1**
18S L1 (1)	-	99.8	99.4	99.6	99.3	60.2	58.9	59
18S L2 (2)	99.8	-	99.7	99.9	99.6	61.3	59	59
18S A1 (3)	99.4	99.7	-	99.7	99.5	61	58.7	58.7
18S L3 (4)	99.6	99.9	99.7	-	99.6	60.8	58.5	58.5
18S A2 (1A)	99.3	99.6	99.5	99.6	-	69.9	59.1	59.1
AF317893.1	60.2	61.3	61	60.8	61.6	-	78.3	77.6
JN869300.1	58.9	59	58.7	58.5	59.1	78.3	-	70.6
JN869301.1	59	59	58.7	58.5	59.1	77.6	99	-

Sequences in full descriptions (corresponding to those listed in the phylogram): 18S L1 Isolate ex *Cyrtobagous salviniae* 1, 18S L2 Isolate *Cyrtobagous salviniae* 2, 18S A1 Isolate *Cyrtobagous salviniae* 3, 18S L3 Isolate *Cyrtobagous salviniae* 4, 18S A2 Isolate *Cyrtobagous salviniae* 1A, AF317893.1 *Helicosporidium* sp. AT-2000 18S Ribosomal RNA gene partial sequence, JN869300.1 *Helicosporidium* sp. ex *Cyrtobagous salviniae* 18S Ribosomal RNA gene partial sequence and JN869301.1 *Helicosporidium* sp. ex *Dendroctonus micans* 18S Ribosomal RNA gene partial sequence. Numbers in the table indicate percentage (%) identity of relatedness between the compared sequences.

2.4. DISCUSSION

The results from the SEM presented in this chapter have confirmed the presence of *Helicosporidium* sp. in *C. salviniae* collected from two mass-rearing facilities. Since all the field released colonies of *C. salviniae* have been sourced from these two facilities, these results imply that the distribution range of this weevil in South Africa has *Helicosporidium* sp. (see chapter 3). However, these results do not indicate the distribution and loads of *Helicosporidium* sp. in the field *C. salviniae*. Further, the processed isolates, excluding the pond water, tested positive for the genomic DNA extraction of *Helicosporidium* sp. The unsuccessful extraction of the DNA from the pool water in which the host plants were growing could mean insufficient samples were processed or this isolate of *Helicosporidium* sp. does not persist for long in the water. The isolates *Helicosporidium* sp. ex *C. salviniae* of the present study and other *Helicosporidium* spp. were placed in a paraphyletic relationship supported with a highly significant 85% bootstrap value, with more than 50% identical support. This paraphyletic relationship involved the placement of *Prototheca* sp. isolates. The incorporation of *Helicosporidium* with *Prototheca* isolates was not a surprising outcome since these genera are both non-photosynthetic trebouxiophyte algae (Mancera *et al*., 2012). The difference of bootstrap support of 15% between the present isolates of *Helicosporidium* sp. ex *C. salviniae* and other *Helicosporidium* spp. could have resulted due to the length and/or quality of the nucleotide sequences.

The ability of *Helicosporidium* sp. to develop in the prepared medium from the contents of the adults of *C. salviniae* makes it easier to investigate life stages of this infection and their response to different temperatures and medium chemistries. Although attempts to extract cells of *Helicosporidium* sp. from the pool water was unsuccessful, the ability of *Helicosporidium* sp. to develop under laboratory conditions implies that some stages of this infection could have a free-living lifestyle in the aquatic environment (Boucias *et al*., 2001). This ability to be both free-living and parasitic has also been reported for *Prototheca* spp. (Boucias *et al*., 2001). The SEM micrographs have generated strong evidence,

depicting additional morphological features of *Helicosporidium* spp. The sizes of the mature cyst and vegetative cells of *Helicosporidium* sp. ex *C. salviniae* were consistent with the sizes of the same cells reported by other researchers (White *et al*., 2007; Bläske-Lietze *et al*., 2006; Bläske & Boucias, 2004). Although no data generated with Transmission Electron Microscopy (TEM) was provided, sufficient diagnostic features were documented from SEM to identify *Helicosporidium* sp. The presence of filamentous cells under the SEM has strongly affirmed *Helicosporidium* sp. was present and not any other algal contaminant.

Observations made under SEM did not show any other cells except those of *Helicosporidium* sp. These observations could also mean that the processed individuals of *C. salviniae* were free from other infectious species or the purification methods have mostly favoured the cells of *Helicosporidium* sp. The extraction and purification of *Helicosporidium* sp. cells were made directly from both the adults and larvae of its host weevil to reduce the chances of contamination. The nucleotide sequence analyses for both the isolates extracted from *in vivo* and *in vitro* of the 18S rDNA gene produced identical DNA sequences, showing the integrity of the genomic DNA of *Helicosporidium* sp. used in this chapter.

Some researchers including, Mancera et al. (2021) and Pombert & Keeling (2010) have reported phylogenetic analyses that have grouped *Helicosporidium* spp. with *Prototheca* spp. with 96% and 100% bootstrap values support, respectively. The occurrence of *Helicosporidium* and *Prototheca* on the common clade is because, species of these genera are both achlorophylous and animal pathogens (Mancera *et al*., 2012). The results of this chapter have also supported Mancera et al. (2012) who reported that there is no genetic evidence that classifies *Helicosporidium* sp. as a member of Protozoa. Phylogenetic analysis of this chapter depicts the grouping of *Helicosporidium* sp. as relative species of *Chlorophyta* and *Prototheca*, suggesting that this infection is a green alga, and not a species of these genera (Mancera *et al*., 2012). This affirmation is based on the presence of filamentous cells from the medium prepared to screen *Helicosporidium* sp. and, no species of these genera, *Prototheca* and *Chlorophyta* were ever reported to have filamentous cells in their life stage developments. This conclusion implies that molecular and morphological evidence should be employed in phylogenetic

reconstructions and refining of the relationships of the species of interest, in particular closely related infectious species.

CHAPTER 3

Distribution and infection load of *Helicosporidium* sp. in *Cyrtobagous salviniae*

3.1. INTRODUCTION

Although insect diseases have their own transmission and replication mechanisms, they still rely on the distribution and prevalence of their host species. Fragmented populations of the host insect species could limit the spread and abundance of diseases, particularly when such diseases are not transmitted in the air (Yaman & Radek, 2007). Moreover, host insect species which have limited dispersal will also delay the spread of diseases over their entire populations in either a localized or broad geographic range.

Field collection and screening of the infected insect species becomes the sole approach to determine the distribution of the particular disease. This procedure determines the geographic areas where the disease is present and can give an indication of the severity of the disease in a particular population. This intensive screening provides the robust infection load in an insect population and further sheds light on the prospects of transmission, replication strategies and impact of such diseases (Yaman & Radek, 2007).

Although the screening for the presence of diseases and their distributions in insect populations is important, this practice remains neglected in biological control programmes across the globe. For instance, in South Africa and elsewhere, there are biocontrol agents that have failed to establish or expand their field population sizes (Winston *et al*., 2014). Such failures are often attributed to the effects of climatic conditions without testing for diseases that could be the main cause.

In South Africa, the biological control programme against *S. molesta* using *C. salviniae* has been highly successful (Coetzee *et al*., 2011). Despite this programme's success, sites where *S. molesta* occur in the shade, or those that experience low winter temperatures, take longer to achieve control (Maseko *et al*., 2017; Martin *et al*., 2018). Since its introduction in 1985, the weevil has been mass reared and distributed throughout the country (Martin *et al.,* 2018), until this insect was shown to be positive for *Helicosporidium* sp. (White *et al*., 2007). *Helicosporidium* sp. could have been inadvertently spread

around the country through mass rearing efforts. And although there has been excellent control of *S. molesta* in the past, it is not known what this disease could do in the future to the biocontrol programme of this plant in South Africa, and beyond. Determining the effect of *Helicosporidium* sp. on *C. salviniae* requires an understanding of the distribution and infection load of this disease in *C. salviniae*. Further, understanding the mechanism that influences the spread of *Helicosporidium* sp. within and between *C. salviniae* populations is crucial in predicting the distribution of this infection and developing possible management interventions, should they be deemed necessary.

This chapter aims to determine the distribution and infection load of *Helicosporidium* sp. in field populations of *C. salviniae* around South Africa and develop the foundation for determining the effects of *Helicosporidium* sp. in the field. The objectives of the study were to determine the occurrence and load of *Helicosporidium* sp. in the inhouse and field colonies of *C. salviniae*.

3.2. MATERIALS AND METHODS

3.2.1. *Cyrtobagous salviniae* collection sites

Records from the post-release evaluation published by Martin *et al*. (2018) and Southern African Plant Invaders Atlas (SAPIA) database were accessed to determine the distribution of *S. molesta* and *C. salviniae* across South Africa. Martin *et al.* (2018) listed some 57 sites with the weed and the agent. Although not all sites were visited, sites that represented the broad climatic zones, that being the subtropical eastern side of the country and the temperate Western Cape with a more Mediterranean type climate, were selected (Table 3.1).

Table 3. 1. Field collection sites of *Cyrtobagous salviniae* on *Salvinia molesta* plant populations.

Province	Site name or description	Coordinates
Limpopo	Selati River (SR)	23˚55'17.89" S 30˚ 51'13.59" E
	Groblersdal Roets Farm (GRF)	25˚12'27.65" S 29˚25'45.15" E
	Tzaneen Orange Farm (TOF)	23˚52'11.55" S 30˚21'23.18" E
KwaZulu-Natal	South African Sugar Research Institute (SASRI)*	29˚42'26.12" S 31˚02'50.99" E
	Kingswood School Dam (KSD)	29˚20'58.17" S 29˚58'57.97" E
	Cato Ridge Farm (CRF)	29˚45.401" S 30˚ 33.444" E
Western Cape	Kogmanskloof River (KR)	33˚52'20.30" S 20˚00'00.13" E
	Taaibos Farm for Compost (TFC)	33˚56'37.08" S 23˚25'52.71" E
	John Stanwix Candle Farm (JSCF)	33˚57'55.68" S 22˚ 58'40.67" E
Eastern Cape	Waainek Rhodes University (WRU)*	33˚19'11.30" S 26˚30'27.48" E

* (Insect mass rearing facility)

3.2.3. Collection and preservation of *Cyrtobagous salviniae*

Field work was conducted between May 2018 and February 2020. At each site, 100 plants were randomly retrieved by hand and adults, pupae, and larvae of *C. salviniae* were collected (Table 3.2). Because

preserving individuals of *C. salviniae* in alcohol (70%) damages some of the *Helicosporidium* sp. life stages, particularly the vegetative cells, the field-collected individuals (adults, larvae and pupae) of *C. salviniae* were placed on ice and transported to the laboratory at the Department of Zoology and Entomology, Rhodes University, and tested upon arrival. Live vegetative cells of many infectious species that attack an insect's hemolymph, like that of *Helicosporidium* sp., turn pink or light purple when stained with Giemsa stain (Rapuntean *et al*., 2009). Damaged vegetative cells of *Helicosporidium* sp. do not turn light pink or purple when stained with Giemsa stain for parasitological and cytological examination of hemolymph smears, making it difficult to detect these cells and quantify them with other cell types (the mature cyst and vegetative cells) when determining the overall infection load of *Helicosporidium* sp. In the laboratory, only live individuals of *C. salviniae* were processed to determine the status of *Helicosporidium* sp. and quantify the infection load.

Table 3. 2. Number of *Cyrtobagous salviniae* adults, larvae, and pupae collected per site.

Site	**No. of adults**	**Sex**		**Larvae**	**Pupae**
		♀	♂		
SR	110	45	65	–	–
GRF	100	41	59	–	–
TOF	105	49	56	11	09
SASRI*	120	53	67	19	13
KSD	73	27	46	–	–
CRF	103	48	55	26	15
KR	67	29	38	–	–
TFC	115	57	58	–	–
JSCF	89	36	53	–	–
WRU*	123	59	64	30	30
Totals	**1005**	**444**	**561**	**86**	**67**

Keys: ♂= female; ♀= male. Abbreviations represent the collection sites of *Cyrtobagous salviniae* throughout South Africa. Abbreviations: SR (Selati River), GRF (Groblersdal Roets Farm), TOF (Tzaneen Orange Farm), SASRI (South African Sugar Research Institute), KSD (Kings School Dam), CRF (Cato Ridge Farm), KR (Kogsmanskloof River), TFC (Taaibos Farm for Compost), JSCF (John Stanwix Candle Farm) and WRU (Waainek Rhodes University). * (Insect mass rearing facility).

3.2.4. *Helicosporidium* sp. infection determination

Each individual adult, larva, or pupa collected from the 10 sites was homogenised separately in a series dilution of 100 µl, 80 µl and 20 µl of distilled water, phosphate buffer (pH 7.4) and Giemsa stain solution, in an Eppendorf tube, using a glass rod. An amount of 100 µl of these smears was mounted on a glass slide, covered with a coverslip, and examined under a compound microscope (Olympus, CX21) at 400x. A total of 450 adults, 86 larvae and 67 pupae were tested, and the outcome was used to determine the status and distribution of *Helicosporidium* sp. at all sites.

The detected cells of *Helicosporidium* sp. were photographed using a built-in camera in an OLYMPUS SC30 microscope. The sizes of the cell samples were measured and analysed with the analySIS docu software [source: www.lre.se (analySISFamily)].

3.2.5. *Helicosporidium* sp. infection load

To quantify the infection load of *Helicosporidium* sp. in *C. salviniae*, only the adults were examined, since they had all the life stages of the disease. The adults of *C. salviniae* were sexed to make a total of 15 pairs (*i.e.* 30 adults) per site. Sexes were processed separately to determine if *Helicosporidium* sp. was more prevalent in males or females.

Subsequently, fresh smears were prepared by homogenising each adult as described above. A hemocytometer (10 x10 1mm grid cells) was used to enumerate the disease particles. The number of cysts, vegetative, or filamentous cells of *Helicosporidium* sp. occurring in each 1 mm^2 block were counted. A total of three (3) counts was made for each 100 µl of the smears mounted on the hemocytometer and an average of these counts was used to determine the infection load of *Helicosporidium* sp. in each adult of *C. salviniae*.

The total number of *Helicosporidium* sp. cells counted from each 100 µl of the smears was converted to determine the infection load of *Helicosporidium* sp., using the following formula adapted from Bitesizebio accessed through Google (https://bitesizebio.com):

$$\text{Infection load} = \frac{\text{Total number of counted cells x dilution factor x 10000 cells/ml}}{\text{Number of squares used on the hemocytometer (25 square blocks)}}$$

Dilution factor = $\underline{V_1/V2}$, where V_1 = initial volume and V_2 = final volume

The equation to determine the infection load of *Helicosporidium* sp. in *C. salviniae* required volumes of each individual of females and males of *C. salviniae*. Therefore, the volumes (V1 & V2) of both the male and female adults of *C. salviniae* were determined using a glass measuring cylinder of 250 ml. A total of 10 ml of water was put into the cylinder, then 10 individuals of either male or female *C. salviniae* were placed in the water. The resultant displacement of the water volume after adding the adults was subtracted from the initial volume and the difference was used to determine the volume of these adults. The volume of each adult was established by dividing the total volume of group of adults by 10 adults.

The initial volumes of the male and female adult *C. salviniae* were 2.02 ml and 2.03 ml, respectively; equivalent to 0.00202 µl and 0.00203 µl, respectively. Therefore, the average volume of other reagents [i.e., water (100 µl), buffer (80 µl, stain (20 µl)] combined with the smears prepared from each adult male or female *C. salviniae,* were both 202µl.

To better understand and visualize the infection load of *Helicosporidium* sp. in *C. salviniae* at each site investigated in South Africa, the infection loads were grouped into five categories: low (0–1 x 10^4), medium (1.1 x 10^4 to 2 x 10^4), high (2.10 x 10^4 to 3 x 10^4), severe (3.10 x 10^4 to 4 x 10^4) and most severe (4.10 x 10^4 to 1 x 10^5) infection loads.

3.2.6. Data analysis

Raw data did not meet the assumptions of the analysis of variance when subjected to Shapiro-Wilk normality tests ($P < 0.05$). Subsequently, a Kruskal-Wallis test was used to compare the *Helicosporidium* sp. infection loads on *C. salviniae* between sites. When statistical differences were observed, the medians were subjected to the Wilcoxon rank-sum test to group the medians which differed significantly from others. The load of *Helicosporidium* sp. between the male and female adults of *C. salviniae* was determined using a Student's *t*-test. A Pearson's Chi-squared test was used to test the differences between the infected males and females. All statistical analyses were performed in R studio (version 1.1.463).

3.3. RESULTS

3.3.1. Distribution of *Helicosporidium* sp.

Helicosporidium sp. infection was recorded in individuals at all of the sites surveyed in this study (Figure 3.2), with 92.15% to 100% of the individuals infected (Table 3.3). All *Helicosporidium* sp. life stages (mature cyst, filamentous, and vegetative cells) (Figure 3.1) were found. *Helicosporidium* sp. cells were detected in both the female and male adults of *C. salviniae* (Table 3.3).

Table 3. 3. Occurrence of *Helicosporidium* sp. infection in *Cyrtobagous salviniae*.

Site	No. of adults screened	No. of adults infected	Sex ♀	Sex ♂	No. of larvae (screened)	No. of pupae (screened)	Infection occurrence (%)
SASRI*	84	84	47	37	19	13	100
WRU*	60	60	37	23	30	30	100
SR	90	90	51	39	–	–	100
KR	37	37	19	11	–	–	100
TFC	68	68	42	26	–	–	100
JSCF	42	42	24	18	–	–	100
KSD	59	59	35	24	–	–	100
CRF	66	66	43	23	26	15	100
GRF	100	100	56	44	–	–	100
TOF	102	94	55	47	11	09	92.15 (adults); 100 (larvae and pupae)

Keys: ♂= female; ♀= male. Abbreviations represent the collection sites of *Cyrtobagous salviniae* throughout South Africa. Abbreviations: SASRI (South African Sugar Research Institute), WRU (Waainek Rhodes University), SR (Selate River), KR (Kogsmagkloof River), TFC (Taaibos Farm for Compost), JSCF (John Stanwix Candle Farm), KSD (Kingswood School Dam), CRF (Cato Ridge Farm), GRF (Grobblersdal Roets Farm), and TOF (Tzaneen Orange Farm). * (insect mass rearing facility).

Of the larvae (86) and pupae (67) screened from only four sites, 100% were infected with *Helicosporidium* sp. Among the three life stages of *Helicosporidium* sp. (Figure 3.1), only mature cyst cells were present in the larvae, while both mature cysts and vegetative cells were detected in the pupae

(Figure 3.1. B & C).

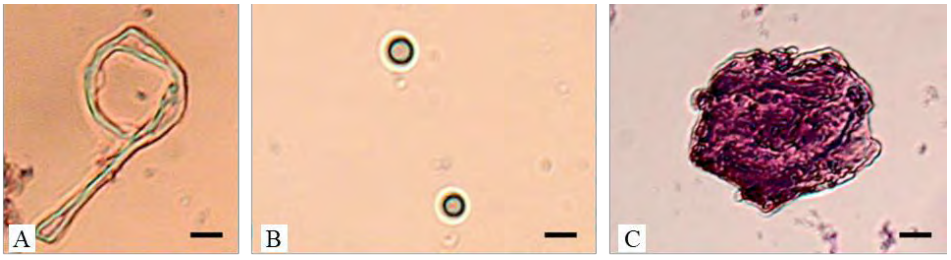

Figure 3. 1. Light micrographs of *Helicosporidium* sp. (Giemsa-stained cells) cells isolated from adult *Cyrtobagous salviniae*. (A) Filamentous cell, (B) Cysts and (C) Vegetative cell. Scale bars: 50 µm.

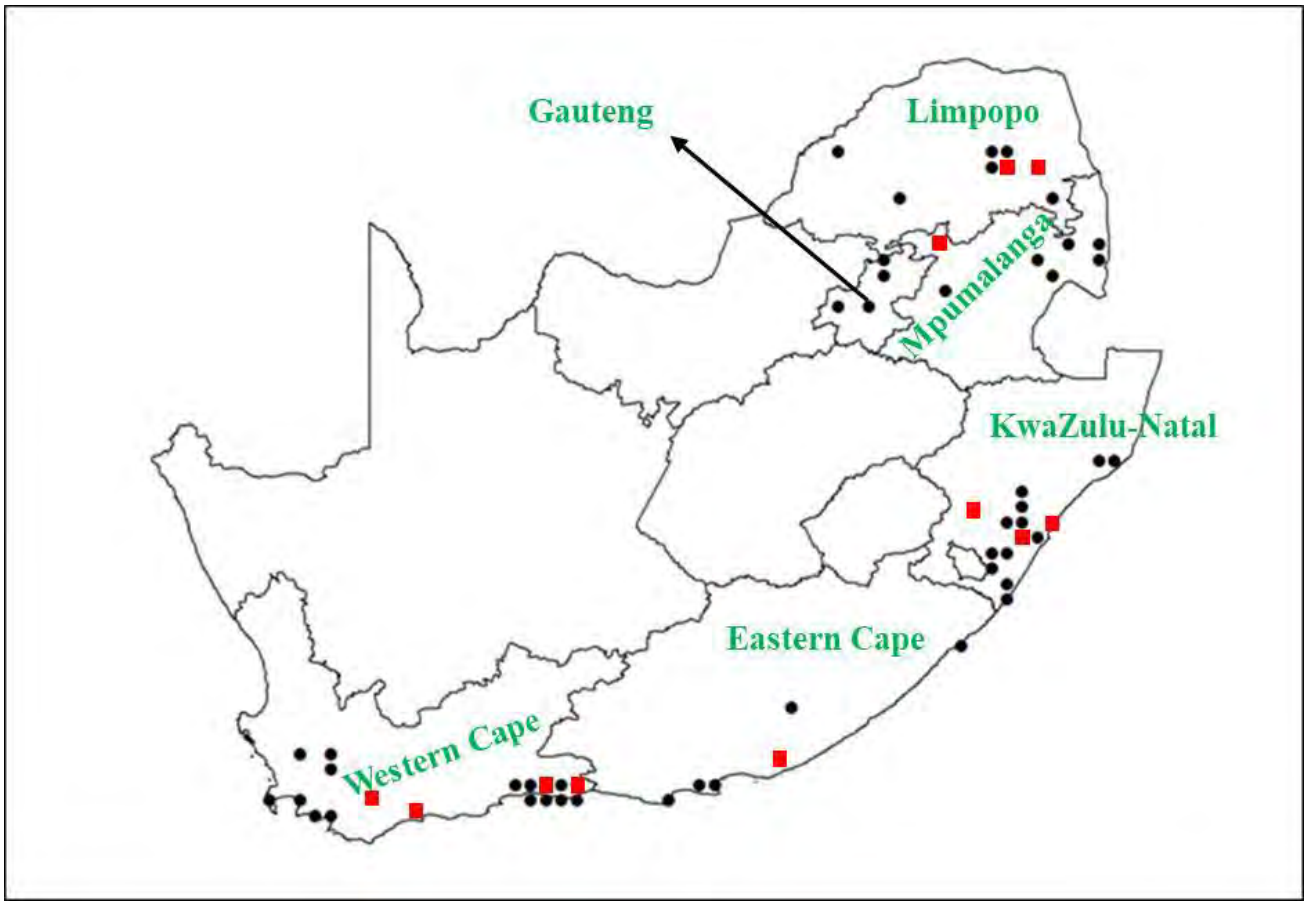

Figure 3. 2. A distribution map of *Helicosporidium* sp. in the field populations of *Cyrtobagous salviniae* distributed on *Salvinia molesta* in South Africa. Black dots (•): Known sites of *Salvinia molesta*. Red squares (■): Sampled sites of *Cyrtobagous salviniae* infected with *Helicosporidium* sp. infection.

3.3.3. *Helicosporidium* sp. infection load

Helicosporidium sp. infection load in adults ranged from low (0 –2 x 10^4 cells per µl) to severe (4.10 x 10^4 –1 x 10^5 cells) (Figures 3.3 and 3.4) throughout South Africa. The infection load of *Helicosporidium* sp. differed significantly between sites ($H_{(19, 280)} = 9.07$; $P < 2.2 \times 10^{-16}$).

A severe infection load of (median ± SD) 3.37 x 10^4 ± 4.29 x 10^3 cells of *Helicosporidium* sp. (per 100 µl) was recorded at Cato Ridge Farm in KwaZulu-Natal. This result was followed by four sites which had a medium infection load of 1.37 x 10^4 ± 1.67 x 10^3; 1.41 x 10^4 ± 975.77; 1.44 x 10^4 ± 597.41, and 1.27 x 10^4 ± 945.23 cells (Groblersdal farm in Limpopo, Kingswood School Dam in KwaZulu-Natal, and two Western Cape sites, John Stanwix Candle Farm and Kogmanskloof River, respectively). The infection loads of *Helicosporidium* sp. did not differ significantly between these four sites. The medium infection loads of 1.71 x 10^4 ± 1.92 x 10^3 (higher) and 1.45 x 10^4 ± 3.11 x 10^3 (lower) cells of *Helicosporidium* sp. which differed significantly from other sites of the same infection category were recorded at the South African Sugar Research Institute in KwaZulu-Natal and Waainek Research Centre (at Rhodes University in Eastern Cape), respectively. Low infection loads of 8.33 x 10^3 ± 468.29 and 6.09 x 10^3 ± 357.25 cells of *Helicosporidium* sp. were recorded at Taaibos Farm for Compost in the Western Cape and Selati River in Limpopo, respectively. No infection load of *Helicosporidium* sp. was considered either severe or most severe in any tested individuals of *C. salviniae* from any of the sites (Figure 3.3).

The infection load of *Helicosporidium* sp. did not differ significantly (t = 0.48; df = 298; P = 0.63) between sexes at any site (Figure 3.4). The means of the infection loads of *Helicosporidium* sp. in the female and male adults were 1.39 x 10^4 ± 1.14 x 10^3 and 1.32 x 10^4 ± 983.16 cells, respectively, at all sites. There was no significant difference in the diseased female and male adults (X^2= 295.2; df = 235; P = 0.005) (Figure 3.4).

Across the investigated sites, the female *C. salviniae* had slightly higher infection loads under medium and high categories than the males (Figure 3.4). The male *C. salviniae* had more low and severe infection categories (Figure 3.4).

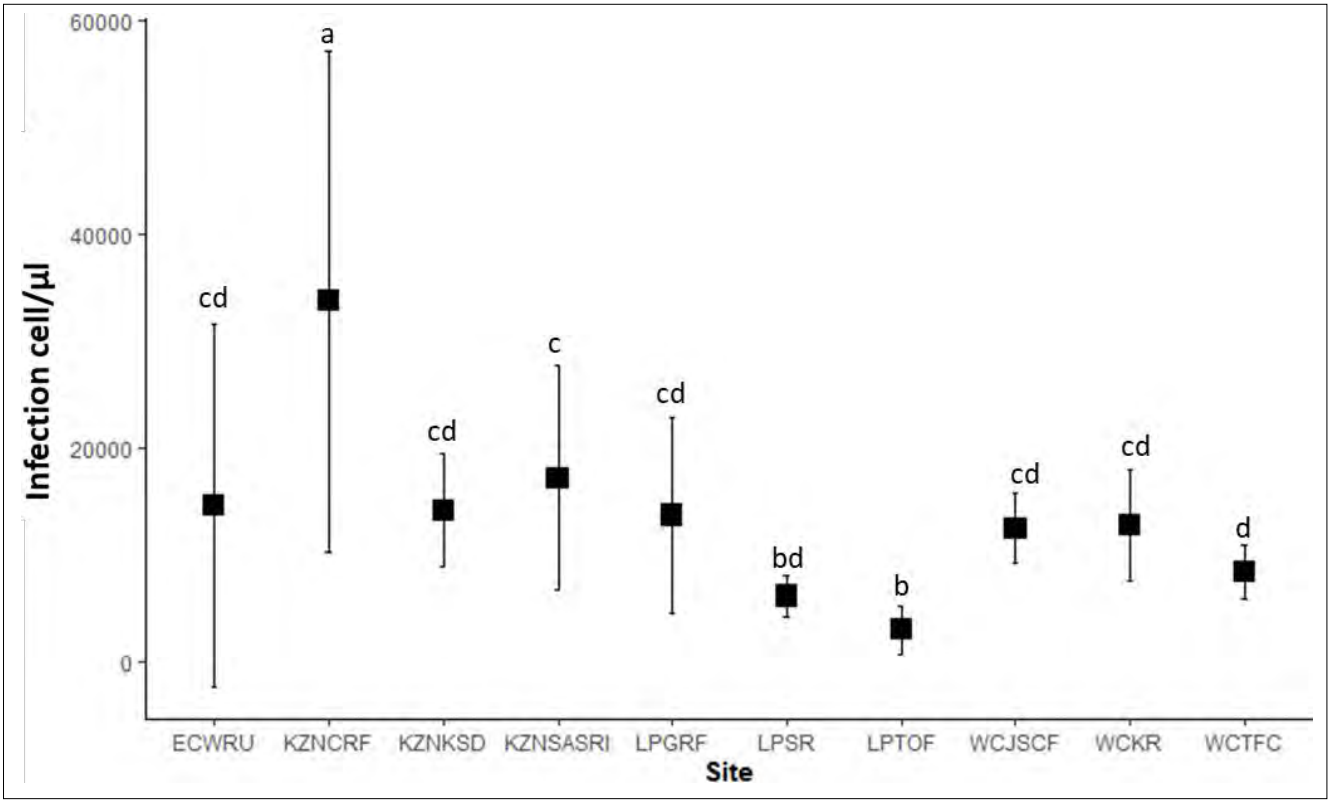

Figure 3. 3. The median infection load of *Helicosporidium* sp. in *Cyrtobagous salviniae* at all sites. Abbreviations: ECWRU (Easter Cape-Waainek Rhodes University), KZNCRF (KwaZulu-Natal-Cato Ridge Farm), KZNKSD (KwaZulu-Natal-Kingswood School Dam), KZNSASRI (KwaZulu-Natal-South African Sugar Research Institute) LPGRF (Grobblersdal Roets Farm), LPSR (Limpopo-Selate River), LPTOF (Limpopo-Tzaneen Orange Farm) WCJSCF (Western Cape-John Stanwix Candle Farm), WCKR (Western Cape-Kogsmagkloof River) and WCTFC (Western Cape-Taaibos Farm for Compost). Box (median) and whisker (standard deviation).

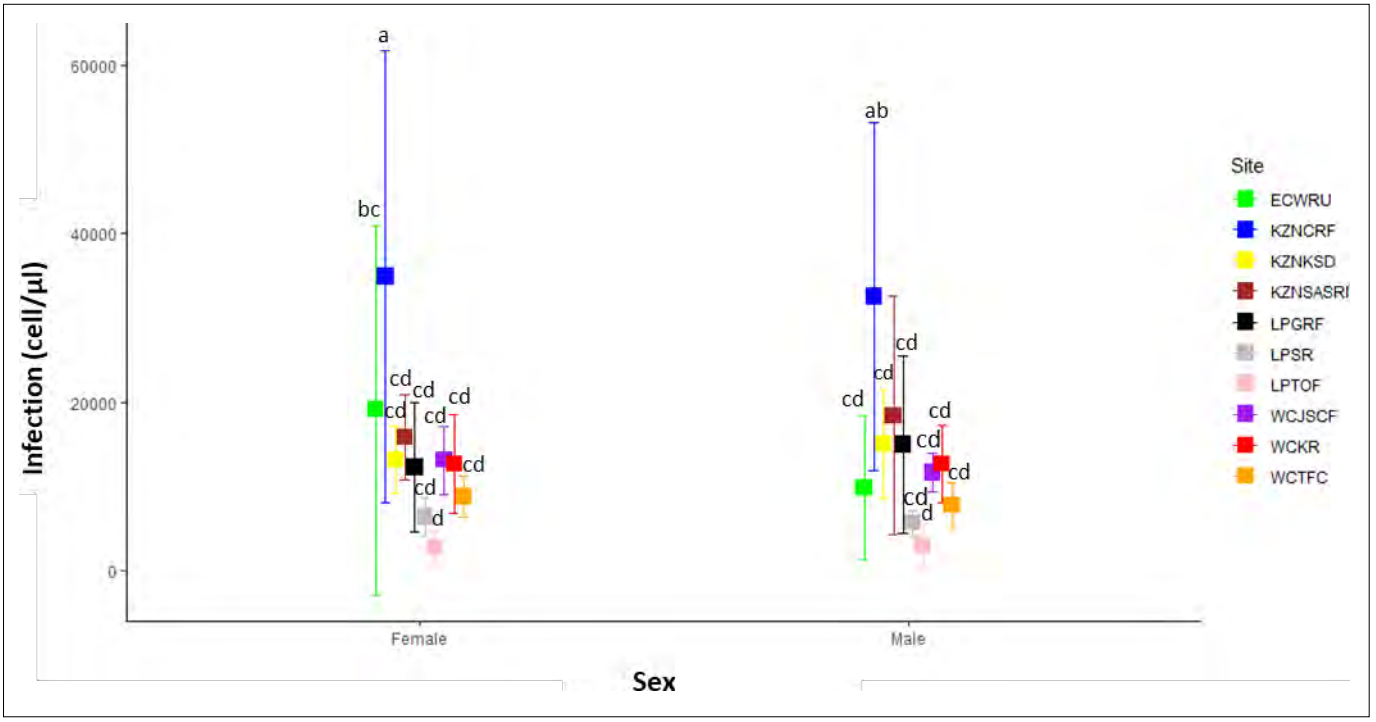

Figure 3. 4. The median infection load of *Helicosporidium* sp. in male and female *Cyrtobagous salviniae*. Abbreviations: ECWRU (Easter Cape-Waainek Rhodes University), KZNCRF (KwaZulu-Natal-Cato Ridge Farm), KZNKSD (KwaZulu-Natal-Kingswood School Dam), KZNSASRI (KwaZulu-Natal-South African Sugar Research Institute) LPGRF (Grobblersdal Roets Farm), LPSR (Limpopo-Selate River), LPTOF (Limpopo-Tzaneen Orange Farm) WCJSCF (Western Cape-John Stanwix Candle Farm), WCKR (Western Cape-Kogsmagkloof River) and WCTFC (Western Cape-Taaibos Farm for Compost). Box (median) and whisker (standard deviation).

3.4. DISCUSSION

Although *Helicosporidium* sp. was first detected from only one mass rearing facility of *C. salviniae* in South Africa (White *et al*., 2007; see Chapter 1), the results of this study show that *Helicosporidium* sp. is distributed throughout the field populations of *C. salviniae* on *S. molesta* with between 92% and 100% of the insects tested infected. *Helicosporidium* sp. was found in all of the different life stages of *C. salviniae*. The infection loads of *Helicosporidium* sp. did not differ significantly between the female and male adults of *C. salviniae*, attesting that *Helicosporidium* sp. has no gender preference in *C. salviniae*. No *C. salviniae* eggs were collected from the field since they are difficult to locate because of their size and because they are deposited in the frond tissue of *S. molesta*.

The commonly known life stages of this infection, the mature cyst, vegetative and filamentous cells matched with those cells isolated as *Helicosporidium* sp. from the field-collected individuals of *C. salviniae*, and other species (Keilin, 1921; Sayre & Clark, 1978; Purrini, 1984; Avery & Undeen, 1987; Pekkarinen, 1993; White *et al*., 2007). Testing different life stages of *C. salviniae* did not only elucidate the infection load of *Helicosporidium* sp. in field populations, but has also provided insights into how *Helicosporidium* sp. could transmit in the weevil because the detection of *Helicosporidium* sp. in different life stages of *C. salviniae* suggests that this disease transmits vertically. These mechanisms are important to understand since they shape the distribution and abundance of the infection in the field populations of *C. salviniae*.

Although some studies, detected higher infection loads of a congeneric isolate of *Helicosporidium* sp. in male rather than female adults of *Dendroctonus micans* (Kugelann) (Coleoptera: Curculionidae) (Yaman 2009), in the present study, both male and female adults of *C. salviniae* were equally vulnerable to infection. The vulnerability of *C. salviniae* to *Helicosporidium* sp. was further attested by the presence of different life cells of *Helicosporidium* sp. in the juvenile life stages, the larvae and pupae of *C. salviniae*, In contrast to this study, *D. micans* larvae showed no infection (Yaman, 2009).

Understanding the implications of the high infection load of *Helicosporidium* sp. recorded in field populations of *C. salviniae* are required to determine the effect of *Helicosporidium* sp. on *C.*

salviniae (see chapter 4). This chapter highlights the need to understand how this infection transmits and replicates to achieve this high abundance and wide distribution in *C. salviniae*. The transmission mechanisms and effects of *Helicosporidium* sp. in the field populations of *C. salviniae* are investigated and discussed in Chapters 3 and 4, respectively.

CHAPTER 4

Transmission of *Helicosporidium* sp. in *Cyrtobagous salviniae*

4.1. INTRODUCTION

Transmission is the fundamental key to replication of diseases in insect populations (Kellen & Lindegren, 1974). Parasitic diseases that attack arthropods transmit most often through horizontal and vertical pathways (Yaman & Radek, 2007). Horizontal transmission occurs through the ingestion of food particles contaminated with the infective cysts of diseases (Yaman, 2008), while vertical transmission occurs through reproduction (Yaman & Radek, 2007). In the vertical transmission process, the infectious disease cells can be mediated either maternally or paternally to transmit from the parental adults to their progeny. In this regard, the most complex studies are to determine whether the male sperm can directly interact with the infective cells and mediate the paternal transmission during mating with female adults.

Mating (rearing both males and females, confined) and non-mating (rearing newly emerged females alone) experiments are often conducted to extract mature eggs or ovaries in either their gravid or pristine status from the adult female insects and tested for the diseases in question (Mao *et al*., 2019). This procedure determines the prospects of either paternal or maternal vertical transmissions in insects (Mao *et al*., 2019). However, some studies argue that certain diseases that are paternally transmitted rarely affect the parental males, or the fitness and feeding of their progeny (Mao *et al*., 2019). Diseases that have these biological attributes are known to have less effect on the reproductive output and this allows the insects to be more efficient in maintaining their populations in nature. It has been reported that paternal transmission becomes less damaging when this transmission occurs through diseases hitchhiking on insect sperm since this method does not disturb sperm functioning, thus allowing a long-term disease epidemic to develop and persist in natural pools.

The key to understanding the transmission mechanisms of diseases lies in their biological cycles and survival attributes because diseases have different life stages and not all are transmittable from one individual insect to another through either vertical or horizontal pathways (Maddox *et al*., 1998; McManus & Solter, 2003; Goertz *et al*., 2004b). However, life histories of adverse isolates of diseases that attack insects have not been thoroughly studied to determine the role of disease life stage interactions with their host insect species (Maddox *et al*., 1998; Goertz *et al*., 2004b). These studies provide crucial information that measure diseases which have potential adverse effects on insects, and shed light on the prospects for, and approaches to, managing and controlling those diseases. Further, this biological approach could be used to assess the suitability of diseases that could possibly be used for biological control against their susceptible host insects in countries of introduction where they are recognised as a disastrous pest species (Maddox *et al*., 1998; McManus & Solter, 2003).

Host insect species face challenges in impeding or limiting transmission of diseases in their populations, but there are other limiting factors, like high or low temperatures and the chemical properties of host bodies which affect transmission and replication of diseases (Goertz *et al*., 2004b). For example, the cyst and vegetative cells of the infective bacteria species, *Clostridium difficile,* Lawson & Rainey (Clostridiales: Peptostreptococcaceae) failed to differentiate under temperatures considered high or unfavourable, and in excessive chemical (toxic) properties conducted in a controlled-environment room (Rodriguez-Palacios & Lejeune, 2011). *Clostridium difficile* has the ability to infect the gut of humans and animals the same way as *Helicosporidium* sp. does, which infects hemolymph via the gut of insect species (Rodriguez-Palacios & Lejeune, 2011). This failure to differentiate under high temperatures implies that animal species susceptible to *C. difficile* infection that are field distributed in high-temperature conditions are unlikely to experience adverse effects of the disease.

This chapter develops on the results presented in Chapter 3 that the disease,

Helicosporidium sp., covers the entire field distribution range of *Cyrtobagous salviniae* in South Africa. The aim of this chapter was to determine how this disease is transmitted. Although both transmission mechanisms have been shown in *Helicosporidium* sp. (Yaman & Radek, 2007; Kellen & Lindegren, 1974), this study has focused on determining the role played by the vertical transmission of *Helicosporidium* sp. in the populations of *C. salviniae*. The detection of *Helicosporidium* sp. in the larval, pupal and adult life stages of *C. salviniae* (Chapter 3) suggests that *Helicosporidium* sp. could be transmitting vertically in the field populations, but the eggs and ovaries of *C. salviniae,* were not screened. Whether the vertical pathway enabled the transmission of *Helicosporidium* sp. in *C. salviniae*, and whether paternal or maternal interactions mediated this transmission mechanism of *Helicosporidium* sp. infection in *C. salviniae* is also not known. Therefore, the aim of this study was to determine the transmission pathway of *Helicosporidium* sp. infection in mass reared populations of *C. salviniae*. The objective of the study was to evaluate the extent of vertical transmission pathway of *Helicosporidium* sp. in *C. salviniae*.

4.2. MATERIALS AND METHODS

4.2.1. Source of *Cyrtobagous salviniae* and *Salvinia molesta*, and experimental conditions

Trials were conducted at the Centre for Biological Control (CBC), Rhodes University. *Salvinia molesta* was obtained from stock cultures maintained at the rearing station, and grown in 5l containers under Controlled Environmental (CE) room conditions, at 25ºC to 26ºC, which is favoured by *C. salviniae* (Forno *et al*., 1983). Relative humidity ranged between 35% and 70%, and a photoperiod of 14L; 10D produced by an OSRAM L 36W/77 FLUORA (grow lux). *Salvinia molesta* plants were supplied with 1-2 small granule(s) of Culterra fertilizer, consisting of nitrogen, phosphorus and potassium (NPK: 8:1:5), every two weeks.

Experimental colonies of the infected adults, larvae and pupae of *C. salviniae* were collected from two mass rearing facilities in South Africa: Waainek Mass rearing Facility in Makhanda, Eastern Cape and SASRI, near Umhlanga, KwaZulu-Natal. 30 newly emerged adult pairs, 100 pupae, and 30 larvae of *C. salviniae* were collected and reared on *S. molesta* for further experiments in the CE room. During the experiments, a small amount of water was sprayed on the fronds of *Salvinia molesta* plants to keep them fresh and to discourage potential predators of *C. salviniae*. A full cover of the plant was placed in each of the containers.

4.2.2. Extraction of eggs and ovaries of *Cyrtobagous salviniae*

For extractions, the plants were searched for eggs and adult females. Mature eggs were successfully extracted from the female adults of *C. salviniae* and not from the *S. molesta* plants. Mature eggs and ovaries of *C. salviniae* (Figure 4.1) were extracted to screen them for *Helicosporidium* sp. cells. The mature eggs and ovaries were collected from both gravid and virgin females. Fresh and cultured smears prepared from mature eggs and ovaries of *C. salviniae* were screened. Fresh smears were body fluids extracted from live adults of *C. salviniae* to screen their status of *Helicosporidium* sp., whilst cultured smears referred to the tissues or fluids of the adults of *C. salviniae* which were preserved in *Helicosporidium* sp.

screening solution (deionised water, phosphate buffer (pH 7.4) and Giemsta stain) to allow the cells of *Helicosporidium* sp. to differentiate and replicate for screening. These were compared to fresh smears to confirm the status of *Helicosporidium* sp. from these insects.

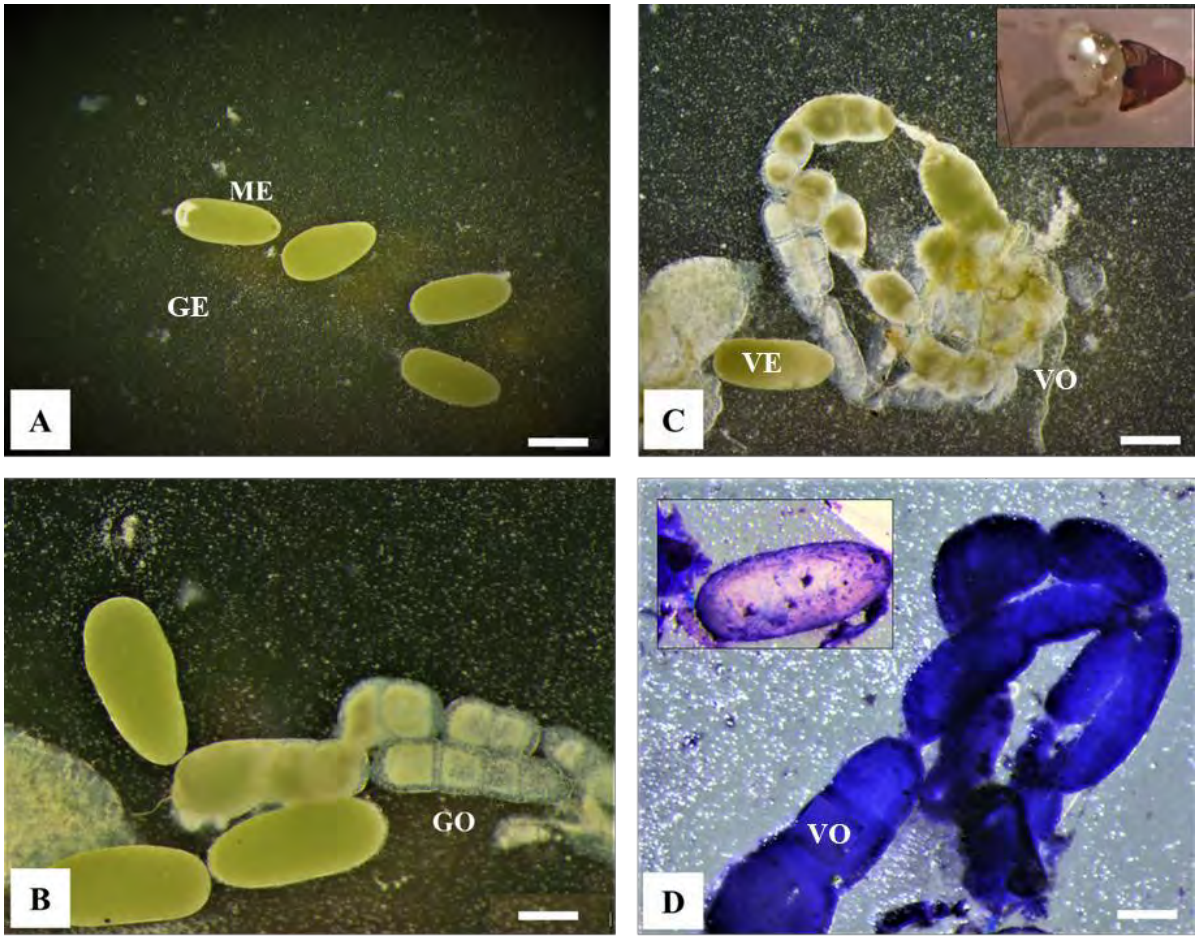

Figure 4. 1. Mature eggs and ovaries isolated from gravid and pristine females of *Cyrtobagous salviniae*. (A) Mature eggs of a gravid female, (B) Ovaries and eggs of a gravid female, (C) Ovaries and eggs of a virgin female and (D) Ovaries of a virgin female. Abbreviations: (GO) Gravid Ovary, (GE) Gravid Egg, (VO) Virgin Ovary, (VE) Virgin Egg and (ME) Matured Egg. Scale bars: 250 µm.

4.2.3. Fresh smears

Fresh smears were prepared from three different colonies of *C. salviniae*. The first colony comprised 30 pairs of newly eclosed *C. salviniae* adults which were reared in three separate 5-litre containers (10 pairs per container) for 21 days in the CE room. After the pre-oviposition period (6 to 14 days after eclosion (Forno *et al*., 1983)), the female adults were removed and dissected to extract their fertilized ovaries and mature eggs. The second colony comprised 100

pupae which were placed in groups of 10 individuals in 1-litre containers, until they eclosed as new adults. Thirty female adults were isolated and placed in groups of five in 1-litre containers for 21 days with no males until they completed their pre-oviposition period. Subsequently, these virgin females were dissected to extract their non-fertilized ovaries and mature eggs. This test of virgin female adults made it possible to determine whether paternal and maternal interaction with *Helicosporidium* sp. causes vertical transmission of the infection. The last colony comprised early to mid-instar *C. salviniae* larvae which were collected directly from culture maintenance pools of infected *C. salviniae*. These infected larvae were processed without being reared further under the CE room conditions. The larvae were collected from the same culture as the pupae used in these experiments. This colony served as a control for this whole experiment. The experimental control cells of *Helicosporidium* sp. were extracted from the infected larvae. These cells were matched with those detected in both the gravid and virgin eggs and ovaries of *C. salviniae* to confirm the presence of *Helicosporidium* sp.

To prepare fresh smears for screening *Helicosporidium* sp. infection in three life stages, eggs, ovaries and larvae of *C. salviniae* were separately washed (five times with distilled water) and crushed on glass slides using ordinary sharp pins under a stereo microscope. A screening solution consisting of 10 µl of deionised water, 8 µl of phosphate buffer (pH 7.4) and 3 µl of Giemsta stain was homogenised with the smears prepared from eggs, ovaries and larva, separately, on the glass slides, and covered with a cover slip. The overall solution (smears and homogenising solution) was 22 µl when counting the number of *Helicosporidium* sp. cells. Counting *Helicosporidium* cells was conducted under a compound microscope at 400X magnification. Counting was done on the overall mounted solution.

4.2.4. Smear culture

This trial was aimed at confirming that the cells found in the fresh smears prepared from both the gravid and virgin mature eggs and ovaries of *C. salviniae* were *Helicosporidium* sp. This

confirmation was done by culturing the same fresh smear to see if the detected cells would differentiate into other life cell stages of *Helicosporidium* sp. Culturing of smears of the gravid and virgin mature eggs and ovaries followed the same procedure used in Chapter 2 (see life cycle of *Helicosporidium* sp. extracted from *C. salviniae*). Smears were prepared from ovaries, eggs and larvae placed at room temperature (which is favourable, see Chapter 1), and mounted on 30 sterilized glass slides. Thus, 30 replicates were run for culturing. As a backup, smears from the same ovaries, eggs, and larvae were placed in 30 sterilized petri dishes. Another solution of smears prepared from both gravid and virgin ovaries, eggs and larvae was cultured to serve as an additional control of the experiments.

Both the glass slides and petri dishes were observed under a compound microscope, at 400X magnification. Observations were made between seven and 21 days after culturing the smears. This duration allowed any cell of the infection to differentiate since the life cycle of *Helicosporidium* sp. infection takes approximately seven days to complete (Keilin, 1921; Chapter 1, section 6.2). Cysts of *Helicosporidium* sp. were expected to differentiate into the vegetative cell stage during the culturing process.

4.2.5. Data analysis

The raw data met the normality assumptions of Analysis of Variance (ANOVA), which was checked by the Shapiro-Wilk normality test ($P > 0.05$). Thus, One-Way ANOVA, followed by Tukey HSD post-hoc tests were performed to compute and compare the means of the occurrence of *Helicosporidium* sp. cysts in ovaries, eggs and instars of *C. salviniae*. All statistical analyses were performed in R Studio (version 1.1.463).

4.3. RESULTS

4.3.1. Fresh smear

The ovaries and mature eggs of virgin and gravid female adults of *C. salviniae* tested positive for *Helicosporidium* sp. infection, as cysts were detected in the fresh smears of eggs and ovaries extracted from both the gravid and virgin female adults (Figure 4.4). The same cyst cells were found in the smears of the larvae.

The cyst counts in smears of gravid and virgin ovaries were significantly higher compared to those counted from larvae ($F_{(2, 58)} = 51.58$; $P = 2.15 \times 10^{-15}$) (Figure 4.2). In contrast, the cyst counts in the gravid and virgin eggs of *C. salviniae* were not statistically different compared to those counted in the larvae ($F_{(2, 42)} = 1.58$; $P = 0.22$) (Figure 4.3.). The cyst cells counted from the fresh smears of the gravid and virgin ovaries and eggs were not affected by the absence of mating between the infected female and male adults of *C. salviniae* (Table 4.1).

Table 4. 1. Statistical details of linear hypothesis based on multiple comparisons (Tukey contrasts) of the occurrence means of *Helicosporidium* sp. cysts in the fresh smears of both gravid and virgin ovaries, mature eggs, and larvae of *Cyrtobagous salviniae*.

Terms	Variables	Estimate	Std. Error	*t*-value	*P*-value
Ovary	GO * EL	-12.59	1.51	-8.31	**1.10 x 10^{-04}**
	VO * EL	-13.63	1.49	-9.17	**1.10 x 10-04**
	VO * GO	-1.05	1.51	-0.69	0.77
Egg	GE * EL	-2.40	1.83	-1.28	0.41
	VE * EL	-3.20	1.88	-1.71	0.21
	VE *GE	-0.80	1.87	-0.43	0.91

* Interactions between variables. *P*-values in bold are statistically different. Abbreviations: (GO) Gravid Ovary, (VO) Virgin Ovary, (EL) Early Larval, (VE) Virgin Egg and (GE) Gravid Egg.

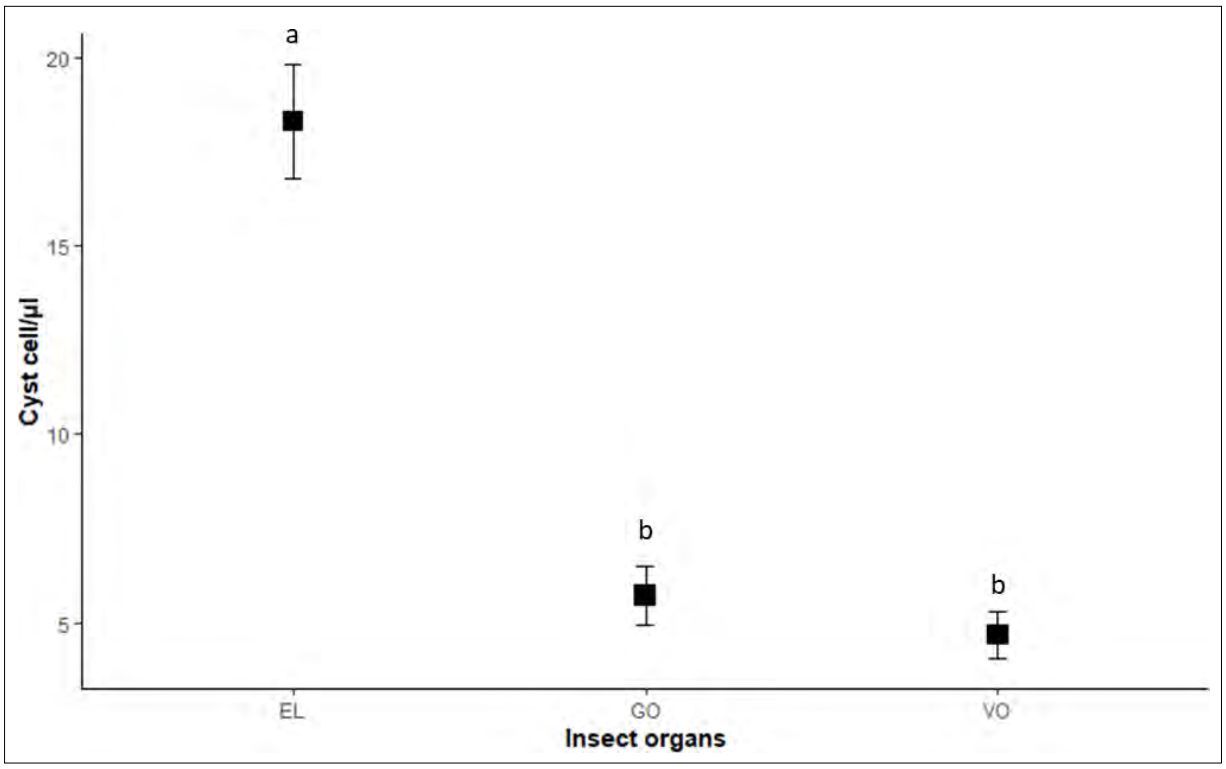

Figure 4. 2. Mean (+SE) counts of *Helicosporidium* sp. cyst cells from smears of larvae, gravid and virgin ovaries of *Cyrtobagous salviniae*. **Abbreviations**: (EL) Early Larval stage, (GO) Gravid Ovary and (VO) Virgin Ovary. Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

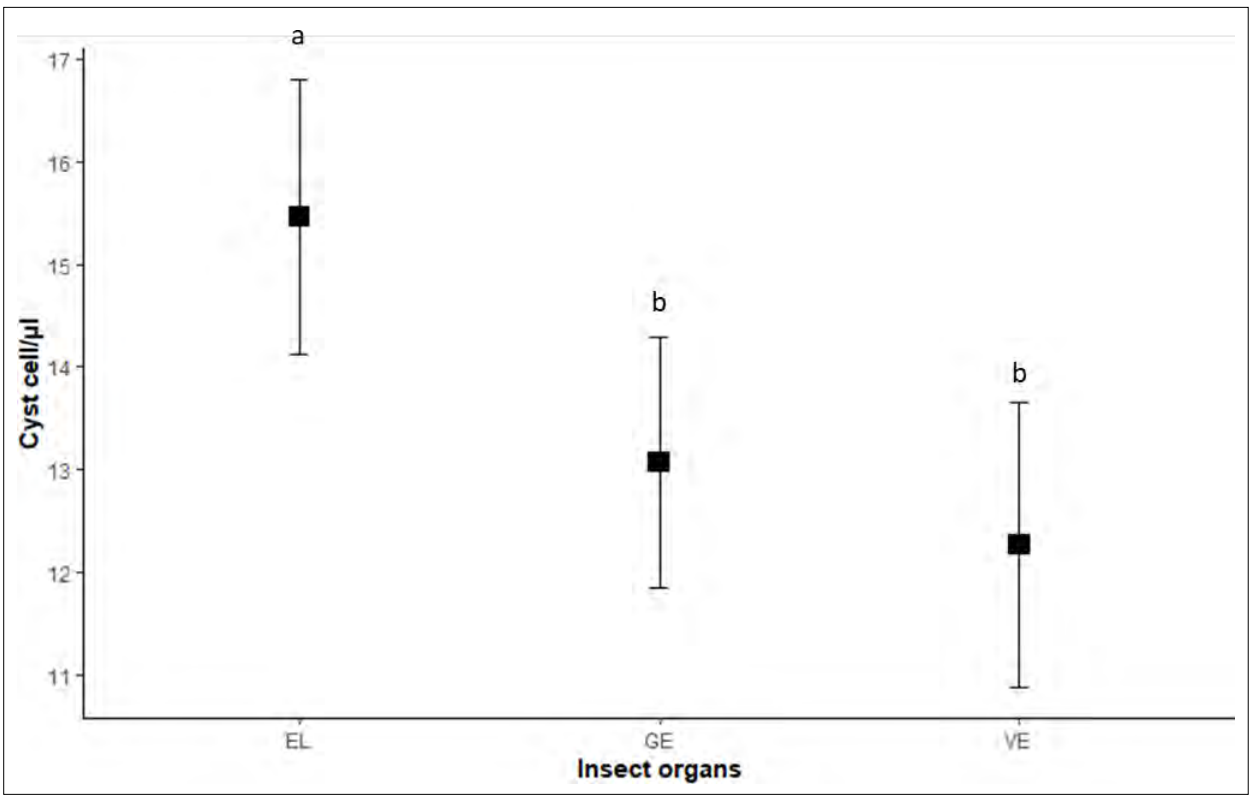

Figure 4. 3. Mean (+SE) counts of *Helicosporidium* sp. cyst cells from smears of the larvae, gravid and

virgin mature eggs, of *Cyrtobagous salviniae*. Abbreviations: (EL) Early Larva, (GE) Gravid Egg and (VE) Virgin Egg. Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

4.3.2. Smear culture

The cultured smears of both ovaries and eggs of either gravid or virgin female adults of *C. salviniae* showed distinct *Helicosporidium* sp. cysts like those detected in the fresh smears prepared from the same organs (Figure 4.4 A). This result confirms that both gravid and virgin ovaries of female *C. salviniae* adults carry and transmit *Helicosporidium* sp. cysts. The counted cysts of *Helicosporidium* sp. in the cultured smears of the gravid and virgin eggs and ovaries were significantly lower than those counted from the larvae of *C. salviniae* ($F_{(2, 85)} = 54.19$; $P = 6.73 \times 10^{-16}$). The mean counts of *Helicosporidium* sp. cysts per 22 µl of the cultured smears of the gravid ovaries, virgin ovaries and larvae of *C. salviniae* were 3.86 ± 0.53 (mean ± SE), 2.77 ± 0.49 and 16.30 ± 1.62, respectively (Figure 4.5).

The mature cysts found in the cultured smears of both the gravid and virgin ovaries and eggs, as well as the larvae of *C. salviniae* all differentiated into the vegetative cells of *Helicosporidium* sp. (Figure 4.4 B & C).

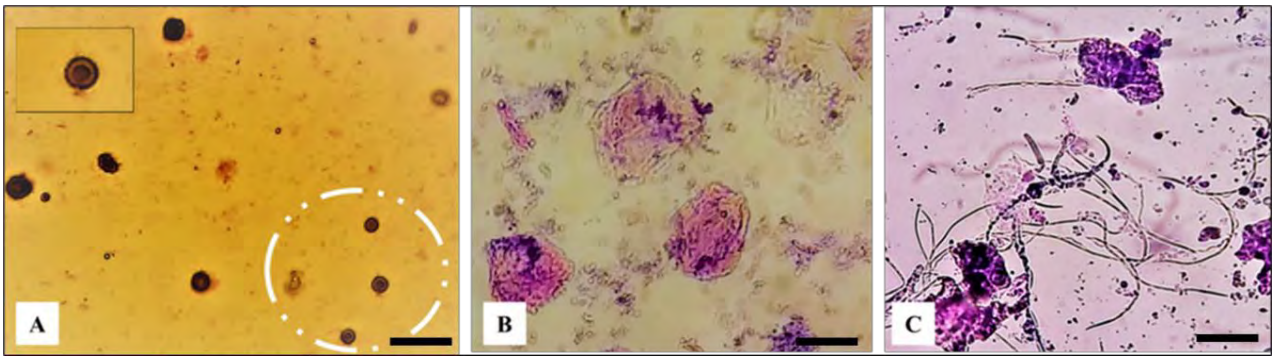

Figure 4. 4. Common life stages of *Helicosporidium* sp. which developed in cultures of smears prepared from gravid and virgin ovaries and mature eggs, as well as the larvae of *Cyrtobagous salviniae*. (A) mature cysts, (B) vegetative cells and (C) vegetative cells rupturing. Micrograph scale: 50 µm.

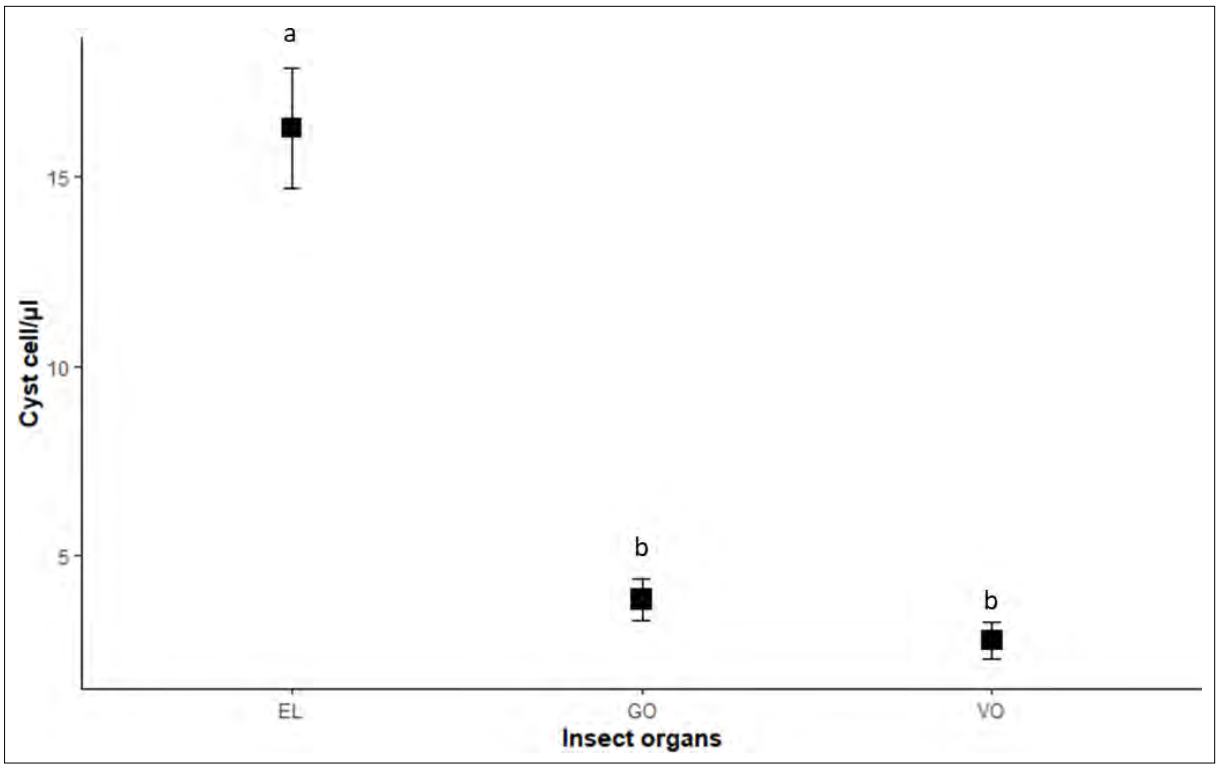

Figure 4. 5. Mean (+SE) counts of *Helicosporidium* sp. cyst cells from cultured smears of gravid and virgin matured eggs, as well as the larvae of *Cyrtobagous salviniae*. **Abbreviations**: (EL) Early Larva, (GO) Gravid Ovary and (VO) Virgin Ovary. Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error)

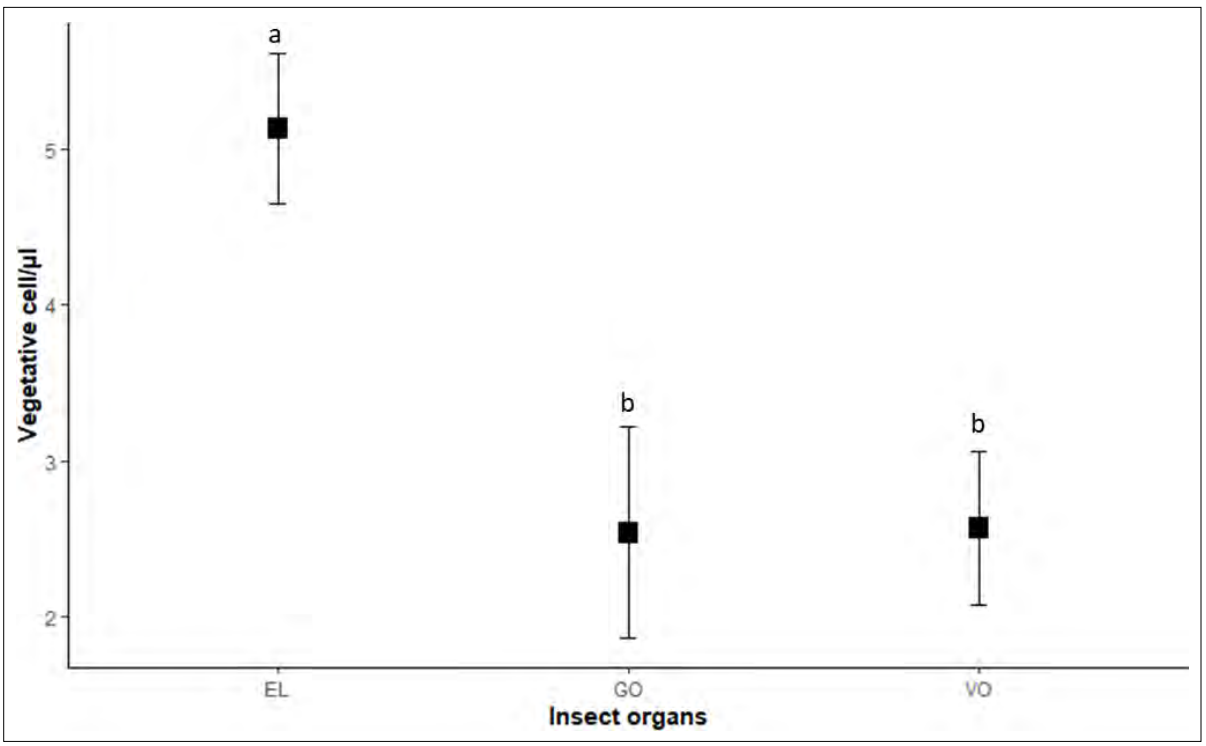

Figure 4. 6. Mean (+SE) counts of *Helicosporidium* sp. vegetative cells from cultured smears of gravid and virgin matured eggs, as well as the larvae of *Cyrtobagous salviniae*. Abbreviations: (EL) Early

Larva, (GO) Gravid Ovary, (VO) Virgin Ovary. Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

The counts of vegetative cells from the cultured smears of the gravid and virgin ovaries were significantly lower than those in the larvae of *C. salviniae* ($F_{(2, 85)} = 7.31$; $P = 0.001$). The vegetative cells developed from cultured smears of the gravid and virgin ovaries were not affected by the absence of mating between the infected male and female adults of *C. salviniae* (Table 4.2).

Table 4. 2. Statistical details of the linear hypothesis based on multiple comparisons (Tukey contrasts) of the mean occurrences of *Helicosporidium* sp. cyst and vegetative cells in cultured smears of both gravid and virgin ovaries and matured eggs, and the larvae of *Cyrtobagous salviniae*.

Terms	Variables	Estimate	Std. Error	*t*-value	*P*-value
Cyst	GO * EL	-12.44	1.51	-8.50	**1 x 10-04**
	VO * EL	-13.53	1.44	-9.41	**1 x 10-04**
	VO * GO	-1.09	1.51	-0.75	0.74
Vegetative	GO * EL	-2.62	0.79	-3.29	**0.004**
	VO * EL	-2.62	0.78	-3.31	**0.003**
	VO * GO	0.03	0.79	0.04	0.99

* Interactions between variables. *P*-value in bold are statistically different. Abbreviations: (GO) Gravid Ovary, (VO) Virgin Ovary and (EL) Early Larval stages.

4.4. DISCUSSION

The results from Chapter 3 suggested that *Helicosporidium* sp. infection was vertically transmitted in field populations of *C. salviniae*, because *Helicosporidium* sp. was detected in field-collected larvae, pupae and adults of *C. salviniae*. This was supported by the occurrence of different life stages of *Helicosporidium* sp. in *C. salviniae* life stages. However, *Helicosporidium* sp., could have been ingested by either the larvae or adults of *C. salviniae*. Furthermore, De Koning (2006) reported that there has never been a case where *Helicosporidium* spp. were observed to have transmitted through reproduction of the host insect species.

The results from this chapter demonstrated that *Helicosporidium* sp. infection does indeed transmit vertically in field populations of *C. salviniae*. The transmission mechanism of *Helicosporidium* sp. in *C. salviniae* occurs maternally because the eggs and ovaries of both gravid and virgin female adults of *C. salviniae* tested positive for *Helicosporidium* sp. Although the status of *Helicosporidium* sp. in sperm of the infected male adults of *C. salviniae* was not determined directly, the male adults cannot be completely ruled out from paternal vertical transmission. Although horizontal transmission of *Helicosporidium* sp. could also occur in *C. salviniae* (Yaman & Radek, 2007), the results of this study strongly suggest that a vertical pathway is the key transmission mechanism of *Helicosporidium* sp. in *C. salviniae* in South Africa.

The transmittable cell of *Helicosporidium* sp. in *C. salviniae* was found to be the mature cysts. 31% and 25% of the cysts were counted from fresh smears of the gravid and virgin ovaries, respectively, which was lower than counts found in the early instars of *C. salviniae*. By contrast, 23.48% and 18.58% of the mature cysts were recorded from the cultured smears prepared from the gravid and virgin ovaries, respectively. The cysts counted in the cultured smears of both the gravid and virgin ovaries had differentiated and formed a considerably high

count of the vegetative cells or spores. These results show that, with or without mating, female adults of *C. salviniae* can transmit *Helicosporidium* sp. infection maternally. The small difference in the mean occurrence of *Helicosporidium* sp. in both gravid and virgin ovaries and eggs could be due to the missing injection of additional cysts paternally from male adults. Thus, these findings do not rule out the possibility that paternal transmission of *Helicosporidium* sp. could occur. Horizontal transmission of *Helicosporidium* sp. was not investigated in this study since there were no uninfected adults of *C. salviniae*. However, in some insects, *Helicosporidium* sp. can transmit horizontally (Yaman, 2009); therefore, the probability of this infection expanding its host range to native or introduced insect species is extremely low because no interbreeding occurs between them and the infected *C. salviniae*.

To date, there are no known isolates of *Helicosporidium* spp. in nature which can be efficiently transmitted maternally or paternally. The prospect of the mature cyst hitchhiking from the female's hemocoel into its ovaries was not investigated and remains uncertain. Investigations into the prospects of *Helicosporidium* spp. transmitting vertically in naturally occurring insect populations remains deficient, but could be an important study to add further explanation to the results of this chapter. Bläske & Boucias (2004) recorded vertical transmission of *Helicosporidium* sp. which was first orally transmitted from *C. salviniae* to *Helipcoverpa zea*, *Spodoptera exigua* and *Trichoplusia ni*, where the infection cells were subsequently detected in 41.7% of their progeny (Bläske & Boucias, 2004). Although this study supports the notion that vertical transmission of *Helicosporidium* sp. occurs, it did not reveal whether transmission was mediated by paternal or maternal pathways.

In summary, this chapter revealed that *Helicosporidium* sp. infection transmits vertically through the maternal pathway in field populations of *C. salviniae*. Although previous studies have found horizontal and vertical transmission to transmit high and low cell loads of *Helicosporidium* sp., respectively (Blaske & Boucias, 2004), based on the increase of

Helicosporidium sp. infectivity showed in Chapter 2, with low or high load of this infection, *Helicosporidium* sp. can replicate rapidly. This finding provides a foundation for further studies on the mechanisms underlying maternal infection transmissions, and the information is useful in providing strategic and efficient approaches to attenuate *Helicosporidium* sp. infection. This study did not determine the effect of *Helicosporidium* sp. in *C. salviniae,* which is investigated and discussed in the next chapter, Chapter 5.

CHAPTER 5

Effect of *Helicosporidium* sp. infection on *Cyrtobagous salviniae*

5.1. INTRODUCTION

Re-uniting alien invasive plant species with their natural enemies is the fundamental focus of both classical and augmentative release biological control programmes (Hoy, 2008). If, however, the re-union is applied with biocontrol agents that are themselves carrying natural enemies, such as diseases, then the programme may not be successful (White *et al*., 2007). Insect diseases could have effects that are negligible, moderate, or highly harmful to their host insect species (Vega & Kaya, 2012). These effects can result in disruptions of biological attributes of their host and lead to mortality, or reduced fitness significantly decreasing their populations (White *et al*., 2007). In insect mass-rearing systems, it is important to continuously screen for diseases, and where necessary, implement treatments to control diseases that can affect the health of insect colonies (White *et al*., 2007).

Some insect diseases (entomopathogens) are important in biological control programmes, specifically in geographic locations where their hosts have established as pests. Diseases that are specific to a single insect species are mostly targeted to control pests or weeds, threatening native and agricultural species. For example, *Bacillus thuringiensis* var. *kurstaki* that has been used to control a leaf defoliator pest species in commercial forest plantations in the USA (Hajek & van Frankenhuyzen, 2017), and the nematode disease caused by *Deladenus siricidicola* Bedding (Tylenchida: Neotylenchidae) has been used to control the invasive pine wood borer *Sirex noctilio* Fabricius (Hymenoptera: Siricidae) in Brazil (Fenili *et al*., 2000). Entomopathogens and plant pathogens are acknowledged as potential biocontrol agents since they are very useful in slowing down the spread of either alien invasive insect pests or weed plant species.

Several studies evaluated impacts of *Helicosporidium* sp. and found it readily killed

several mosquito and lepidopteran species (Avery & Undeen, 1987). Based on the track record of these impacts, a study to evaluate the microbial potential of *Helicosporidium* sp. isolated from *Aedes aegypti* and *Culex quinqefasciatus* (Diptera: Culicidae) originated from Thailand was conducted in the USA (Hembree, 1981). In this evaluation, *Helicosporidium* sp. showed no potential to be a cost-effective microbial control agent of the targeted insect species, *Spodoptera frugiperda*, *A. dirus*, *A. maculatus*, *A. taeniorhynchus* and *Toxorhynchites splendens* (Diptera: Culicidae) (Hembree, 1981). The lack of the microbial control potential of *Helicosporidium* sp. was concluded despite its high infectivity rates that were retrieved from the exposed insects (Hembree, 1981). Nonetheless, the results of this study do not imply that, *Helicosporidium* sp. cannot have the desired microbial impact on other susceptible insect species (Solter & Vávra, 2012). Based on the recorded impacts of *Helicosporidium* sp. (Yaman, 2008; Bläske-lietze *et al*., 2006; Bläske-lietze & Boucias, 2005; Avery & Undeen, 1987), it is surprising that, there is no a single research programme that has successfully controlled a pest insect species using *Helicosporidium* spp. The lack of such programmes across the globe suggests that, either *Helicosporidium* sp. have not been fully explored or this *Helicosporidium* sp. cannot be a potential biological control agent as reported by Hembree (1981). The poor potential of *Helicosporidium* sp. as a biological control agent could be due to its ability to infect multiple insects.

Further, *Helicosporidium* sp. isolates of mosquito and black fly larva species have been found to infect other insect species of lepidoptera and coleoptera (Avery & Undeed, 1987; Boucias *et al*., 2001). Coupling these findings and other results on the pathogenicity of *Helicosporidium* sp. isolates (Hembree, 1981; Tartar, 2013; Conklin *et al*., 2005), this clearly depicts a very low specificity degree of this infection.

Despite the widespread and high infection load of *Helicosporidium* sp. in field populations of *C. salviniae* established on *S. molesta*, the insect is still regarded as an effective

agent for the control of *S. molesta* in South Africa (Martin *et al*., 2018; Hill & Coetzee, 2017). In South Africa, the role of *Helicosporidium* sp. disease on the biology of *C. salviniae* has not been fully elucidated, and this chapter focuses on determining the effect of *Helicosporidium* sp. on this weevil, and updates the absolute effectiveness of the infected *C. salviniae* under field conditions. The objectives of the study were to determine the impact and reproductive output of the infected *C. salviniae* on *S. molesta*.

5.2. MATERIALS AND METHODS

To determine the impact of *Helicosporidium* sp. on the biology of *C. salviniae*, a disease-free culture of the insect was required. As it was not possible to obtain this in South Africa, a parallel set of trials were conducted in the laboratory at Rhodes University with a diseased culture, and at Louisiana State University, Louisiana, USA with a disease-free culture. Although preliminary tests conducted in South Africa alone have indicated that, high and low impact on *S. molesta* were caused by the adults of *C. salviniae* with low/minimal and high loads of *Helicosporidium* sp., respectively (Mphephu *et al*., 2019), a disease-free culture was sought.

Before the start of the experiment in Louisiana, trials were conducted to check if the *C. salviniae* were indeed disease-free. Thirty adult *C. salviniae* were collected from St. Gabriel, LA field mass rearing ponds and homogenised separately in a solution of a few drops of deionised water (likely 100 µl) and one drop (likely 20 µl) of Giemsa stain solution, in an Eppendorf tube, using a still rod. Three days later, the solution was mounted on a glass slide, covered with a coverslip, and examined under a compound microscope at 400X. The tests revealed a non-prominent load of mature cyst-like and vegetative cells from fresh smears of these weevils, which looked like those of *Helicosporidium* sp. reported by Boucias *et al*, (2001). The vegetative cells ruptured and released the filamentous cells, which eventually begun releasing cysts. Zero (in 23 adults) to fewer than 10 (in 7 adults) of these cells were recorded per one drop of the prepared smears of these weevils, using an ordinary pipette. However, cells detected in the LSU *C. salviniae* were compared with *Helicosporidium* sp. cells from *C. salviniae* from South Africa reported by White *et al*, (2007), and were found not to be *Helicosporidium* sp., hence the LSU culture was clear of the disease.

5.2.1. Source of *Cyrtobagous salviniae* and experimental conditions

Experimental colonies of both the diseased and disease-free adults of *C. salviniae* were collected from two of the weevil's introduced ranges, South Africa and Louisiana, United

States of America, respectively. Tests of the feeding impact and reproductive output of the diseased *C. salviniae* adults were conducted at the Department of Zoology and Entomology, Centre for Biological Control (CBC), Rhodes University, South Africa. These tests were subsequently repeated with disease-free *C. salviniae* adults on *S. molesta* at the Department of Entomology, Louisiana State University (LSU), USA. Disease-free and diseased adults of *C. salviniae* were maintained under natural conditions in artificial pools. Newly eclosed adults were used to test the impact of infection on *C. salviniae* feeding on *S. molesta* biomass, while mature adults were used to conduct the reproduction trials. 512 *C. salviniae* adults (males:females = 1:1) were collected for each of the two experiments (diseased and disease-free) to determine the impact of infection on adult feeding on the host plant biomass, and reproductive output of *C. salviniae*.

The experiments were conducted in 5-litre containers under Controlled Environmental (CE) room conditions with temperatures maintained at 25ºC to 26ºC, (a temperature range favourable for *C. salviniae* (Forno *et al*., 1983)), relative humidity of 35% to 70%, and a photoperiod of 14L: 10 D (grow lux lights

5.2.2. Source and culture maintenance of *Salvinia molesta* plants

Salvinia molesta plants were collected from their culture maintenance pools at both LSU and CBC. 750g of *S. molesta* plants were collected for the experiment, including controls. Before setting up the experiments, the *S. molesta* plants were thoroughly washed under running tap water for five minutes to remove any potential pest species (e.g. spiders, mites). Each replicate used 25 g of *S. molesta*. During the experiments, the plants were supplied with one to two small granule(s) of Culterra fertilizer, consisting of nitrogen, phosphorus, and potassium (NPK: 8:1:5)), every two weeks. During the experiments, a small amount of water was sprayed on the leaves of *S. molesta* plants to keep them fresh and to discourage potential predators.

5.2.3. Impact of *Cyrtobagous salviniae* on *Salvinia molesta* biomass

This trial was conducted to determine the impact of the diseased and disease-free adults of *C. salviniae* on *S. molesta* plant biomass (Figure 5.1). Biomass was used as a measure of efficacy of *C. salviniae* on *S. molesta* (Mphephu, 2015). Two colonies of the diseased and disease-free (newly emerged) adults of *C. salviniae* were given equal amounts of *S. molesta*, 25 g each, in 10-litre containers. A gauze covering on each container prevented escape of the insects. A series of *C. salviniae* adult pairs (one, three, five and seven) were separately confined to each 25 g of *S. molesta*, for seven (7) days. An insect free control was also set up.

Figure 5. 1. Experimental setup to determine the effect of *Helicosporidium* sp. infection on the reproductive output and feeding of *Cyrtobagous salviniae* on *Salvinia molesta*.

At the end of the experiment, the remaining plant material was harvested and weighed to determine the amount of damage inflicted by both the diseased and disease-free colonies of *C. salviniae*. The number of fronds damaged by *C. salviniae* influencing the resultant biomass of *S. molesta* were assessed and scored. Frond damage was scored from 0 to 3, where 0 = no feeding; 1 = exploratory feeding; 2 = minor feeding and 3 = normal feeding. Lastly, each adult pair of the diseased colony of *C. salviniae* were screened to determine the infection load of *Helicosporidium* sp. that influenced their feeding performance on *S. molesta*. This screening followed the same procedure that was described in Chapter 2.

5.2.5. Reproductive output of *Cyrtobagous salviniae* pilot study

To determine the effect of *Helicosporidium* sp. infection on the reproductive output of *C. salviniae*, a series of the gravid and ovipositing adult pairs, ranging from one, three, five and seven were confined to 25 grams of *S. molesta* in 10-litre containers, separately. The selection of this stage of adults was motivated by the reported biology which shows that each female *C. salviniae* adult can deposit one or two eggs per day, and 208–374 eggs during a lifespan that lasts for 20–36 weeks (Sands *et al*., 1986; Jayanth, 1989; Eisenberg, 2011). Therefore, the experimental adults had equal opportunities for oviposition. Each adult pair consisted of either diseased or disease free, gravid adults of *C. salviniae*. Each pair of either the diseased or disease-free adults was exposed to *C. salviniae* for 24, 48 and 72 hours, separately. At the end of the allocated times of exposure, the exposed adult pairs were gently removed from the *S. molesta* plants. The plants were then monitored until new adults emerged, which determined the reproductive output of the diseased and disease-free *C. salviniae* adults at their different colony sizes and oviposition duration. The experimental and newly produced adults of *C. salviniae* from the diseased colony were screened to determine the infection load of *Helicosporidium* sp. that influenced their reproductive output.

As an additional control, the duration of this experiment was guided by duration of a generation, including life stages of *C. salviniae* recorded on *S. molesta* (see Chapter 1). Under favourable conditions (25.5°C), the female adults of *C. salviniae* have a pre-oviposition period that ranges between six and 14 days after they have emerged as new adults (Forno *et al*., 1983). Each female can deposit an average of 208–374 eggs during its lifetime and can live for approximately 20–36 weeks (Sands *et al*., 1986; Elsenberg, 2011). The incubation period of each egg is about 10 days (Forno *et al*., 1983). Given these biological attributes of *C. salviniae*, the duration of the study was sufficient to determine the reproductive output of both the diseased and diseased free adults of *C. salviniae* on *S. molesta*.

In order to determine whether *Helicosporidium* sp. infection influences body size of *C. salviniae* adults, newly eclosed adults body size (length by breadth) was measured using a light microscope and these measurements were analysed with analySIS docu software (source: analysis-docu.software.informer.com, accessed 2020).

5.2.6. Data analysis

General linear model (GLM) full factorial analyses of variance (ANOVA), followed by Tukey HSD post-hoc tests were performed to compare a.) *S. molesta* biomass, and b.) reproductive output of the diseased and disease-free *C. salviniae* adults. Linear regression models tested for significant correlations between the resultant plant biomass and reproductive output by disease-free and diseased adult pairs of *C. salviniae*. A Student's *t*-test compared the body sizes or areas of both the diseased and disease-free adults of *C. salviniae*. All statistical analyses were conducted in R Studio (version 1.1.463).

5.3. RESULTS

5.3.1. Impact of *Cyrtobagous salviniae* on *Salvinia molesta* biomass

Disease free pairs of *C. salviniae* significantly reduced the fresh biomass of *S. molesta* plants, at higher densities, compared to the infected insects ($F_{(1, 28)} = 70.66$; $P = 9.42 \times 10^{-7}$) (Figure 5.2). Biomass of *S. molesta* was significantly lower on the plants which were exposed to disease-free rather than diseased adult pairs of *C. salviniae* (Table 5.1). The increase in the number of diseased adult pairs of *C. salviniae* did not significantly improve the impact on *S. molesta* biomass (Figure 5.2; Table 5.2), but the increase in the number of disease-free adult pairs of *C. salviniae* did significantly reduce *S. molesta* biomass (Figure 5.2; Table 5.2).

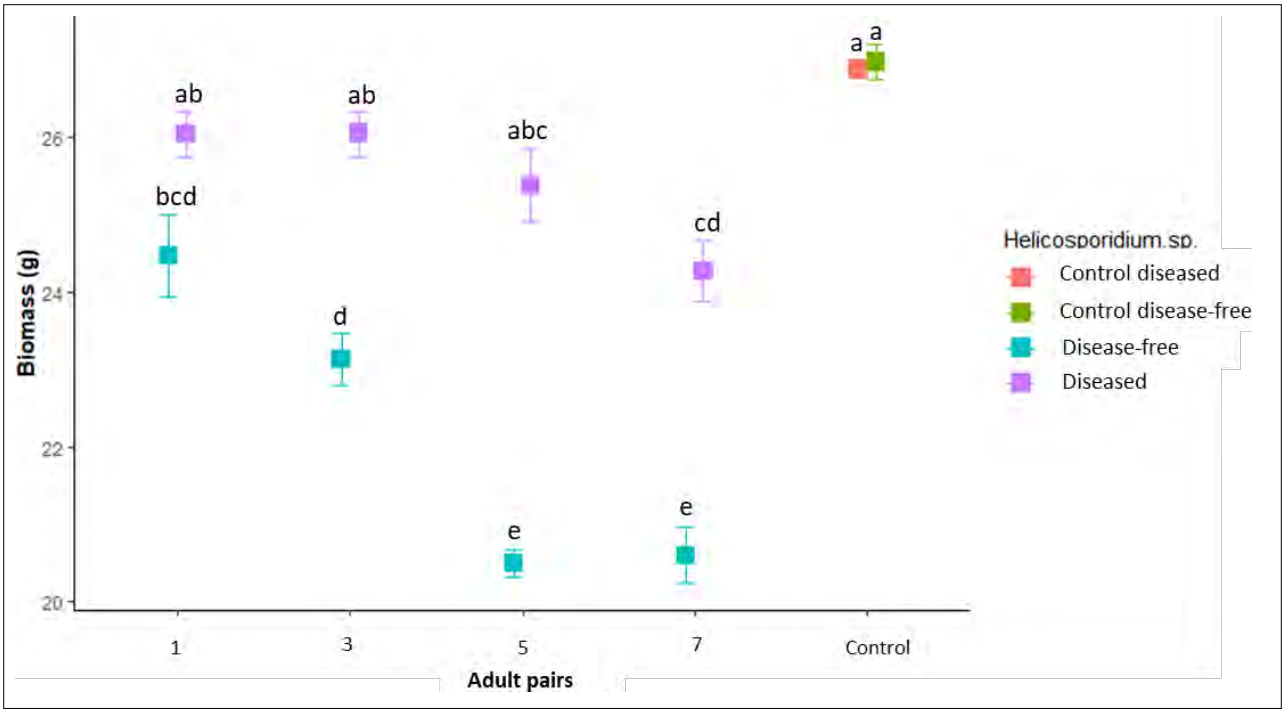

Figure 5. 2. Patterns of *Salvinia molesta* biomass changes by the impact of the diseased and disease-free adult pairs of *Cyrtobagous salviniae* in a seven-day experimental test ($n = 4$). Box (mean) and whisker (standard error).

Table 5. 1. Statistical details on the mean (mean ± SE) of the biomass, leaf damage and adult survival recorded from the diseased and disease-free adult pairs of *Cyrtobagous salviniae* on *Salvinia molesta* for seven days.

Adult pairs	Biomass (grams)	Leaf damage	Adult survival	Adult produced
Diseased				
One	$26 \pm 0.29^{*}$	10 ± 1.15	$1 \pm 0.58^{*}$	$1 \pm 0.24^{*}$
Three	$26.30 \pm 0.29^{***}$	$17 \pm 2.31^{***}$	3.33 ± 0.33	2.8 ± 0.32
Five	25.40 ± 0.51^{x}	27.7 ± 1.33^{x}	7 ± 0.58	3.4 ± 0.38^{x}
Seven	$24.90 \pm 0.11^{x**}$	$56 \pm 7.21^{x**}$	12 ± 0.58	$5.7 \pm 0.47^{x**}$
Disease free				
One	$23.80 \pm 0.17^{*}$	13 ± 2.31	2^{*}	$2 \pm 0.29^{*}$
Three	$23.10 \pm 0.34^{***}$	$34.3 \pm 3.18^{***}$	5.33 ± 0.67	3.6 ± 0.29
Five	20.50 ± 0.17^{x}	53 ± 4.58^{x}	8.33 ± 0.88	6.2 ± 0.40^{x}
Seven	$20.60 \pm 0.36^{x**}$	$79.3 \pm 4.91^{x**}$	13.67 ± 0.33	$7.6 \pm 1.12^{x**}$

Significant differences on the impact of *Salvinia molesta* biomass by the adult pairs were matched with these keys: one pair (*); three pairs (***); five pairs (x), and seven pairs (x**).

Among the infected adult pairs of *C. salviniae*, an increase in the infection loads of *Helicosporidium* sp. influenced the impact of the adult pairs of *C. salviniae* and resulted in less reduction in *S. molesta* biomass (Table 5.2). *Helicosporidium* sp. infection loads ranged from a medium ($10 – 14 \times 10^4$) to high ($15 \times 10^4 – 20 \times 10^5$) number of cells per 100 µl of smears prepared from each adult of the diseased *C. salviniae.* These infection load categories were adopted from those formulated in Chapter 2.

The performance of the diseased and disease-free insects was not affected by the time of exposure on *S. molesta*. At the end of the experiment, the biomass of plant components of *S. molesta* (rhizomes, fronds, and leaves) exposed to the diseased colony of *C. salviniae* were substantially reduced by 2.48%, 2.51%, 6% and 7.89% at one, three, five and seven adult pair treatments, respectively. The disease-free colony of *C. salviniae* caused 11.85%, 14%, 24% and 23.70% of *S. molesta* biomass reduction at one, three, five and seven adult pairs of *C. salviniae*, respectively.

Table 5. 2. Statistical details (GLM) of the effect of *Helicosporidium* sp. infection on the impact of *Cyrtobagous salviniae* on fresh *Salvinia molesta* biomass.

Variables	Terms	Estimate	Std. Error	*t*-value	*P*-value
Intercept	-	2.73×10^{01}	5.26×10^{-01}	51.95	**$< 2 \times 10^{-16}$**
Pairs	-	-7.75×10^{-01}	9.26×10^{-02}	-8.38	**1.01×10^{-08}**
Infection	-	-4.33×10^{-05}	5.36×10^{-05}	-0.81	0.43
Site	Diseased (reference)	-	-	-	-
	Disease-free	-1.86×10^{00}	6.19×10^{-01}	-3.01	**0.01**
Pairs: Infection	Pairs: Infection	3×10^{-05}	1.03×10^{-05}	2.91	**0.01**

Significant *P*-values are in bold.

5.3.2. Frond damage

The frond damage caused by disease-free adult pairs of *C. salviniae* was significantly greater than that of the diseased pairs ($F_{(1, 28)} = 373.55$; $P = 2.2 \times 10^{-16}$) (Figure 5.3). Although there was noticeable frond damage on *S. molesta* plants by the diseased adult pairs of *C. salviniae*, the damage ranged between exploratory to minor [1 to 1.70 ± 0.33 (mean ± SE)] feeding scores, where the frond damage by disease-free adult pairs of *C. salviniae* on *S. molesta* was rated between minor and normal (2.70 ± 0.33 and 3) feeding scores (Figures 5.3 and 4.5; Table 5.3).

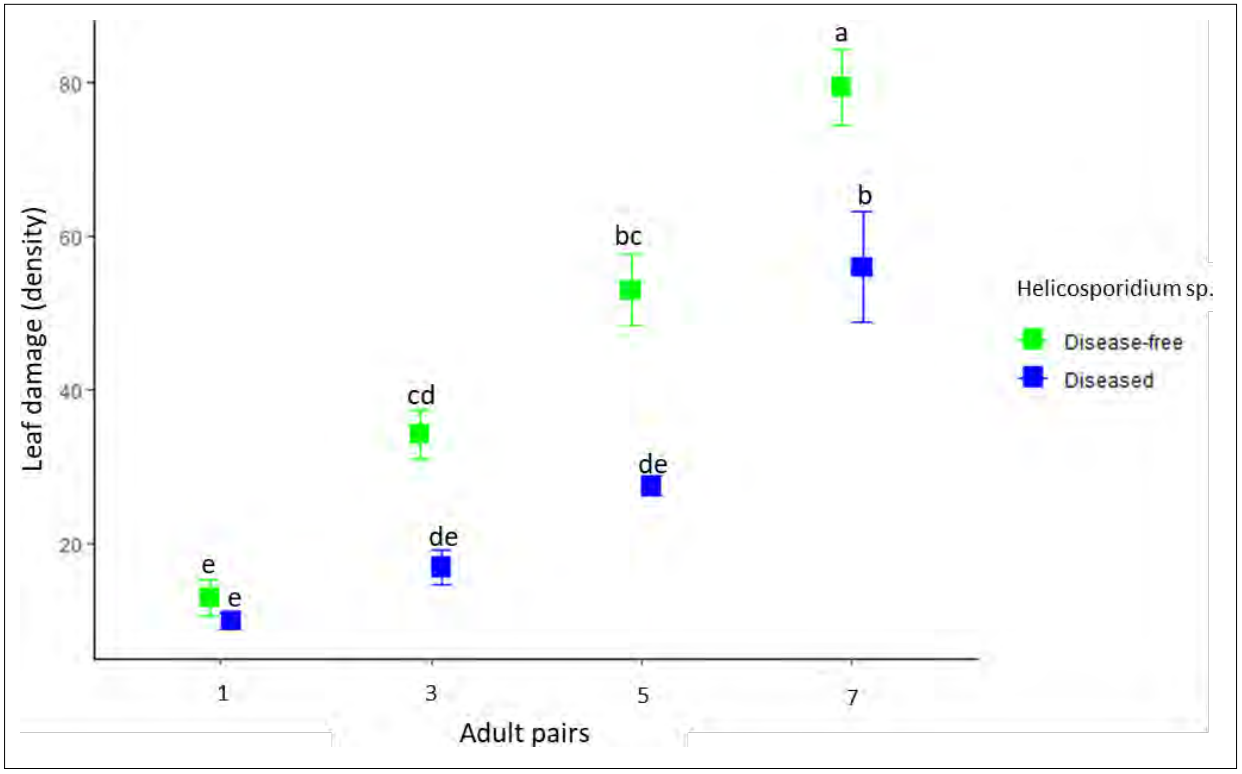

Figure 5. 3. Mean impact of the diseased and disease-free adult pairs of *Cyrtobagous salviniae* on the frond density of *Salvinia molesta*. Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

Increasing the number of the diseased *C. salviniae* adult pairs did not improve the impact on the frond density of *S. molesta* compared to that caused by disease-free pairs (Figure 5.3). Only increasing the number of adult pairs of *C. salviniae* slightly increased the damage on the frond density of *S. molesta*. The frond damage by adult pairs of *C. salviniae* was affected by the presence of *Helicosporidium* sp. disease (Table 5.3). Overall, the amount of damage diseased *C. salviniae* adults caused to the frond density was more limited than fronds exposed to disease-free weevils (Table 5.3).

Table 5. 3. Statistical details (GLM) of the effect of *Helicosporidium* sp. on the impact of *Cyrtobagous salviniae* on frond densities of *Salvinia molesta*.

Variables	Terms	Estimate	Std. Error	*t*-value	*P*-value
Intercept	-	-1.01	2.74	-0.37	0.72
Pairs	-	7.23	0.67	10.83	**3.92×10^{-11}**
Infection	-	-4.33×10^{-05}	5.36×10^{-05}	-0.81	0.43
Site	Diseased	-	-	-	-
	Disease-free	1.68	3.87	0.44	0.67
Pairs: Infection	Pairs: Infection	3×10^{-05}	1.03×10^{-05}	2.91	**0.01**
Pairs: Sites	Pairs: Sites	3.79	0.94	4.01	**0.01**

Significant *P*-values are in bold.

5.3.3. Adult survival

A significantly higher number of diseased adults died over the course of the trials in comparison to the disease-free insects ($F_{(1, 28)} = 577.22$; $P = 2.20 \times 10^{-16}$) (Figure 5.4) (Table 5.4).

Table 5. 4. Statistical details (GLM) on the effect of *Helicosporidium* sp. infection on the adult survival of *Cyrtobagous salviniae* from the adult pairs exposed to *Salvinia molesta*.

Variables	Terms	Estimate	Std. Error	*t*-value	*P*-value
Intercept	-	-1.03	0.36	-2.88	**0.01**
Pairs	-	1.78	0.07	24.03	**2×10^{-16}**
Infection	-	-4.33×10^{-05}	5.36×10^{-05}	-0.81	0.43
Site	Diseased	-	-	-	-
	Disease-free	1.20	0.38	3.16	**0.004**
Pairs: Infection	Pairs: Infection	3×10^{-05}	1.03×10^{-05}	2.91	**0.01**
Pairs: Sites	Pairs: Sites	3.79	0.94	4.01	**0.001**

Significant *P*-values are in bold.

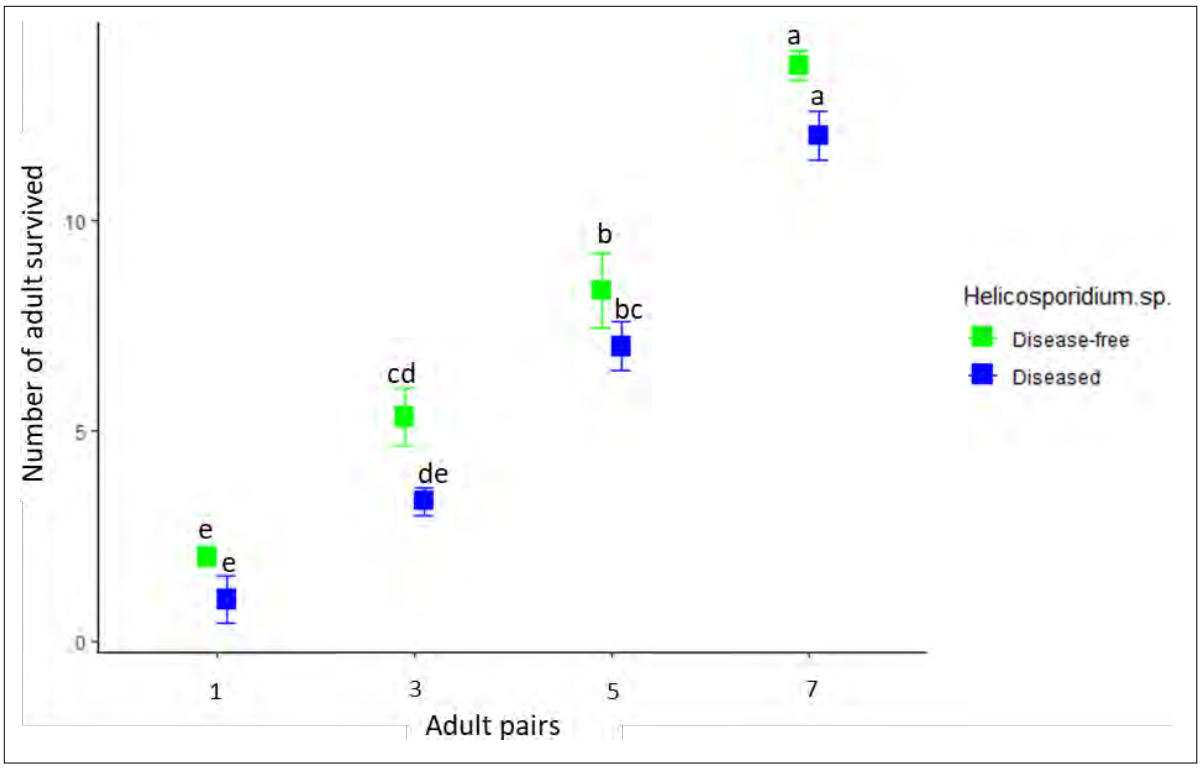

Figure 5. 4. The mean number of surviving diseased and disease-free adults of *Cyrtobagous salviniae* pairs exposed to *Salvinia molesta*. Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

5.3.4. Reproductive output of *Cyrtobagous salviniae*

The mean number of adults produced by the diseased adult pairs was significantly less than the number produced by disease-free adult pairs of *C. salviniae* ($F_{(3, 67)} = 41.19$; $P = 4.04 \times 10^{-15}$) (Figure 5.7). Comparing the diseased to the disease-free colony, the time of the exposure of the diseased adult pairs of *C. salviniae* to oviposit affected their overall reproductive output on *S. molesta* ($F_{(1, 70)} = 11.44$; $P = 0.001$) (Table 5.1).

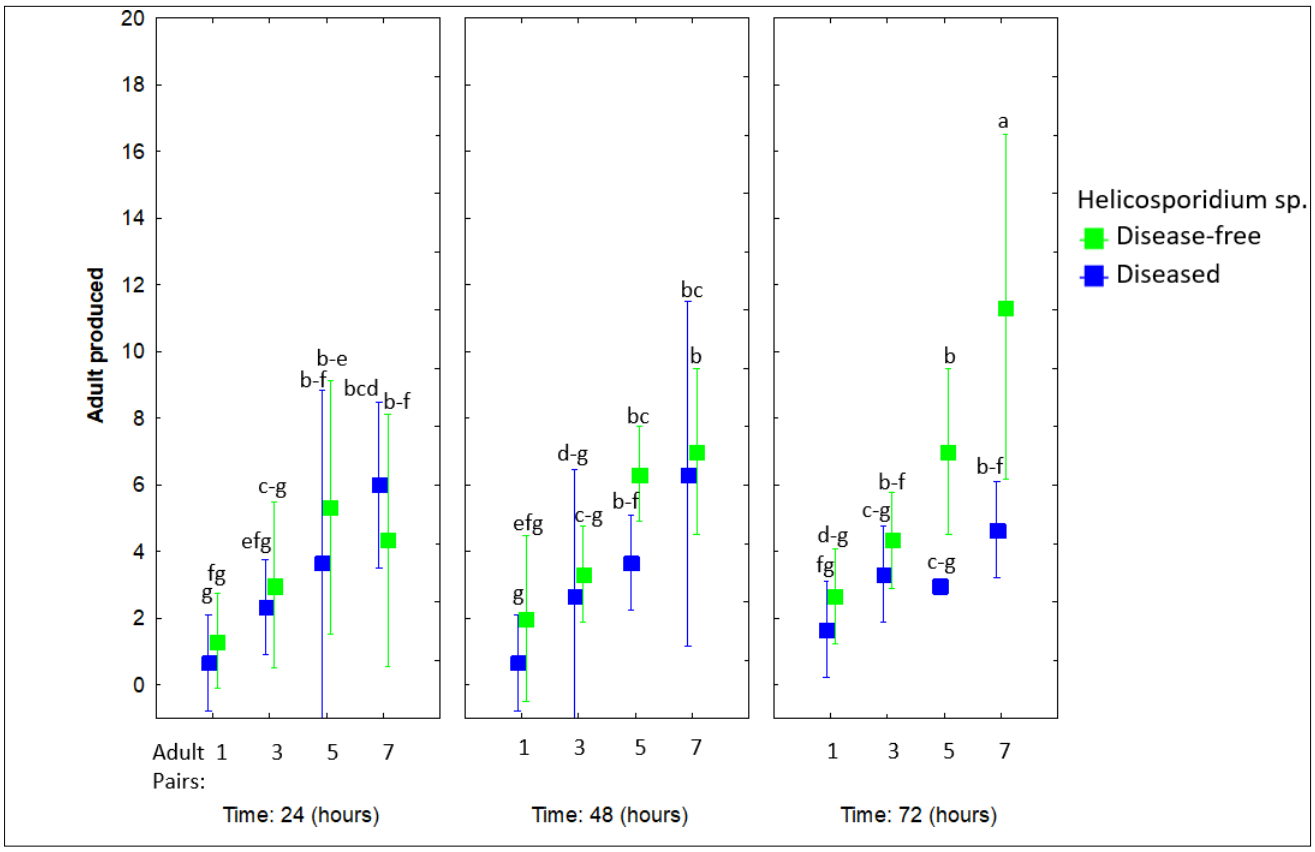

Figure 5. 5. Mean reproductive output of diseased (SA) and disease-free (US) adult pairs of *Cyrtobagous salviniae* exposed to *Salvinia molesta* for different times. Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

Diseased adult pairs of *C. salviniae* produced adults with medium (10 –14 x 10^4 cells) and high (i.e., 15 x 10^4 –20 x 10^5 cells) loads of *Helicosporidium* sp. Increased time of exposure to *S. molesta* produced no significant difference between the number of newly produced adults by the diseased and disease-free adult pairs of *C. salviniae*. Both the time of exposure and the presence of *Helicosporidium* sp. affected the reproductive output of diseased adult pairs of *C. salviniae* (Table 5.5).

Table 5. 5. Statistical details (GLM) on the effects of *Helicosporidium* sp. infection on the reproductive output of the adult pairs of *Cyrtobagous salviniae* exposed to *Salvinia molesta* during different periods.

Variables	Terms	Estimate	Std. Error	*t*-value	*P*-value
Intercept	-	3.73×10^{0}	1.29×10^{0}	2.88	**0.005**
Pairs	Five (reference)	-	-	-	-
	One	-3.29×10^{0}	4.86×10^{-1}	-6.78	**4.25×10^{-9}**
	Seven	1.82×10^{0}	4.84×10^{-1}	3.71	**0.001**
	Three	-1.64×10^{0}	4.85×10^{-1}	-3.39	**0.001**
Infection	-	-9.02×10^{-5}	9.36×10^{-5}	-0.96	0.34
Site	Diseased	-	-	-	-
	Disease-free	4.81×10^{-1}	1.22×10^{0}	0.39	0.72
Time	-	2.93×10^{-02}	8.74×10^{-03}	3.35	**0.001**
Time: Sites	Time: Sites	3.79	0.94	4.01	**0.001**

Significant *P*-values are in bold.

At the end of the experiment, no larvae or pupae were present when counting the number of the new adults produced by the diseased or the disease-free adult pairs of *C. salviniae*.

5.3.5. Adult size

The was no significant difference between the body sizes of the diseased and disease-free adult genders of *C. salviniae* ($t = 5.18$; $df = 38$; $P = 7.49 \times 10\text{-}6$) (Figure 5.8). However, the sizes of the disease-free adults were slightly bigger than those of the diseased adults of *C. salviniae*.

5.3.6. Insect developmental period

The mean insect developmental time from egg to newly eclosed adult, differed significantly between diseased 57.2 ± 1.11 (mean ± SE) (range = 55–61 days) and disease-free 37.2 ± 1.43 (range = 33–41 days) *C. salviniae* on *S. molesta*.

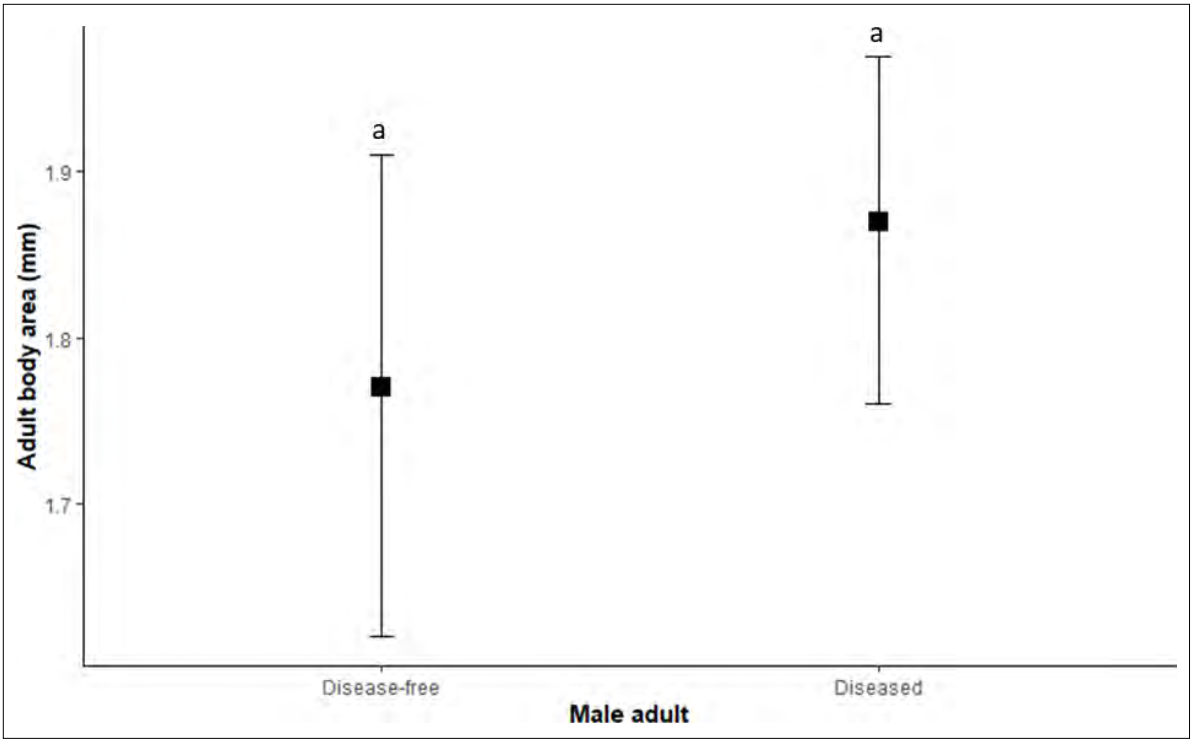

Figure 5. 6. Body area or size in millimeters (mm) of the newly produced disease-free and the diseased male adults of *Crytobagous salviniae*. Same letters indicate no significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

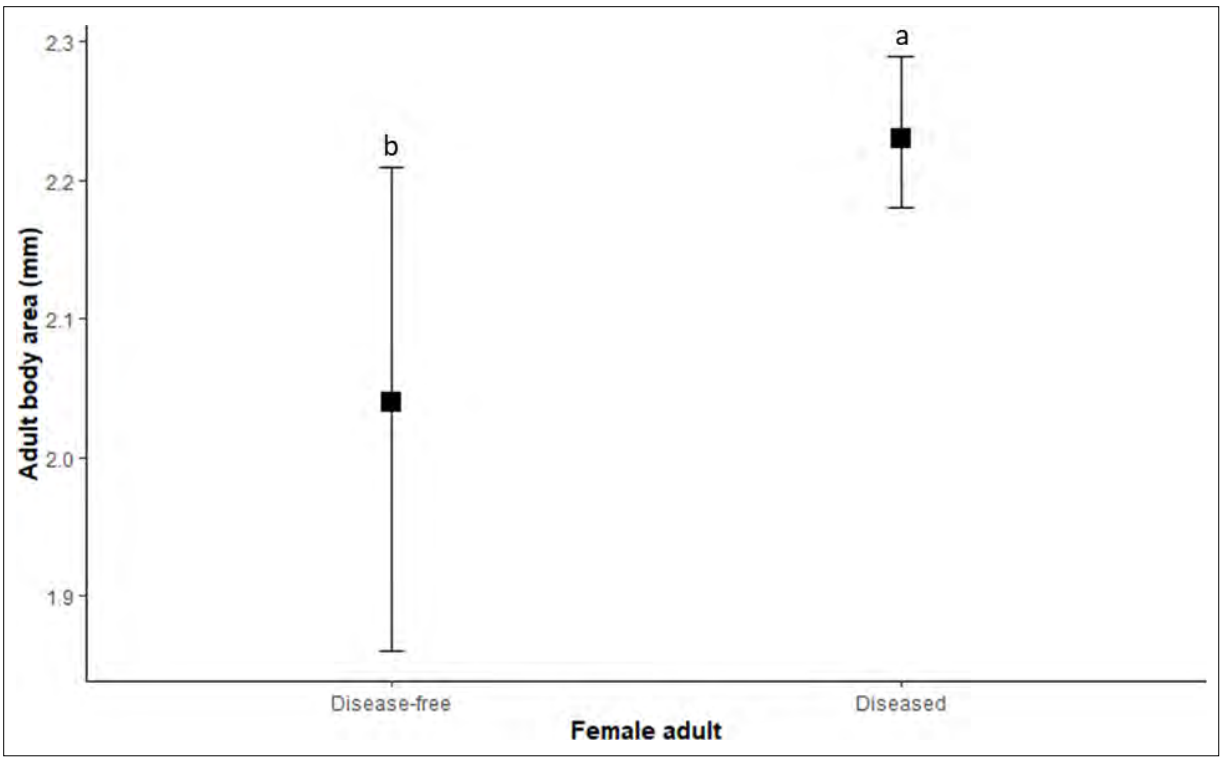

Figure 5. 7. Body area or size in millimeters (mm) of the newly produced disease-free and the diseased female adults of *Crytobagous salviniae*. Different letters indicate significant differences ($P < 0.05$). Box

(mean) and whisker (standard error).

5.4. DISCUSSION

The results of this chapter showed that *Helicosporidium* sp. infection reduced the effectiveness of *C. salviniae* to control *S. molesta* when compared to a disease-free culture under laboratory conditions. However, when comparing the density of the diseased adults of *C. salviniae* only, increasing the number of adult pairs slightly increased the impact on *S. molesta* biomass. The feeding and reproductive output of the diseased *C. salviniae* on *S. molesta* showed a slight difference when carrying medium and high infection loads of *Helicosporidium* sp. This performance of the diseased weevils implies that, at medium and high infection loads of *Helicosporidium* sp., the biological response of *C. salviniae* is similar. Both the diseased female and male adults were slightly larger than their respective genders of disease-free adults. The developmental periods of the diseased and disease-free adults of *C. salviniae*, from egg to the newly emerged adult stages, were completed at approximately 55 and 33 days, respectively. Therefore, the results of this study imply that, under field conditions, the prolonged developmental period of the diseased *C. salviniae* with less impact on *S. molesta* could allow the weed to proliferate.

The general implication of these results is that the presence of *Helicosporidium* sp. in *C. salviniae* threatens the control of *S. molesta* in South Africa. Although increasing the number of diseased adult pairs of *C. salvinia* slightly increased the reduction of *S. molesta* biomasses, this resultant biomass was lower than the biomass gained by *S. molesta* plants that were insect-free. This is not the first case of an isolate of *Helicosporidium* sp. threatening a biocontrol agent used to control an alien pest or weed species. For example, an isolated congener of *Helicosporidium* sp. reduced the effectiveness of the biocontrol agent, *Rhizophagus grandis,* released to control *Dendroctonus micans,* which was considered a disastrous pest to spruce trees (Yaman & Radek, 2007; Yaman, 2008). In another related case, the microsporidian infection, *Nosema cactoblastis,* greatly reduced the field populations and inhouse cultures of the cactus moth, *Cactoblastis cactorum* (Berg, 1885) (Lepidoptera: Pyralidae) which is a

biocontrol agent of the invasive prickly pear cactus plant species in Australia (Pettey, 1947). Further, some diseases of Microsporidia reduce the fecundity and predatory (feeding impact) potential of the mites, *Amyblyseius* spp., to control other mite's species which were considered disastrous to important plant species (Van Der Geest, 2000; Bjørnson & Schütte, 2003). Under field conditions, *Helicosporidium* sp. could be having more impact on *C. salviniae* than what has been discovered under laboratory conditions. Similarly, *Megamelus scutellaris* Berg (Hemiptera: Delphacidae), a biological control agent of *Pontederia crassipes* (Martius) [≡*Eichhornia crassipes* (Martius) Solms-Laubach] (Pontederiaceae) was discovered being parasitized by *Echthrodelphax migratorius* Benoit (Hymenoptera: Dryinidae) in South Africa (Kraus *et al*., 2019). This parasitism is suspected to have repercussions for the efficacy of *M. scutellaris* to control *P. crassipes* (Kraus *et al*., 2019). Although a beneficial pathogen, *Nosema pyrausta* infection has significantly reduced the field populations of *Ostrinia nubilalis*, a serious pest of corn in the United States and Canada (Zimmack & Brindley, 1957). Another example is the red imported fire ant, *Solenopsis invicta*, that also feeds on the adults of *C. salviniae* (Parys & Johnson, 2012), which was controlled by the pathogens, *Kneallhazia solenopsae* and *Vairimorpha invictae* in the United States (Williams *et al*., 1998). These beneficial scenarios attest that pathogens have the potential to affect and reduce insect populations, including those under biocontrol programmes.

The lower performance of the diseased *C. salviniae* was also evident in the localised dispersal of these weevils which fed only on a little leaf density compared to those fed on by disease-free weevils. The low dispersal of the diseased adult pairs of *C. salviniae* was observed on the tests, and this was measured by the number of leaves that were fed by these weevils. Likewise, Keilin (1921) and Hembree (1981) reported that insects that are infected with *Helicosporidium* sp. are conspicuously slower and usually have retarded growth. As observed, the diseased adults of *C. salviniae* were slightly larger than those of disease-free *C. salviniae* which could contribute to the poor dispersal over the entire leaf densities of *S. molesta*. These

findings imply that, continuous mass releases of the diseased adults of *C. salviniae*, should also disperse the insects over the entire population of *S. molesta* established to any extent in the aquatic system. However, based on the noticeable differences between feeding damage caused by the adult pairs of *C. salviniae* which were under the influence of low and high infection load of *Helicosporidium* sp., it is likely that, in the field, *C. salviniae* will control *S. molesta* slightly better where infection loads of *Helicosporidium* sp. are low.

In summary, comparing the diseased and disease-free *C. salviniae*, the results of this study show that *Helicosporidium* sp. has reduced the effectiveness of *C. salviniae* to control *S. molesta* in South Africa. Based on the slight improvement on the impact of the diseased *C. salviniae* following increasing the number of pairs, this study advocates that more effort should be put on the mass-rearing, and releasing large numbers of *C. salviniae* on *S. molesta* populations. To achieve the amount of impact caused by the disease-free *C. salviniae*, more diseased adults of *C. salviniae* were required. An alternative way of improving the impact of *C. salviniae* could be subjecting the diseased weevils to antibiotic treatments and determine if these weevils could be cured. If *Helicosporidium* sp. infection can be cured, then the feeding and reproductive output of *C. salviniae* at zero percent of the disease load should be tested to assess improvements to the weevil's performance on *S. molesta*. However, the failure of treating *Helicosporidium* sp. infection in *C. salviniae* would not mean that the diseased *C. salviniae* will not continue to control *S. molesta* in South Africa. But dissociation of *C. salviniae* from *Helicosporidium* sp. could improve the current impact of *C. salviniae* on *S. molesta*, and this could also ease mass rearing efforts (and labour costs) of these weevils. However, no *Helicosporidium* sp. isolates have ever been subjected to antibiotic treatment. The prospects of the treatment of *Helicosporidium* sp. in *C. salviniae* are discussed in the next chapter.

CHAPTER 6

Prospects for the antibiotic treatment of *Helicosporidium sp.* in *Cyrtobagous salviniae*

6.1. INTRODUCTION

Insects can be screened and tested genetically for diseases, or standard microscopic examination of smears of their body fluids or tissues can be used. Screening for, and testing of diseases in insects should be done on all life stages (eggs, larvae, pupae and adults) since not every stage may be equally susceptible to such diseases. Although many pest and disease management programmes have used biocontrol agents, there are only a few instances where antibiotics have been used to ensure healthy colonies, particularly in the case of infected insects in mass-rearing facilities. For example, colonies of the honeybee, *Apis mellifera,* and the silkworm, *Bombyx mori,* which are mass-reared, are constantly screened for diseases and when necessary, they are treated with antibiotics (Bassi, 1835, 1836; James & Li, 2012; Pasteur, 1870).

The present study investigated a possible treatment for the *Helicosporidium* sp. disease in *C. salviniae* since the disease was shown to reduce the feeding and reproductive output of *C. salviniae* (Chapter 4). No antibiotic has been used to treat an isolate of *Helicosporidium* spp. infection in insects. Therefore, the only option was to try antibiotics used to treat disease organisms that are closely related to *Helicosporidium* sp. A genomic study by Tarta (2002) indicated that *Prototheca* spp. (Chlorophyta: Trebouxiophyceae) were most genetically closely related to *Helicosporidium* spp (see Chapter 2). Among the species in the genus *Prototheca*, the closest infective species is *P. zopffii* which shares the same clade as *Helicosporidium* sp. It is likely that antibiotics used to treat the disease caused by *P. zopffii* could also treat *Helicosporidium* sp. in *C. salviniae*. There are two other species, *P. wickerhamii* and *P.*

blaschkeae, which are not too distantly related to *Helicosporidium* sp. and cause common diseases in the blood cells of mammals, including humans (Tarta, 2002; Meinke *et al*., 2017). The most effective antibiotics against *P. zopffii, P. wickerhamii* and *P. blaschkeae* are ketoconazole, itraconazole, fluconazole, conventional amphotericin B and liposomal amphotericin B (Ahbel *et al*., 1980; Kim *et al*., 1996; Meinke *et al*., 2017). Of these antibiotics, ketoconazole and amphotericin B are the most effective and widely used to treat *Prototheca* spp. infections (Meineke *et al*., 2017). However, these antibiotics may have side effects when administered. For this study, ketoconazole was selected as the most promising (and easily accessible) antibiotic to treat *Helicosporidium* sp. in *C. salviniae*.

The main aim of this study was to investigate whether *Helicosporidium* sp. in *C. salviniae* could be treated with ketoconazole and if this would improve the performance of *C. salviniae.* This study also aimed to determine whether the treated *C. salviniae* adults could be re-infected when re-introduced to *S. molesta* field sites.

6.2. MATERIALS AND METHODS

The experiments outlined below required infected adults of *C. salviniae*. The adults, unlike the larvae, have all the cell types of *Helicosporidium* sp. and were thus used for testing the effectiveness of ketoconazole (antibiotic) treatment of this disease under both *in vivo* and *in vitro* conditions.

6.2.1. Source of *Cyrtobagous salviniae* and experimental conditions

Experimental colonies of the infected adults of *C. salviniae* were sourced from the SASRI mass-rearing facility. The antibiotic treatment trials of *Helicosporidium* sp. disease were conducted at the Department of Zoology and Entomology, Centre for Biological Control (DZE-CBC), Rhodes University. The treatment trials were conducted in sterilised Petri dishes and when necessary, on glass slides, under CE room conditions maintained at a temperature of 25 ºC, with a relative humidity of 35% to 70% and a photoperiod of 14L; 10 D (grow lux lights). 300 diseased adults of *C. salviniae* were collected for these trials.

6.2.2. Extraction and maintenance of *Helicosporidium* sp. culture

Different cell types of *Helicosporidium* sp. were sourced from the diseased adults of *C. salviniae*. Both mature cyst and vegetative cells of *Helicosporidium* sp. were successfully isolated. To isolate enough *Helicosporidium* sp. cells for the *in vitro* treatment trials, 15 *C. salviniae* adults (randomly mixed females and males) were homogenised in 500µl of deionised water, 375 µl of phosphate buffer (pH 7.4) and 125µl of Giemsa stain, and this was replicated 12 times to make a stock solution of 12 000µl of smears (ample for the *in vitro* treatment trials). This stock solution of the smears of *C. salviniae* with the cells of *Helicosporidium* sp. was maintained in sterilised Petri dishes at CE room conditions for further culturing of the required cells of this disease. The presence and development of *Helicosporidium* sp. cells in the cultured smears of *C. salviniae* were confirmed by observing 10µl of the smears mounted on sterilised glass slides covered with coverslips under a compound microscope, Olympus, CX21, at 40X

magnification.

6.2.3. Source and preparation of antibiotic treatment solution

Ketoconazole was selected as the most promising antibiotic to treat *Helicosporidium* sp. in *C. salviniae*. The raw form of this antibiotic was sourced from the Department of Pharmacy at Rhodes University.

Since there has not been any treatment of *Helicosporidium* sp. in any insect species, there was no reported concentration that could be followed to formulate effective doses of ketoconazole. Therefore, dosages of other antibiotics used to treat other diseases in insects were reviewed to guide the preparation of the ketoconazole concentration which could treat *Helicosporidium* sp. (Lin *et al*., 2015). However, this review only guided the present study in the preparation of ketoconazole concentrations that could be effective.

Lin *et al.,* (2015) dissolved 1.5g of raw antibiotic powder in 1ml of methanol (solvent) and 7.5ml of deionised water. Following these preparations, 1.5g of ketoconazole was first dissolved in 1ml of methanol (solvent), and more methanol was added when ketoconazole was not completely dissolved. The most efficient solvent concentration for 1.5g of ketoconazole was 3ml of methanol, which was placed in a sterilised Petri dish and left open for 9 minutes to evaporate the methanol. Thereafter, 8ml of deionised water was added just before the ketoconazole solution dried up and agitated using a glass rod until the solution turned from milky white to pale. This preparation was replicated to make ample stock solution for the *in vitro* trials.

6.2.4. *In vitro* treatment of *Helicosporidium* sp.

6.2.4.1. Single treatment

2000 µl of stock solution made from smears of *C. salviniae* infected with *Helicosporidium* sp. was used to test the effectiveness of a single application of the antibiotic. This response guided further preparations of other doses (if deemed necessary). Therefore, 100µl of the antibiotic

was mixed with 100µl of the smear solution, and mounted on glass slides and covered with coverslips. Observations and counts of *Helicosporidium* sp. cells were made once on the glass slide under a compound microscope, Olympus, CX21, using 40X and 10X magnification. Only live *Helicosporidium* sp. cells were counted and recorded. The duration of this trial was set to end at day 21 but, it was also planned to be terminated when the count of the newly formed cells exceeded the number of cells at which the experiment started. This 21-day period allowed *Helicosporidium* sp. to complete three life cycles (Chapters 1 & 2).

6.2.4.2. Multiple treatments

8 400µl of stock solution was then used to test the effectiveness of multiple applications of the antibiotic. Guided by the response of *Helicosporidium* sp. to the single application of the antibiotic treatment, the dose of the antibiotic used in the single treatment was further applied to *Helicosporidium* sp. at different concentrations. The formulated concentrations of the antibiotic dose were; 100µl of the antibiotic (A) plus 100µl of the smear solution (S) (high antibiotic concentration); 100µl of the smear solution plus 80µl of the antibiotic (medium concentration); and 100µl of the smear solution plus 50µl of the antibiotic (low concentration), thoroughly mixed with thin steel rod in tubes and mounted on glass slides. These glass slides were then put into sterilised Petri dishes and closed for further developments. This trial was run for 21 days.

Each antibiotic concentration was applied at 5, 10, 15, or 20 days after their first application, and this was replicated four times. The response of *Helicosporidium* sp. was then quantified (live *Helicosporidium* sp. cell count) at 7, 14, and 21 days post application. Observations and cell counts of *Helicosporidium* sp. on the entire mount were made under an Olympus CX21compound microscope, at 40X and 10X magnification.

6.2.5. *In vivo* treatment of *Helicosporidium* sp.

6.2.5.1. Application of the antibiotic

To test the possible toxicity of antibiotic application to five adult *C. salviniae*, several antibiotic concentrations were applied as a thin film to *S. molesta* fronds, and survival of the weevils was compared between concentrations and against a control in which water was applied to the plants. The antibiotic formulation used in the *in vitro* treatments was first tested on five *C. salviniae* adults to determine the safety of this antibiotic concentration. Adult survival was measured over three days. When necessary, the antibiotic concentration was diluted to lower concentrations until a good number of adults survived. The most appropriate formulation of the antibiotic was used in these experiments.

Thereafter, the efficacy of the antibiotic in treating the *Helicosporidium* sp. infection was evaluated. To effectively administer the antibiotic to the adults of *C. salviniae*, the fronds of *S. molesta* were punctured several times (making small pores in the fronds) with a thin pointed rod (pin) (Figure 6.1. A), and submerged in the prepared antibiotic in a small glass vile for 3 hours (Figure 6.1. B). The pores in the fronds allowed the antibiotic to sink into the cells to be consumed by the adults (Figure 6.1. B).

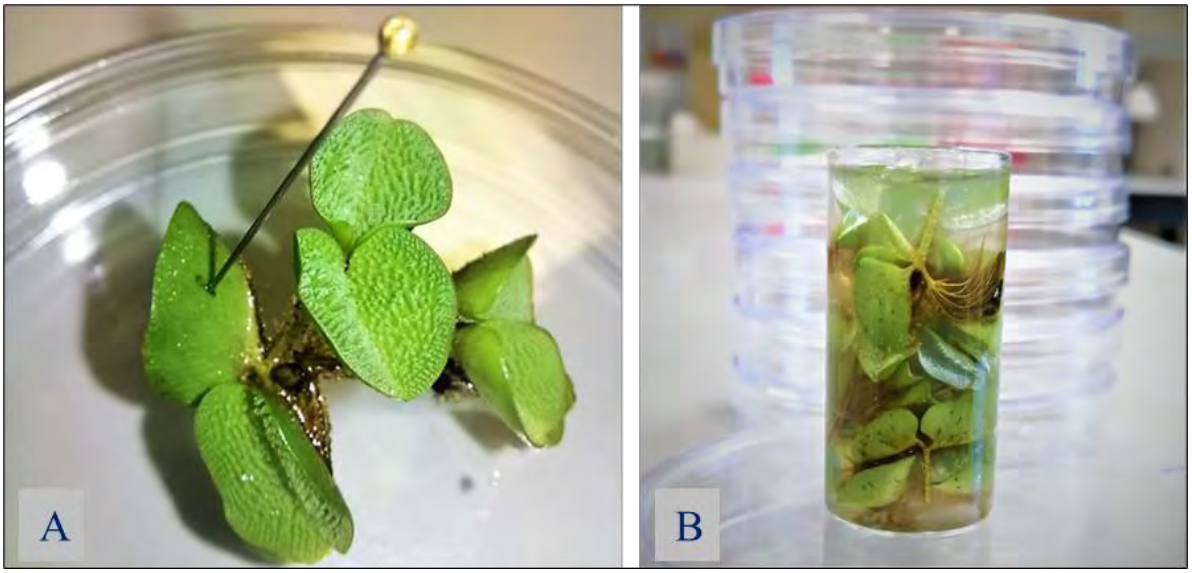

Figure 6. 1. Images of *Salvinia molesta* fronds inoculated with the antibiotic. (A) Puncturing of *Salvinia molesta* fronds and (B) Drenching of *Salvinia molesta* fronds into the antibiotic solution.

To maximise the consumption of the antibiotic by the adults, a *S. molesta* plant, with two fronds inoculated with the antibiotic, was exposed to one *C. salviniae* adult in a separate Petri dish. A film of this antibiotic (ample for the adult to submerge in) was also supplied to each *S. molesta* plant for further consumption by the adult. This trial was run for 21 days. After 5, 10, 15, and 20 days, the *C. salviniae* adults were carefully transferred onto newly inoculated fronds of *S. molesta* and in new films of the antibiotic, at the same concentration. Transferring the adults ensured that they were subjected to multiple treatments of the same concentration, and maximised the chances of the consumption of antibiotic.

72 adults of *C. salviniae* (mixed females and males) were divided into 12 groups, each group consisted of 6 adults per antibiotic treatment trial. Within each group (with six adults), in either the treatment (single or multiple) or control, one adult of *C. salviniae* was separately introduced onto *S. molesta* fronds. The effectiveness and safety of each antibiotic concentration was measured by recording feeding and survival of the adults.

Feeding and survival assessments were conducted to determine if the adults in the treatment were repelled by the presence of the antibiotic. Feeding was measured by counting the number of feeding holes in the fronds of *S. molesta* by *C. salviniae*. The number of holes were scored as 0 = no feeding, 1 (1–3 holes) = exploratory feeding, 2 (4–6 holes) = minor feeding, and 3 (7–9 holes = normal feeding).

6.2.5.2. Screening of the treated *Cyrtobagous salviniae*

Adult *C. salviniae* treated with antibiotics were screened to determine whether they were free of infection. 21 treated and 21 control (untreated) *C. salviniae* adults were randomly screened, using the same procedure described in Chapter 2. One hundred microliters (100µl) of fresh smears were prepared from each adult, and 50µl of each smear was mounted on a glass slide and covered with a coverslip for observations and counting of cells. Thereafter, 20µl from the remaining fresh smears was mounted on glass slides, and placed in Petri dishes at room

temperature and cultured for 7 days, to determine the viability of *Helicosporidium* sp. All the *Helicosporidium* sp. cells were counted before culturing (pre-culture screening), and after culturing (post-culture screening). The viability of *Helicosporidium* sp. cells was determined as the difference between the number of *Helicosporidium* sp. cells counted pre- and post-culture screenings.

6.2.6. Data Analysis

A Simple Linear Model (LM) was used to explore the relationship between antibiotic treatment and time on the survival of *Helicosporidium* sp. cells during the *in vivo* trials. A Generalized Least Squares (GLS) model with repeated measures was used to explore the most influential model of the antibiotic treatment on the survival of *Helicosporidium* sp. cells. The raw data sets on the *in vitro* tests met the normality assumptions of Analysis of Variance (ANOVA), which was checked by the Shapiro-Wilk normality test ($P > 0.05$). Thus, either One-Way or Two-Way ANOVA, followed by Tukey HSD post-hoc tests were performed to compute and compare the means of the variables (cysts and vegetative cells, adult feeding and survival) measured during and after the *in vivo* trials. The means of the cysts and vegetative cells of the fresh and cultured smears, post-adult survival and feeding were analysed with the student *t-test*. All statistical analyses were conducted in R Studio (version 1.1.463).

6.3. RESULTS

6.3.1. *In vitro*

6.3.1.1. Single treatment

There were more *Helicosporidium* sp. cells following the single antibiotic treatment than before the treatment (Figure 6.2 & 6.3). Significantly more *Helicosporidium* sp. (100% plus) were produced by those that survived the single application of the antibiotic (R^2 = 0.39; P = 2.52 x 10-7) (Table 6.1; Figure 6.3). The single application of the antibiotic dosage did not affect the survival of *Helicosporidium* sp. cells over time (Table 6.1) and the cells were able to replicate (Figures 6.2 & 6.3; Table 6.1).

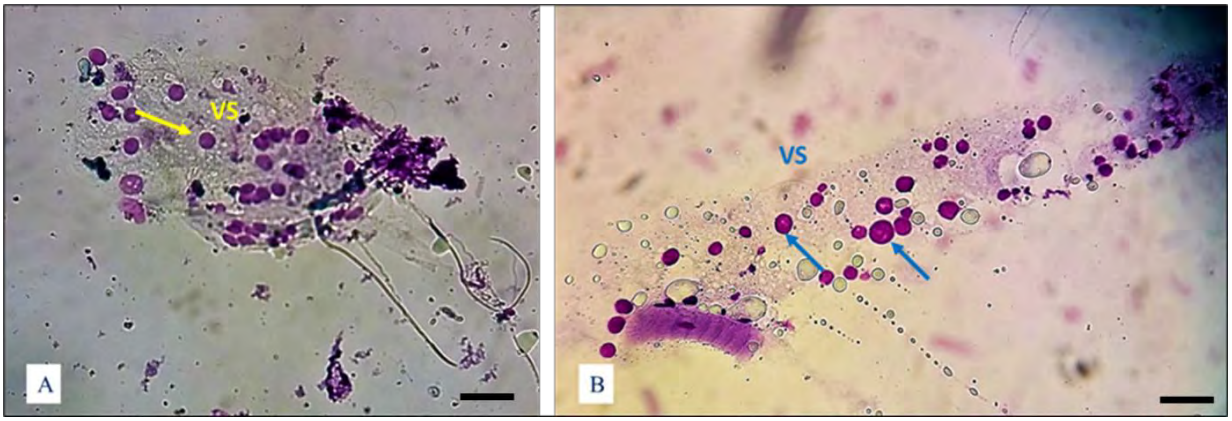

Figure 6. 2. The response of *Helicosporidium* sp. to the single applications of the antibiotic concentration. (A) Vegetative cell rupturing and releasing vegetative spores and (B) Released and developing vegetative spores. (VS) Vegetative Spores. Micrograph scale: 50 µm.

Table 6. 1. Generalised Least Squares model summary of *Helicosporidium* sp. cells counts following a single dose of antibiotic treatment over time. Values in bold are statistically significant.

Terms	Variables	Value	Std. Error	*t*-value	*P*-value
-	(Intercept)	28.93	5.78	5.01	**0.01**
Treatment (Control) (C)	Treatment (Single) (S)	-8.83	8.35	-1.06	0.29
Treatment	Time (T)	1.64	0.62	2.65	**0.01**
Treatment (C) * (T)	Treatment (S) * (T)	-0.53	0.9	-0.59	0.56
	Phi	0.52			
	Residual Standard Error	12.74			
	Degrees of freedom	60			
	Residuals DF	56			
	AIC	453.94			
	Log-Likelihood	-220.97			

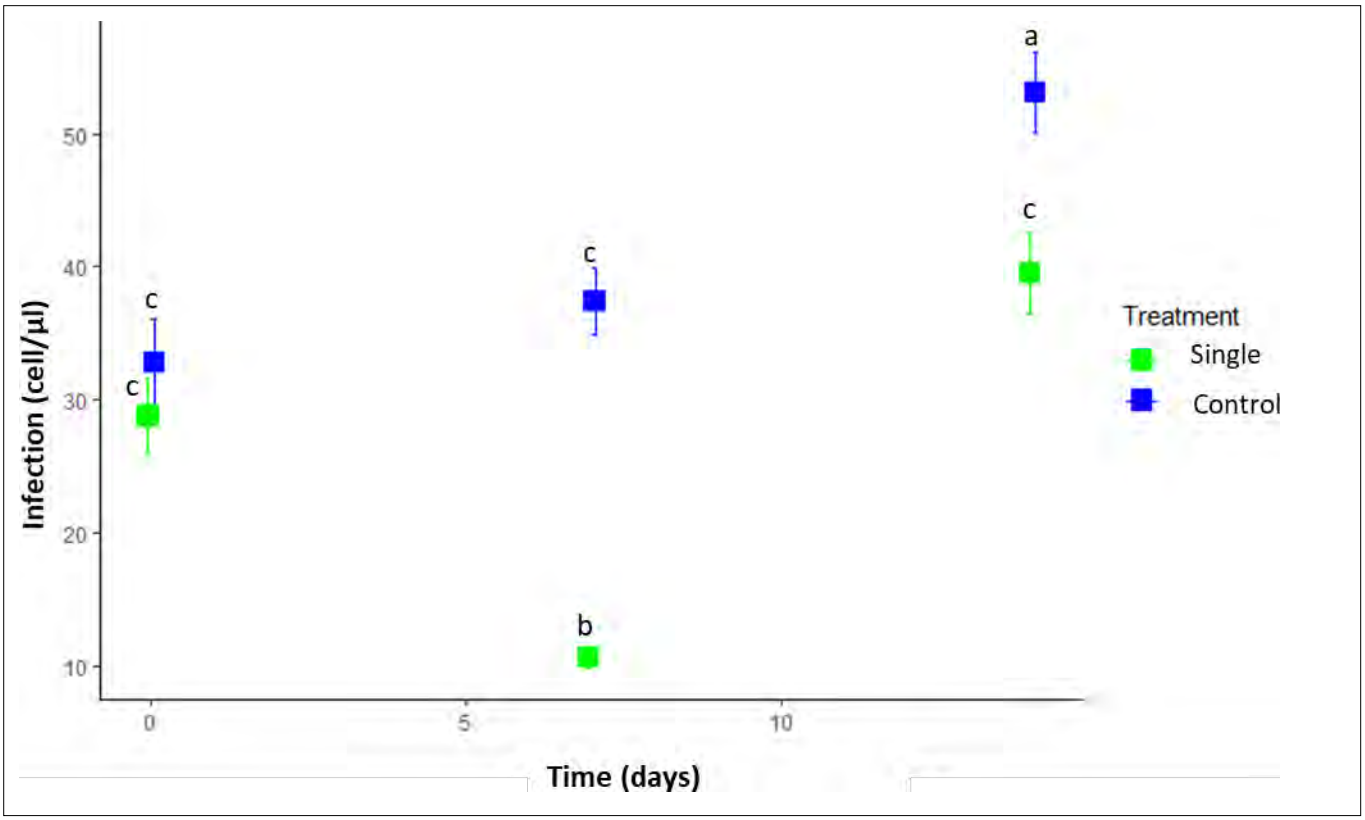

Figure 6. 3. *Helicosporidium* sp. cell concentration in response to the single-dose application of the antibiotic. Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

6.3.1.2. Multiple treatments

There was a strong relationship between multiple applications of the antibiotic and time (R^2 = 0.66; $P < 2.20 \times 10^{-16}$) (Table 6.2; Figure 6.5). With an increase in time and more applications of the antibiotic, up to 100% mortality of *Helicosporidium* sp. cells was achieved (Table 6.2; Figure 6.4)

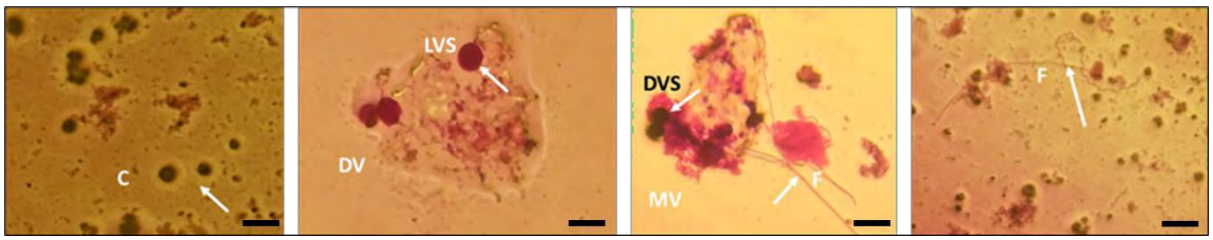

Figure 6. 4. The response of *Helicosporidium* sp. to multiple applications of the antibiotic concentrations. (C) Cysts, (DV) Developing Vegetative cell, (DVS) Dead Vegetative Spore, (LVS) Live Vegetative Spore, (F) Filamentous cell and (MV) Mature Vegetative cell. Micrograph scale: 50 µm.

Multiple applications of the antibiotic at different concentrations significantly reduced the survival of *Helicosporidium* sp. cells (Table 6.2; Figure 6.5). Both the multiple antibiotic applications and the time of the antibiotic application had a significant effect on the cells of *Helicosporidium* sp. (Table 6.4). Application of the low antibiotic concentration resulted in significantly fewer *Helicosporidium* sp. cells compared to those from the high and medium concentrations (Table 6.2). Over time, all the antibiotic concentration treatments resulted in a significantly lower survival of *Helicosporidium* sp. (Table 6.4).

Table 6. 2. Generalised Least Squares model summary on the treatment of *Helicosporidium* sp. cells using different dosages of the antibiotic over time.

Terms	**Variables**	**Value**	**Std. Error**	***t*-value**	***P*-value**
-	(Intercept)	28.15	2.96	9.52	**0.01**
Treatment Control (TC)	Treatment High (TH)	-7.56	4.23	-1.79	0.08
TC	Treatment Low (TL)	-8.62	4.21	-2.04	**0.04**
TC	Treatment Medium (TM)	-6.22	4.19	-1.49	0.14
TC; TH; TL; TM	Time (T)	1.94	0.22	8.68	**0.01**
TC	TH * T	-.3.01	0.31	-9.54	**0.01**
TC	TL * T	-3.01	0.32	-9.37	**0.01**
TC	TM * T	-3.13	0.31	-9.97	**0.01**
	Phi	0.41			
	Residual Standard Error	7.69			
	Degrees of freedom	159			
	Residuals DF	151			
	AIC	1076.34			
	Log-Likelihood	-528.17			

(*) means interactions between variables. Values in bold are statistically significant.

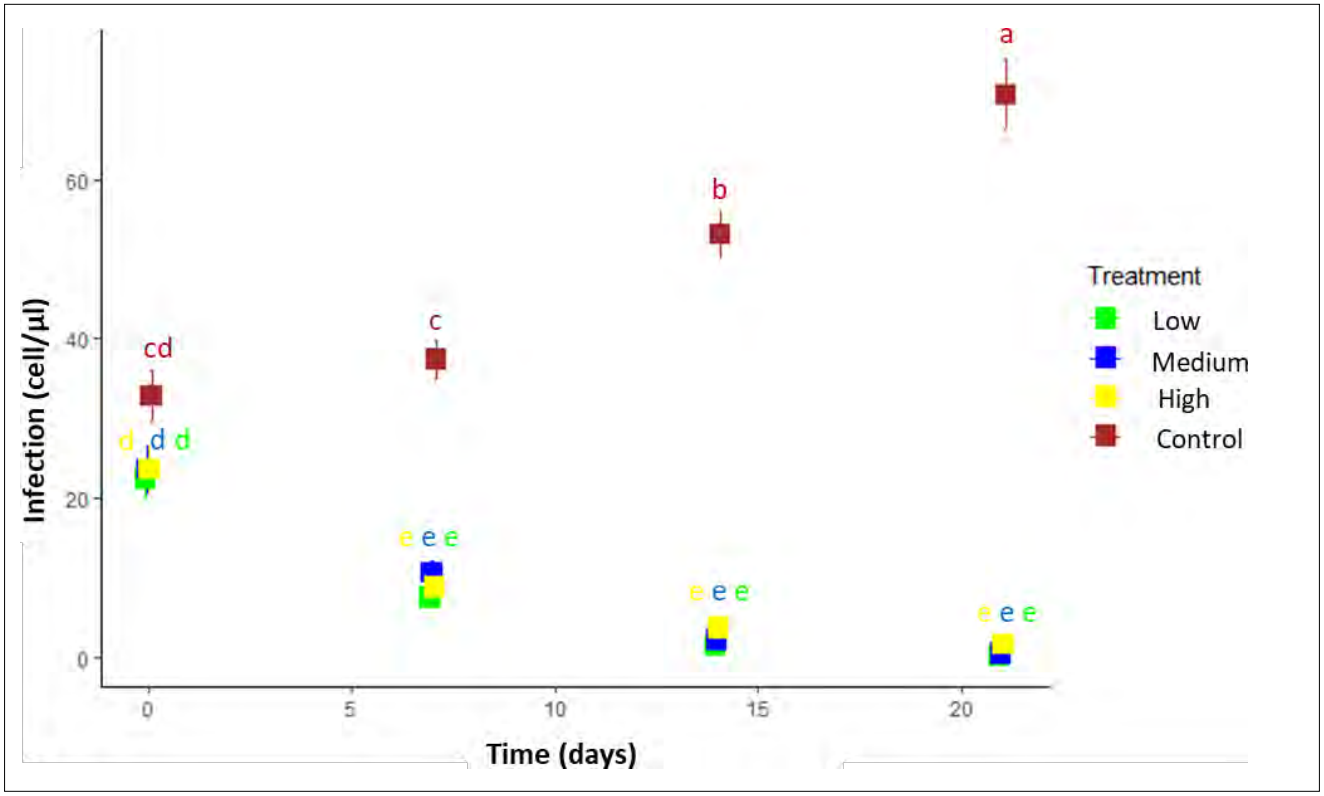

Figure 6. 5. *Helicosporidium* sp. cell concentration in response to the multiple applications of different antibiotic concentrations for 21 days. Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

6.3.2. *In* vivo

6.3.2.1. Formulation of ketoconazole concentration

The most appropriate formulation of the antibiotic was 0.5g of ketoconazole dissolved in 1.5ml of methanol and titrated with 8ml of deionised water (Table 6.3).

Table 6. 3. Details on the preparation and selection of the safest formulation of the antibiotic, ketoconazole, for application in in vivo treatments.

Ketoconazole (g)	Methanol (ml)	Water (ml)	Adults treated	Adult survived	Antibiotic formulation
1.5	3	8	5	0	Lethal
1	2.5	8	5	1	Lethal
0.5	2	8	5	3	Moderate lethal
0.5	1.5	8	5	5	Non-lethal

6.3.2.2. Screening of *Cyrtobagous salviniae* treated with an antibiotic for *Helicosporidium* sp. infection

The antibiotic significantly reduced *Helicosporidium* sp. infection in antibiotic-treated *C. salviniae*. The total *Helicosporidium* cell count in the treated adults (71.15 ± 3.73 (mean ± SE) per 50µl) was significantly lower than in either the pre-treated (146.30 ± 18.74) or control (166.60 ± 22.16) ($F_{(1, 57)} = 8.87$; $P = 0.004$) (Figure 6.6), representing a 48.63% reduction in infected cell load.

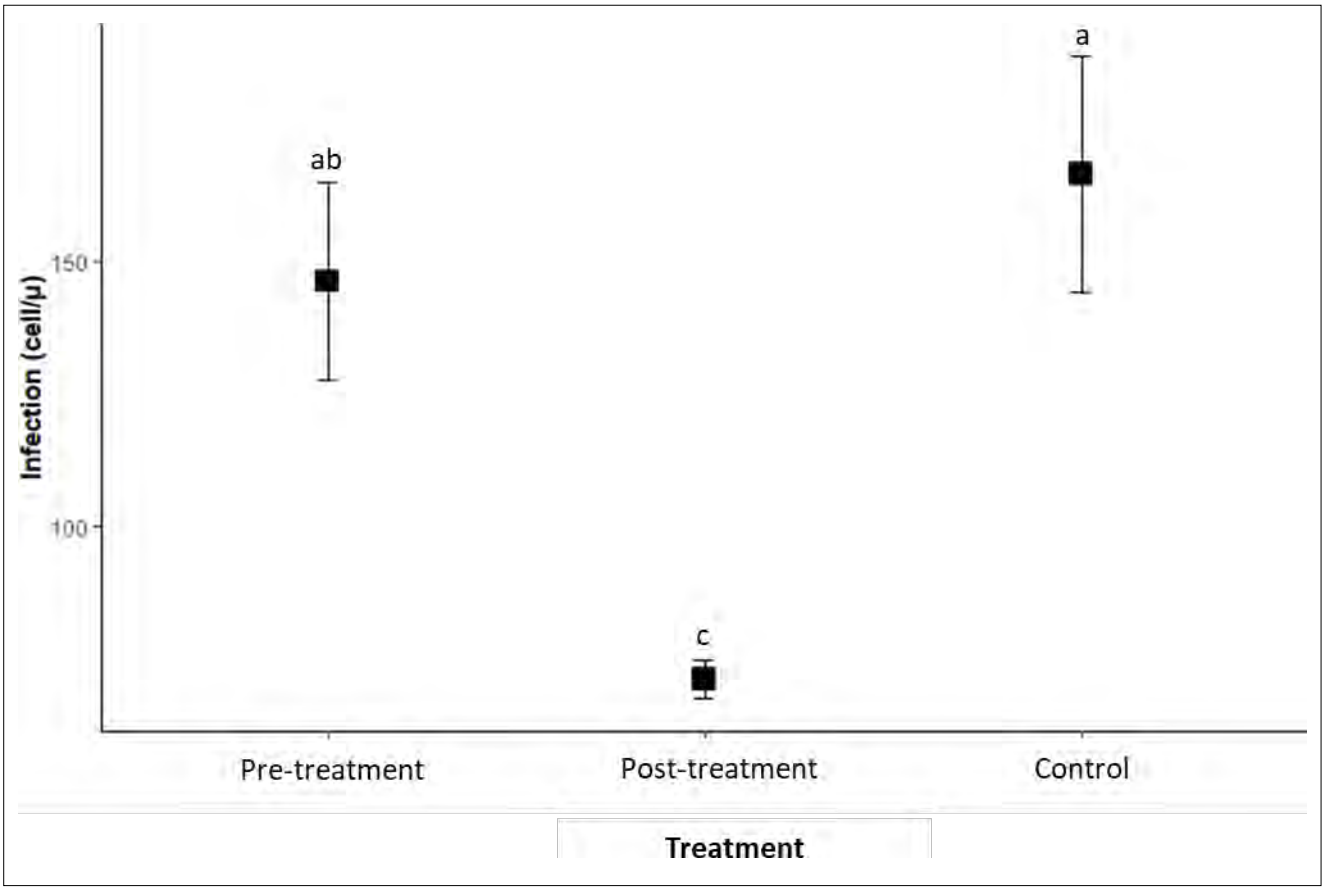

Figure 6. 6. The number (mean ± SE) of *Helicosporidium* sp. cells from the homogenised smears of both the treated and untreated (control) adults of *Cyrtobagous salviniae*. Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

6.3.2.3. *Helicosporidium* sp. cell viability from the antibiotic treated *Cyrtobagous salviniae*

There were significantly more *Helicosporidium* sp. cells in the freshly prepared smears (pre-cultured) of the antibiotic-treated adults than in the post-cultured smears (Figures 6.7; 6.8; 6.9 and 6.10). The pre-cultured and post-cultured cysts and vegetative cells were significantly different ($t = 0.09$; $df = 2$; $P = 0.01$ and $t = -5.44$; $df = 26$; $P = 1.13$ x 10-5, respectively). The pre-cultured smears of the treated adults of *C. salviniae* contained 70.86% and 57.46% of the cyst, and vegetative cells of *Helicosporidium* sp., respectively, compared to those of the untreated adults (Figures 6.7; 6.8; 6.9 and 6.10). Only 58.88% and 46.58% of cyst and vegetative cells, respectively, were found viable post-cultured (Figures 6.7; 6.8; 6.9 and 6.10).

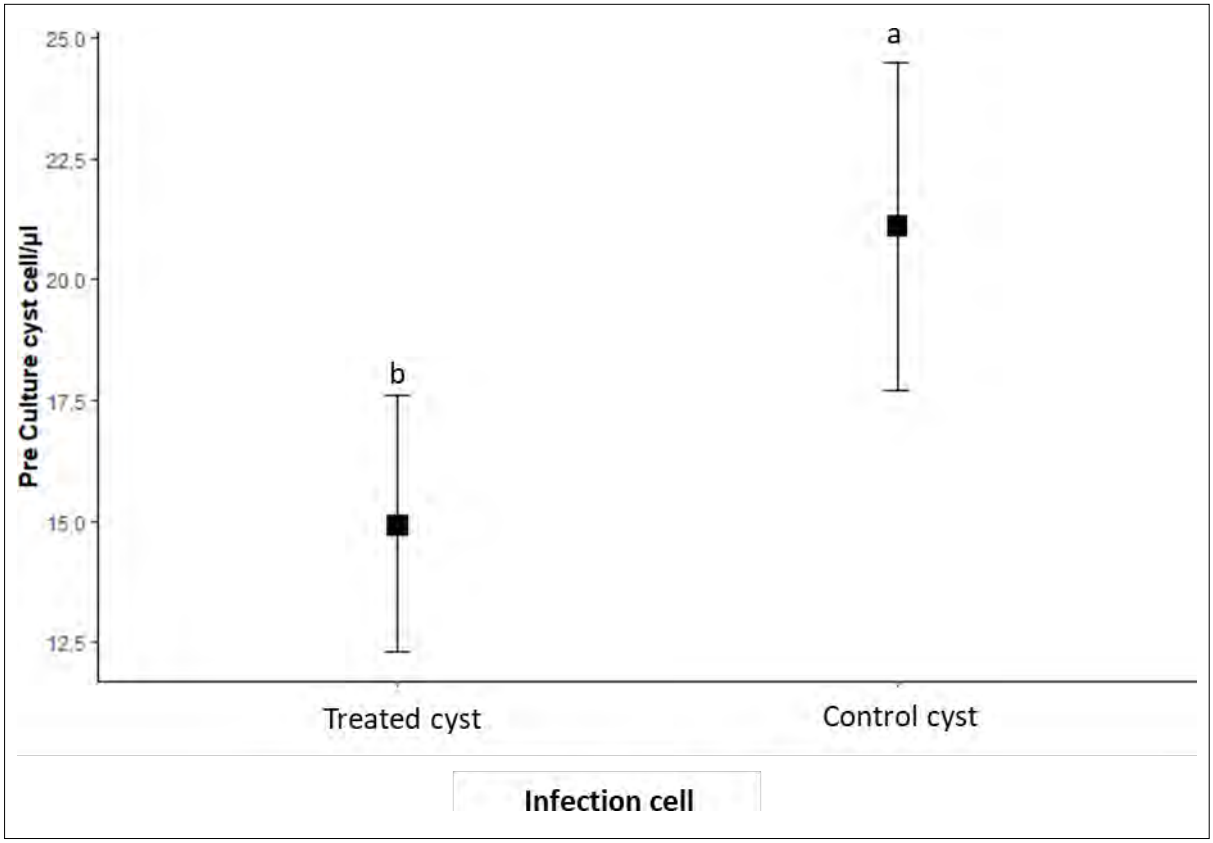

Figure 6. 7. The number (mean ± SE) of cyst cells from the homogenised smears of both the treated and untreated (control) adults of *Cyrtobagous salviniae*. Different letters indicate significant differences ($P < 0.05$).

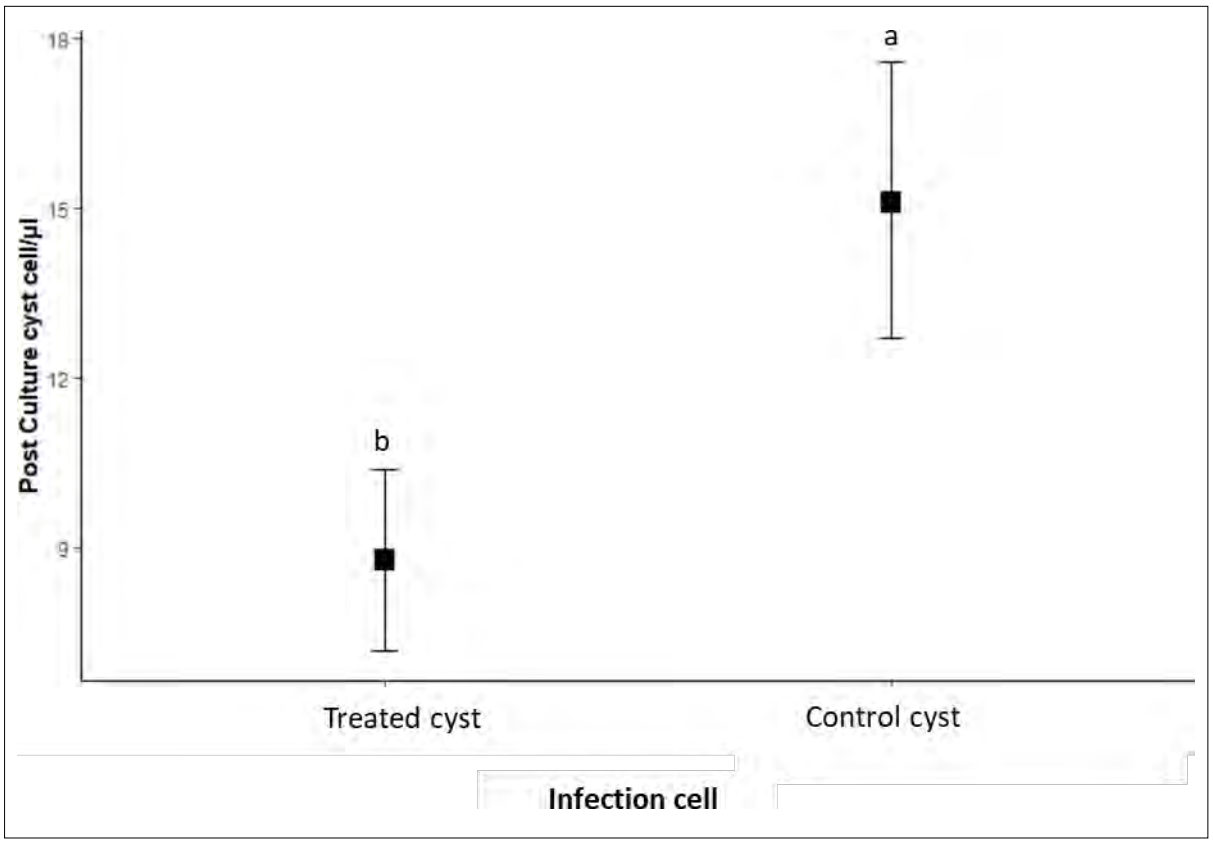

Figure 6. 8. The number (mean ± SE) of cyst cells from the cultured smears of both the treated and untreated (control) adults of *Cyrtobagous salviniae*. Different letters indicate significant differences ($P < 0.05$).

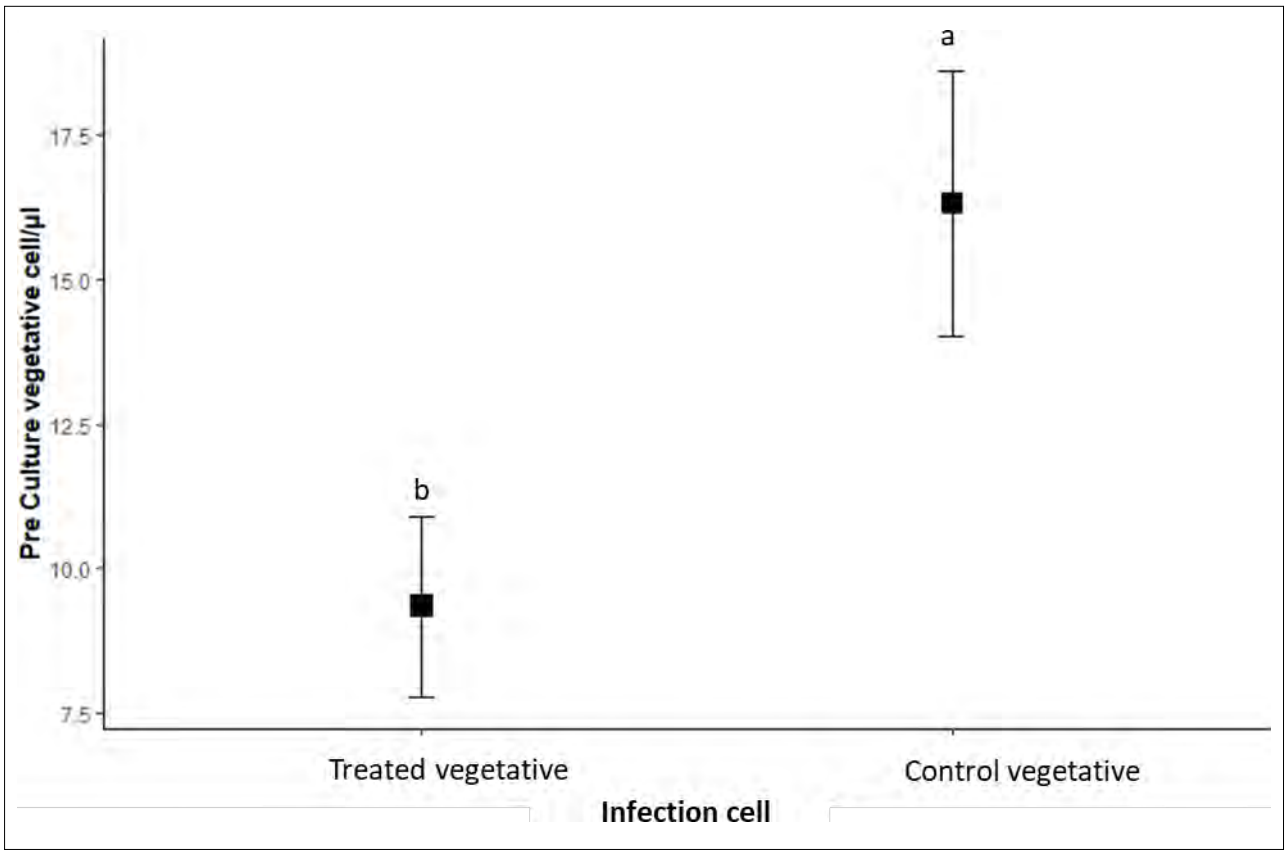

Figure 6. 9. The number (mean ± SE) of vegetative cells from the homogenized smears of both the treated and untreated (control) adults of *Cyrtobagous salviniae*. Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

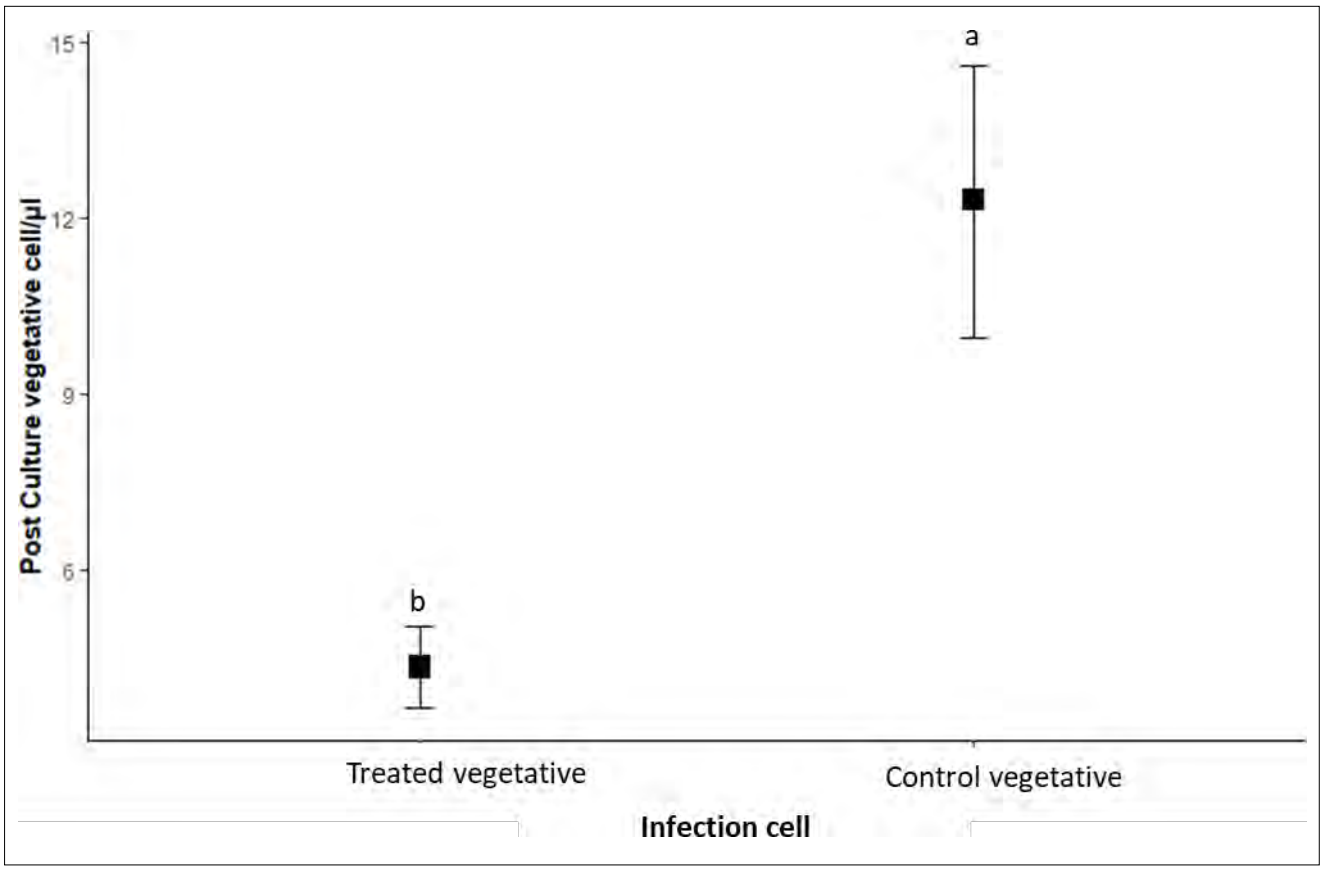

Figure 6. 10. The number (mean ± SE) of vegetative cells from the cultured smears of both the treated and untreated (control) adults of *Cyrtobagous salviniae*. Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

6.3.3. Adult feeding

Adult feeding by *C. salviniae* was not significantly different between the treatment and control ($t = 0$; $df = 40$; $P = 1$) (Figures 6.11 & 6.12).

Figure 6. 11. An adult *Cyrtobagous salviniae* feeding on fronds of *Salvinia molesta* inoculated with ketoconazole.

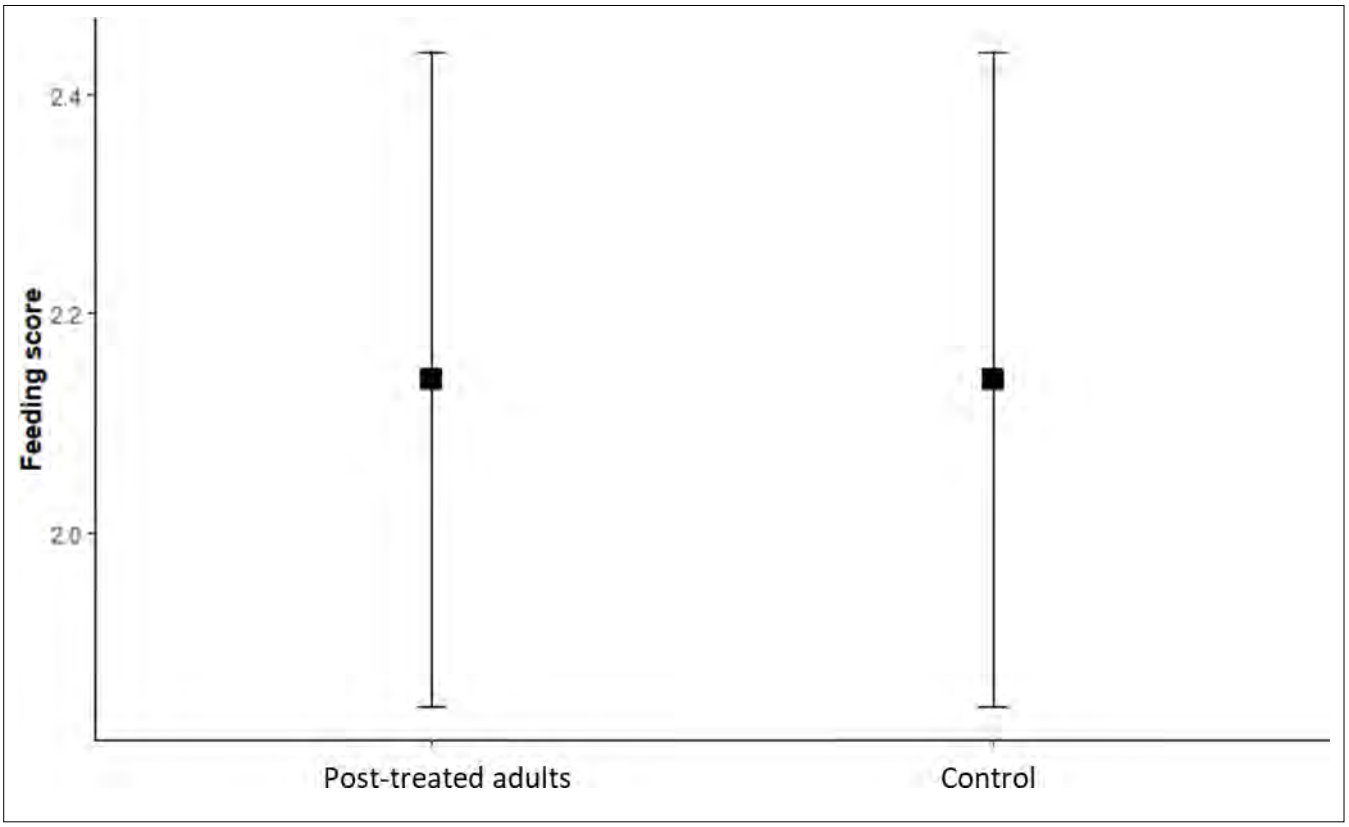

Figure 6. 12. The feeding score (mean ± SE) of the treated and untreated (control) adults of *Cyrtobagous salviniae* on *Salvinia molesta* fronds inoculated with the antibiotic. Box (mean) and whisker (standard error).

6.3.4. Adult survival

There was no significant difference in mortality between adults treated with the antibiotic and the untreated control (t = 0.73; df =10; P = 0.48) (Figure 6.13). At the end of the antibiotic treatment trial, 4.5 ± 0.34 and 5.33 ± 0.33 of the six treated and control adults of *C. salviniae* survived per replicate.

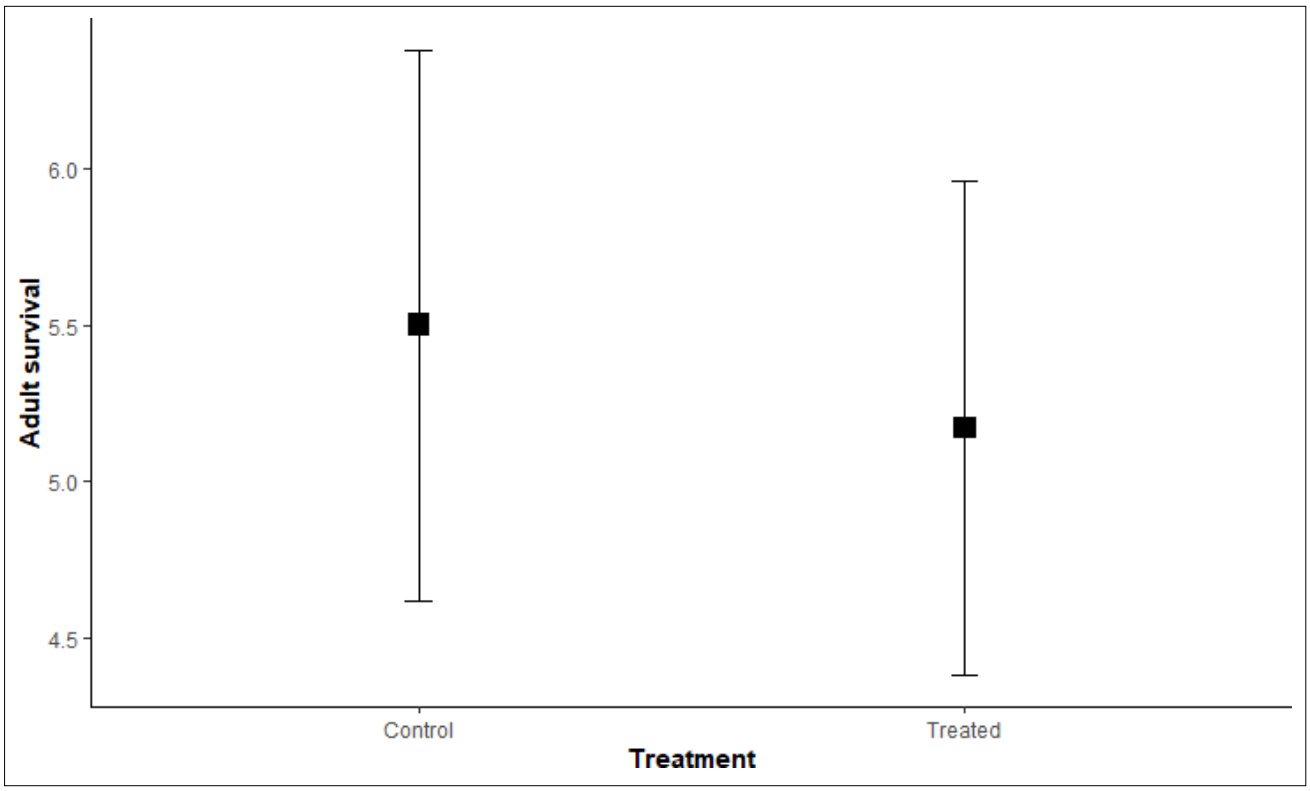

Figure 6. 13. The number (mean ± SE) of *Cyrtobagous salviniae* adult survival following antibiotic treatment. Box (mean) and whisker (standard error).

6.4. DISCUSSION

The results from Chapter 4 showed that *Helicosporidium* sp. reduced the effectiveness of *C. salviniae* against *S. molesta* in South Africa. The present chapter investigated whether *Helicosporidium* sp. could be treated in *C. salviniae*, and if this would improve their performance on *S. molesta*. Interestingly, the results of the study showed that culturable *Helicosporidium* sp. cells could be significantly reduced or eliminated with at all antibiotic treatment concentrations. Further, as treatment time increased, and at the lowest antibiotic concentration, the antibiotic was still highly effective, and compared well with the high and medium concentrations. Although ~100% of *Helicosporidium* sp. was eliminated in the *in vitro* experiment, this was not the case when treating *C. salviniae* adults with a similar low concentration of the antibiotic for the same period. Approximately 22.86% of the *Helicosporidium* sp. cells were eliminated from the adults treated with the low concentration of antibiotics in comparison to the untreated adults. Therefore, it is likely that administering low concentrations of the antibiotic in the adults of *C. salviniae* for a long period could eliminate *Helicosporidium* sp. in these adults. The low concentration of the antibiotic caused no significant harm to *C. salviniae* adults as shown by similar adult survival compared to those of the untreated adults. Further, the treated adults fed as much as the untreated controls. Thus, the antibiotic had no deleterious effects on adult weevil survival and feeding.

Some studies have reported the possibility of eliminating diseases in insect species and establishing disease-free colonies (Koga *et al*., 2007; Raymond *et al*., 2009). For example, symbiont elimination has been possible in aphid species by rearing their adults sequentially on an artificial diet inoculated with low concentrations of antibiotics (Raymond *et al.*, 2009; Frankenhuyzen *et al.*, 2010). Another study by Lin *et al*., (2015), which evaluated five antibiotics on larval gut bacterial diversity of *Plutella xylostella* (Lepidoptera: Plutellidae), suggested that subjecting diseased insects to low concentrations of antibiotics could eliminate

such diseases over a long period. These findings (Raymond *et al.*, 2009; Frankenhuyzen *et al.*, 2010; Lin *et al*., 2015) match the results of the present study, which showed a considerable reduction of *Helicosporidium* sp. cells in *C. salviniae* with the application of a low concentration of an antibiotic. Subjecting *C. salviniae* adults to a low concentration of the antibiotic could eventually eliminate *Helicosporidium* sp. in *C. salviniae*. Thus, improvement of feeding and reproductive output of *C. salviniae* could be tested on *S. molesta*.

In summary, based on the findings of this study, the selected antibiotic ketoconazole showed the potential to treat *Helicosporidium* sp. in *C. salviniae*. Therefore, the present study suggests that a long period of treatment of *C. salviniae* with ketoconazole could eliminate *Helicosporidium* sp. and this is expected to improve this weevil's feeding and reproductive output on *S. molesta*. It is not certain just how practical ketoconazole would be in a large-scale mass-rearing facility. Furthermore, the antibiotic is expensive, approximately $152.53 to $203.38 per kilogram (Xi'an Henrikang Co., Ltd.). Thus, it is recommended that the antibiotic is applied on a small-scale (the size of a single 1mx1mx1m tub) to determine if it is viable at that level and scale up once the proof of concept has been shown.

It is also important to evaluate other factors that could be used in conjunction with ketoconazole to quickly eliminate *Helicosporidium* sp. in *C. salviniae*, for example, a heat stress treatment. A heat stress treatment has been frequently and successfully applied to eliminate some life stages of symbionts in aphids (Montllor *et al.*, 2002; Russell & Moran, 2006). Therefore, the combination of heat stress or treatment with ketoconazole is worth trying on the diseased adults of *C. salviniae*. Lastly, understanding the interrelations between *Helicosporidium* sp., and the efficacy of ketoconazole in *C. salviniae*, could lead to the formulation of more effective doses of the antibiotic and this will remain useful for integrated pest management of *C. salviniae* in the future.

CHAPTER 7

Effect of temperature on *Helicosporidium* sp. load in *Cyrtobagous salviniae*

7.1. INTRODUCTION

The previous chapter showed that there was a possibility of reducing the disease load of *Helicosporidium* sp. in *C. salviniae* through the application of antibiotics. However, based on other studies, antibiotic treatments are known to be more effective when they are applied in conjunction with other factors such as high or low temperatures that also reduce the viability of such diseases (Montllor *et al.*, 2002; Russell & Moran, 2006). *Cyrtobagous salviniae* has a wide range of temperature tolerance (Stone, 1973; Cozad *et al*., 2019), but it is not known if *Helicosporidium* sp. has the same temperature tolerance. Some studies have reported that the mature *Helicosporidium* cysts are tolerant of both extreme cold and hot temperatures (Evans *et al*., 1975). But evidence that these cysts can germinate or differentiate into other life stages after exposure to high or low temperatures remains elusive (Evans *et al*., 1975).

In the field, the load of *Helicosporidium* sp. in *C. salviniae* was low and high in warm-hot and cold areas in South Africa, respectively (see Chapter 3). Although these differences were not quantified and correlated with the field temperatures, it was assumed that, temperature had a role in determining the loads of *Helicosporidium* sp. However, whether temperature alone or with other factors have an influence on *Helicosporidium* sp. loads is unknown. However, *in vitro* experiments demonstrated that, the cells of *Helicosporidium* sp. cannot replicate at temperatures above 35°C (Hembree, 1981). These results suggest that, field temperatures could be a factor on the load of *Helicosporidium* sp. in *C. salviniae*. Hembree (1981) showed that, under *in vitro* trials, *Helicosporidium* sp. cells could survive with 0% loss of infectivity in buffer solutions with pH ranging between 10.5 and 3 at 4°C, but again, this study showed that infectivity was recorded between 37°C and 50°C (Hembree, 1981). Based on this evidence,

temperature was then assumed to have had an influence on the loads of *Helicosporidium* sp. in the field populations of *C. salviniae* (see Chapter 3).

Similarly, field populations of grasshoppers, locusts, and crickets are active behavioral thermoregulators and sometimes rely on behavioral fever when associated with pathogens (Uvarov, 1977; Chappell & Whitman, 1990; Roxburgh *et al*., 1996). In some instances, the ability to elevate body temperatures by the infected insects depends on the type and cell loads of pathogens and their thermal tolerance (Gardner & Thomas, 2002). For example, Adamo (1998) reported that the cricket, *Acheta domesticus* (Orthoptera: Gryidae) was incapable of elevating its body temperature when infected with the bacterium, *Serratia marcescens* and protozoan gut parasites or when attacked with the tachinid fly parasitoid, *Ormia ochracea* (Diptera: Tachinidae). But, *A. domesticus* was capable of raising its body temperature when infected with the prokaryotic parasite, *Rickettsiella grylli* (Legionellales: Coxiellaceae) (Adamo, 1998). Most importantly, thermoregulation and behavioral fever usually result in a partial or complete reduction of

loads of pathogenic cells in grasshoppers (Boorstein & Ewald, 1987; Carruthers *et al*., 1992; Inglis *et al*., 1996), locusts (Arthurs & Thomas, 2000; Blanford & Thomas 2001; Ellliot *et al*., 2002; Ouedraogo *et al*., 2003), and crickets (Louis *et al*., 1986; Adamo, 1998). These examples show that, the potential of pathogens to impact insects has a limit, that is governed by the climatic conditions.

Therefore, the effect of temperature on *Helicosporidium* sp. should be evaluated to determine its potential to affect the infection load in *C. salviniae* and possibly improve the weevil's performance. Further, the response of diseased *C. salviniae* to the different temperatures should also be determined. Therefore, the aim of this chapter was to determine the effect of temperature on *Helicosporidium* sp. load in *C. salviniae*.

7.2. MATERIALS AND METHODS

7.2.1. Experimental setup

The effect of *Helicosporidium sp.* infection on *C. salviniae* feeding and reproductive output was tested at four different temperatures (Figure 7.1). *Salvinia molesta* plants were collected from culture maintenance pools at Waainek Mass Rearing Facility, Rhodes University. The plants were thoroughly washed under running tap water for five minutes to remove any potential pest species (e.g. spiders, mites), and then placed in 1 litre containers, and covered with gauze. The plants were supplied with one granule of Cultera fertiliser, consisting of nitrogen, phosphorus, and potassium (NPK: 8:1:5), every 10 days. Four replicates per temperature treatment were set up. Five pairs of *C. salviniae* adults, sourced from SASRI, were placed on 5g of *S. molesta* in CE rooms set at the four temperatures, 15ºC, 20ºC, 26ºC and 30ºC, and a photoperiod of 14L: 10 D (grow lux lights). The selection of these temperatures was based on the recorded optimal temperature requirements of both *S. molesta* and *C. salviniae*, which were 30ºC and 33ºC, respectively (Room *et al*., 1989; 1986). During the experiments, a small amount of water was sprayed on the fronds to keep them fresh and to discourage potential predators. The experiments were run for 21 days.

At 7, 14, and 21 days of this experiment, the exposed adult pairs of *C. salviniae* were gently removed from the exposed plants of *S. molesta* and transferred onto another 5 grams (new) *S. molesta* plants and kept under their respective temperatures. The previously exposed *S. molesta* plants, including those of the control, were weighed to test for changes caused by the impact of *C. salviniae*. *Salvinia molesta* frond damage was measured by counting the number fronds that had feeding scars and compared to the total number of *S. molesta* fronds (fed and unfed), where 0% (no feeding = 0), 1–19% (exploratory feeding = 1), 20–49% (minor feeding = 2) and 50–100% (normal feeding = 3) fed fronds of the total fronds of *S. molesta* were scored.

Figure 7. 1. Experimental setup of feeding and reproductive output of *Cyrtobagous salviniae* on *Salvinia molesta*.

The survival of adults was recorded. When necessary, new *C. salviniae* adult (either males or females) sourced from the respective temperature treatment were supplied to the experiments to replace the dead ones. Fertility (i.e. larval production) of *C. salviniae* was quantified. To do this, plant material that had been weighed was returned into the respective containers without the insects and monitored until the emergence of the larvae. After the emergence of the first larva, nine days of the monitoring was added to allow all the deposited eggs in different days to have equal incubation period (Figure 7.2). The reproductive output of *C. salviniae* was determined between day 10 and 20 of the initial exposure of these weevils to *S. molesta* under different temperatures. This duration of the larval hatching was adopted from the incubation period of eggs of healthy adults of *C. salviniae* (Forno *et al.*, 1983).

Throughout the experiments, the infection load of *Helicosporidium* sp. was monitored and recorded. At day 0, 7, and 14 days of the experiment, 3 groups consisting of 5 adult pairs of *C. salviniae* were collected from each culture maintained at all the temperature interval and screened to track *Helicosporidium* sp. cell changes in *C. salviniae*. On day 21 of the

experiment, the experiment was terminated and all the experimental adults of *C. salviniae* were also screened. The smear preparation from the screened adults followed the procedure used in Chapter 2. *Helicosporidium* sp. cells were counted from 50µl of the smears prepared from these adults of *C. salviniae*.

Figure 7. 2. Different stages of *Cyrtobagous salviniae* larvae detected on different parts of *Salvinia molesta* plants.

7.2.2. Data analysis

Linear Model (LM) was used to explore the relationship between the response of the variables of the adults of *C. salviniae* to the times of exposure to the different temperature intervals. Generalized Least Squares (GLS) model with repeated measures was used to explore the most influential model of the response of the adults of *C. salviniae* between the times of exposure and different temperature intervals. The raw data of the variables on the response of the adults of *C. salviniae* to the times of exposure to the different temperature intervals met the normality assumptions of Analysis of Variance (ANOVA), which was checked by the Shapiro-Wilk normality test ($P > 0.05$). Thus, Two-way ANOVA was used to separate and compare the means of the measured variables, infection cells, adult feeding and survival, larva production

and biomass changes of *S. molesta*. All statistical analyses were conducted in the R Studio (version 1.1.463).

7.3. RESULTS

7.3.1. *Helicosporidium* sp. cell count

There were more *Helicosporidium* sp. cells counted at lower than higher temperatures post the time of exposure (Figure 7.3). Significantly more *Helicosporidium* sp. were produced at 15˚C; 20˚C and 26˚C compared to 30˚C than the initial loads of *Helicosporidium* sp. Changes in temperature had a significant influence on the number of disease cells (R^2 = 0.06; P = 0.05) (Table 7.1; Figures 7.3).

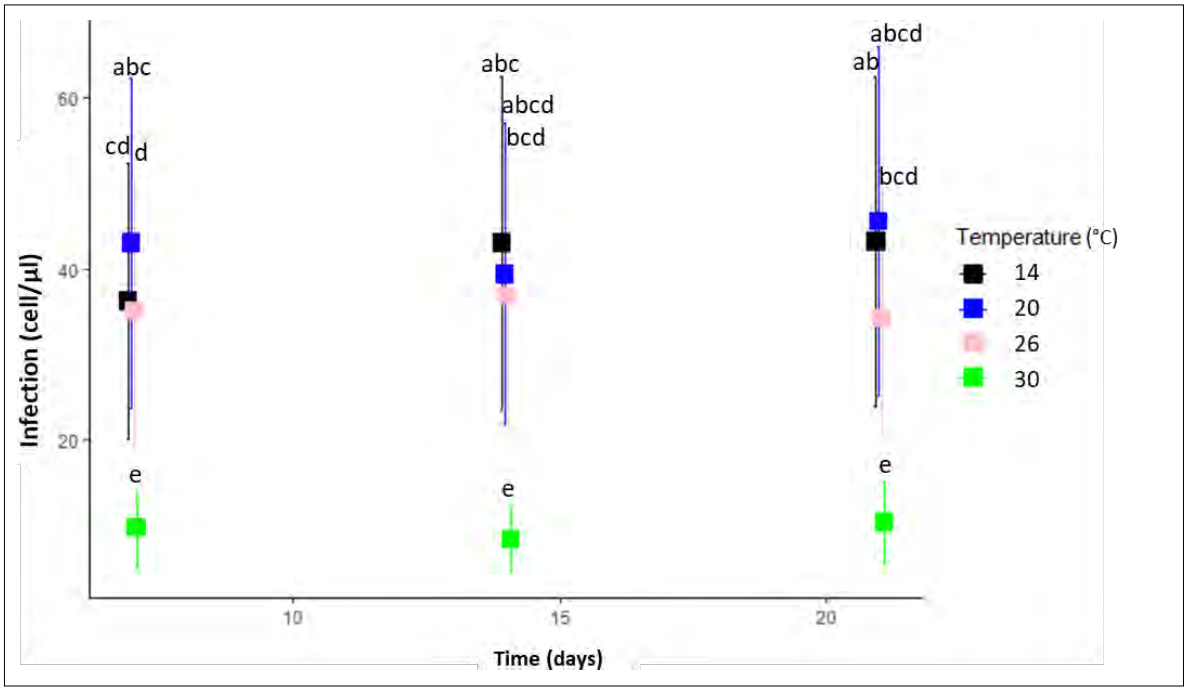

Figure 7. 3. Concentration of *Helicosporidium* sp. cells in response to times of exposure to different temperatures (n = 4). Different letters indicate significant differences (P < 0.05). Box (mean) and whisker (standard error).

Table 7. 1. Generalised Least Squares model summary on the response of *Helicosporidium* sp. cells to the exposure times at different temperatures.

Terms	**Variables**	**Value**	**Std. Error**	***t*-value**	***P*-value**
-	(Intercept)	45.86	74232.70	0.00	0.99
Temperature (T)	Temperature (T)	-1.12	0.65	-1.72	0.09
TC	Time (TH)	-1.13	0.83	-1.37	0.17
	T*TH	0.08	0.04	2.24	**0.03**
	Phi	1			
	Residual Standard Error	74232.71			
	Degrees of freedom	72			
	Residuals DF	68			
	AIC	515.73			
	Log-Likelihood	-251.87			

7.3.2. *Salvinia molesta* frond damage

The highest frond damage of 50% was recorded at 26˚C and 30˚C over 21 days (Table 7.2; Figure 7.4). The lowest frond damage of 5.67% was recorded at 14˚C over 21 days (Table 7.2; Figure 7.4). Significantly more leaf was damaged with the increase in temperature and time of exposure (R^2 = 0.53; P = 1.04 x 10^{-10}) (Figure 7.4). The relationship between the times of exposure to the different temperatures was significant (Table 7.2; Figure 7.4).

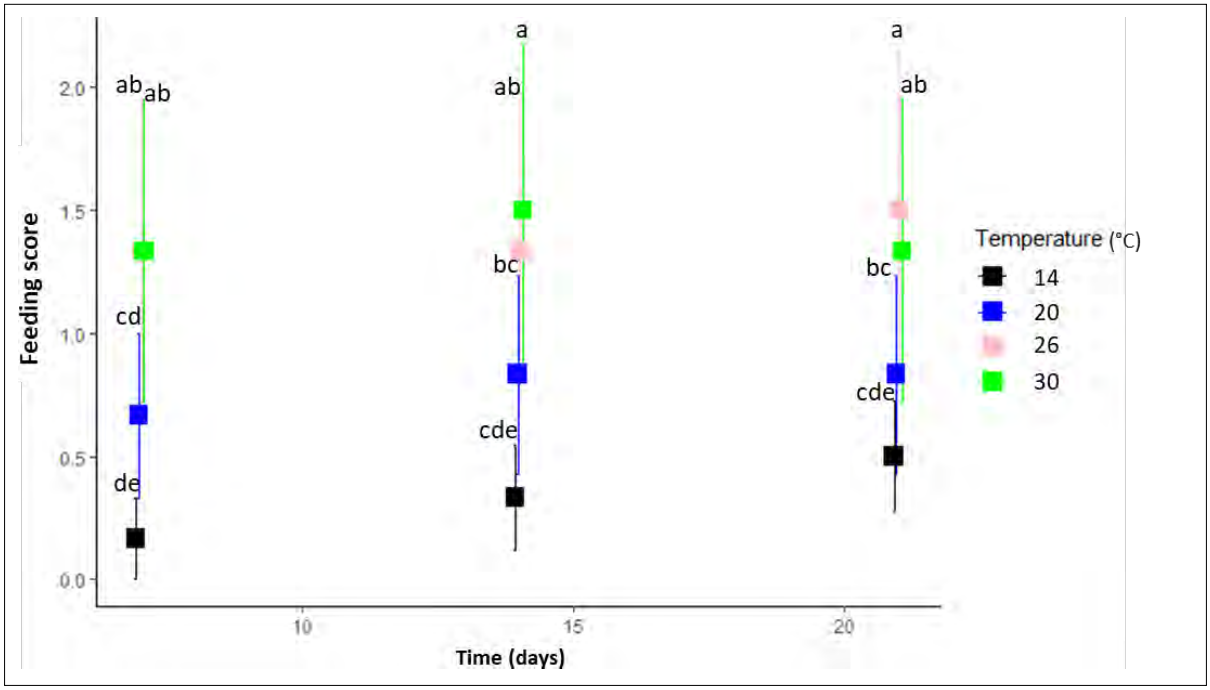

Figure 7. 4. Mean *Salvinia molesta* frond damage by *Crytobagous salviniae* exposed to different temperatures for different times ($n = 4$). Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

Table 7. 2. Generalised Least Squares model summary on the frond damage of *Salvinia molesta* confined to *Cyrtobagous salviniae* at different temperatures and times of exposure.

Terms	Variables	Value	Std. Error	*t*-value	*P*-value
-	(Intercept)	-2.82	1.08	-2.60	**0.01**
Feeding	Temperature (T)	0.12	0.04	2.81	**0.01**
	Time (TH)	0.06	0.06	0.91	0.37
	Infection (I)	-0.05	0.03	-2.04	**0.05**
	T*TH	-0.01	0.01	-0.47	0.64
	T*I	0.01	0.01	2.89	**0.01**
	TH*I	0.01	0.01	1.98	**0.05**
	T*TH*I	-0.01	0.01	-2.04	**0.05**
	Phi	0.88			
	Residual Standard Error	0.92			
	Degrees of freedom	72			
	Residuals DF	64			
	AIC	175.22			
	Log-Likelihood	-77.61			

(*) means interactions between variables. Values in bold are statistically significant.

7.3.3. *Salvinia molesta* biomass

The highest biomass reduction of 52.90% was recorded at 30˚C over 21 days (Figure 7.5; Table 7.3). Significantly more biomass reduced was recorded with the increase in temperature and time of exposure ($R^2 = 0.18$; $P < 0.001$) (Figure 6.5). The relationship between the times of exposure to the different temperatures was not significant (Figure 7.6; Table 7.3).

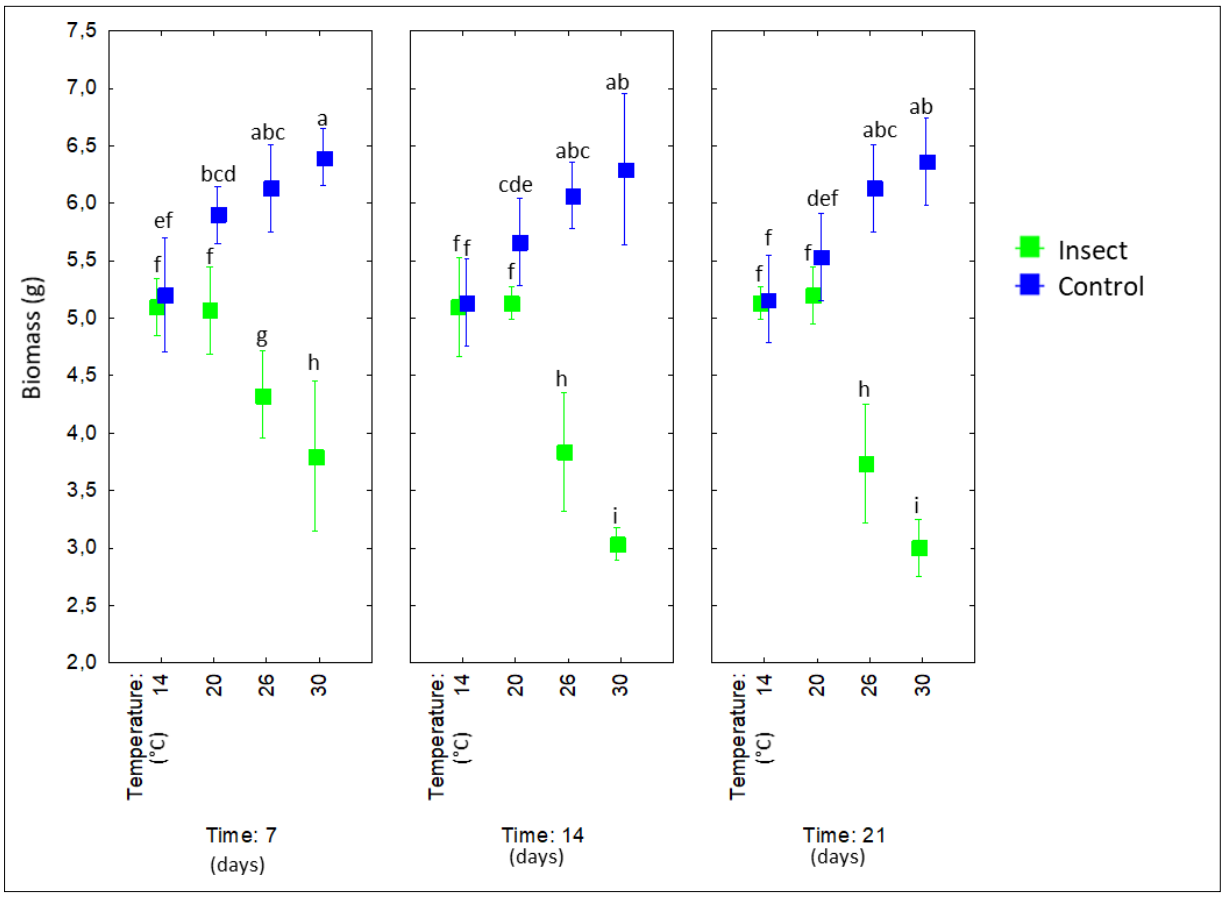

Figure 7. 5. Patterns of *Salvinia molesta* biomass changes by the impact of *Cyrtobagous salviniae* exposed at different temperatures for different durations ($n = 4$).

Table 7. 3. Generalised Least Squares model summary on the changes of *Salvinia molesta* biomass confined to *Cyrtobagous salviniae* at different temperatures and times of exposure.

Terms	**Variables**	**Value**	**Std. Error**	***t*-value**	***P*-value**
-	(Intercept)	8.52	9007.96	0.00	0.99
Temperature (T)	Temperature (T)	-0.09	0.02	-4.39	**0.01**
TC	Time (TH)	-0.08	0.03	-2.69	**0.01**
	Infection (I)	0.01	0.01	0.77	0.44
	T*TH	0.00	0.00	0.98	0.33
	T*I	-0.00	0.00	-1.48	0.14
	TH*I	-0.00	0.00	-0.03	0.97
	T*TH*I	0.00	0.00	0.75	0.45
	Phi	1			
	Residual Standard Error	9007.96			
	Degrees of freedom	72			
	Residuals DF	64			
	AIC	89.21			
	Log-Likelihood	-34.60			

(*) means interactions between variables. Values in bold are statistically significant.

7.3.4. Adult survival

Temperature and time of exposure did not significantly affect adult survival, although more died at 14˚C (Figures 7.6; Table 7.4).

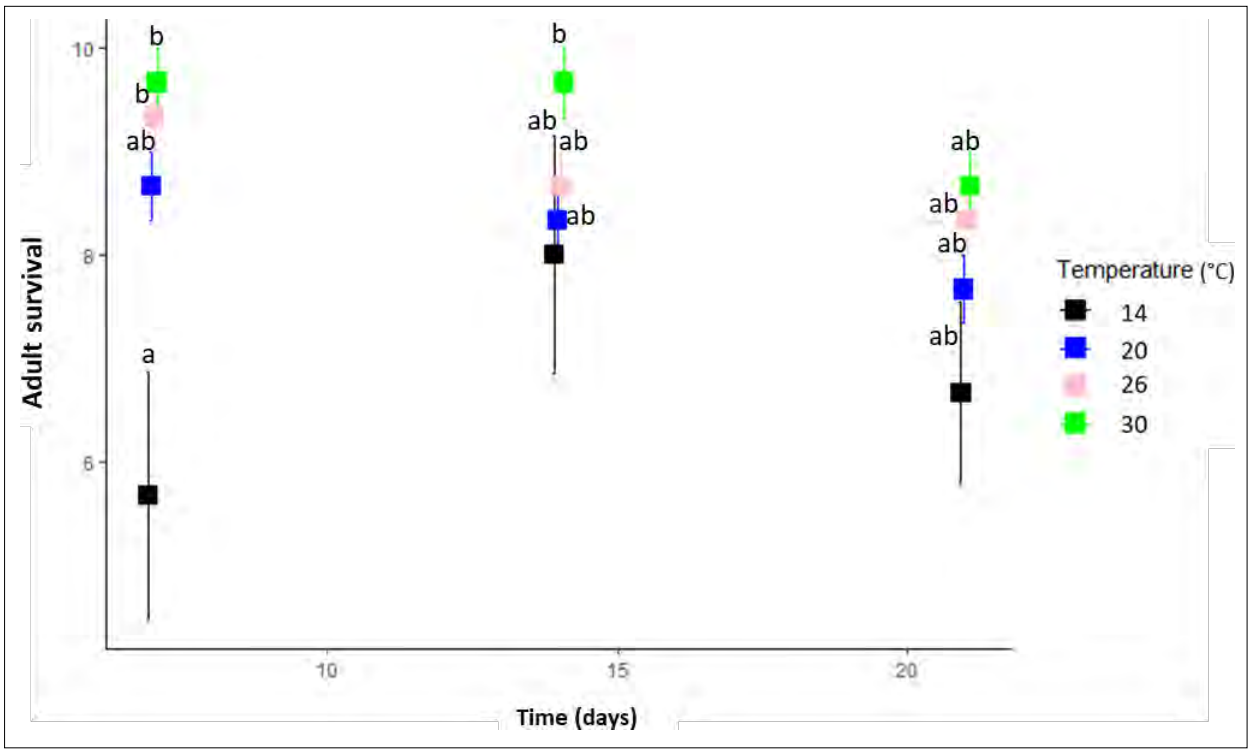

Figure 7. 6. Mean adult *Cyrtobagous salviniae* survival in response to duration of exposure at different temperatures (n = 4). Different letters indicate significant differences ($P < 0.05$). Box (mean) and whisker (standard error).

Table 7. 4. Generalised Least Squares model summary on the survival of the adults of *Cyrtobagous salviniae* in response to the exposure times at different temperatures.

Terms	Variables	Value	Std. Error	*t*-value	*P*-value
-	(Intercept)	-6.61	28594.95	-0.00	0.99
Temperature (T)	Temperature (T)	0.42	0.09	4.82	**0.01**
TC	Time (TH)	0.21	0.13	1.56	**0.12**
	Infection (I)	-0.08	0.06	-1.39	0.17
	T*TH	-0.01	0.01	-0.82	0.41
	T*I	0.00	0.00	1.27	0.21
	TH*I	-0.00	0.00	-0.19	0.84
	T*TH*I	0.00	0.00	0.3	0.97
	Phi	1			
	Residual Standard Error	28594.95			
	Degrees of freedom	72			
	Residuals DF	64			
	AIC	270.88			
	Log-Likelihood	-125.44			

(*) means interactions between variables. Values in bold are statistically significant.

7.3.5. Larval production

Larval production was only recorded at 26˚C and 30˚C. The mean number of larvae produced were 1.00 ± 0.56 (mean ± SE, n = 4) and 6.78 ± 0.64 (n = 4) at 26˚C and 30˚C, respectively, thus increased temperature and time of exposure had a significant effect on larval production (R^2 = 0.41; P = 1.40 x 10^{-8}) (Figures 7.7; Table 7.5).

Table 7. 5. Generalised Least Squares model summary on the larval production by the adults of *Cyrtobagous salviniae* exposed to different temperatures for different times.

Terms	Variables	Value	Std. Error	*t*-value	*P*-value
-	(Intercept)	2.21	2.48	0.89	0.38
Temperature (T)	Temperature (T)	-0.09	0.11	-0.85	0.41
TC	Time (TH)	-0.46	0.21	-2.32	**0.02**
	T*TH	0.02	0.00	2.88	**0.01**
	Phi	0.57			
	Residual Standard Error	1.90			
	Degrees of freedom	76			
	Residuals DF	72			
	AIC	307.16			
	Log-Likelihood	-147.58			

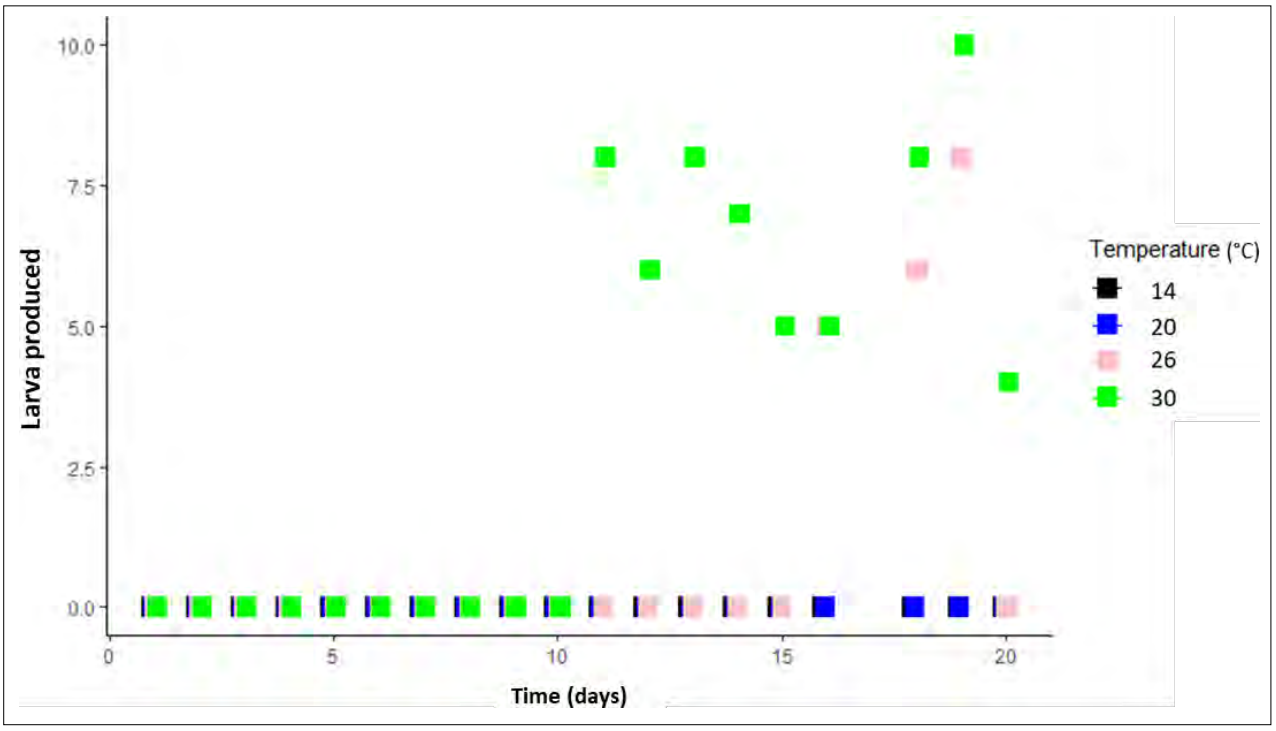

Figure 7. 7. Mean larval production by the adults of *Cyrtobagous salviniae* in response to the duration of exposure at different temperatures. Box (mean) and whisker (standard error).

7.4. DISCUSSION

The present chapter investigated whether heat treatment alone could reduce a load of *Helicosporidium* sp. disease in *C. salviniae*. The experiments showed that long-term exposure to high temperatures had significantly reduced *Helicosporidium* sp. cell load in adults *C. salviniae*. A 21day exposure to 30˚C induced a significant reduction in the disease load of *Helicosporidium* sp. in *C. salviniae.* Further, higher temperatures also improved adult survival and their reproductive output. The low feeding and reproductive output of *C. salviniae* at the lower temperatures (i.e., 14˚C and 20˚C) could have also been due to the sensitivity of *C. salviniae* to cold temperatures (Forno *et al*., 1983; Sands *et al*., 1983), and possibly not due to *Helicosporidium* sp.

Nonetheless, high temperatures have been shown to influence insect diseases, for example, locusts infected with a pathogenic fungus survived better in warmer areas of their distribution range (Blanford *et al*., 1998; Elliot *et al*., 2002;). Indeed, locusts infected with fungi bask in the sun to reduce the conidial yields (Thomas & Blanford, 2003). Further, many diseased insects were found to have a high tolerance to heat treatment (Montllor *et al.*, 2002; Russell & Moran, 2006).

Like other studies, the present study showed a significant and positive relationship between insect's feeding and reproductive output with temperature, which is what we would anticipate, but it also showed an inverse relationship between increasing temperature and *Helicosporidium* sp. load in *C. salviniae*. Heat treatment has been frequently and successfully applied to eliminate some life stages of symbionts in aphids (Montllor *et al.*, 2002; Russell & Moran, 2006). However, it should be noted that the heat treatment could not be beneficial for every infected insect species (Guinnee & Moore, 2004). For example, the fecundity of infected cockroaches was significantly reduced when these cockroaches were subjected to heat treatment (Guinnee & Moore, 2004). Similarly, the survival and fecundity of bumblebee

infected with the parasitic conopid fly was improved at cold and not hot temperatures (Muller & Schmid-Hempel, 1993). The combined effects of these treatments are expected not only to reduce the load of *Helicosporidium* sp., but to also reduce the rate of transmission (Chapter 3) of the mature cyst of *Helicosporidium* sp. from the parental adults of *C. salviniae* to their progeny. The individual contribution from each of these treatments when applied together can be difficult to determine, but additive effects can make a significant impact on the final eradication success of *Helicosporidium* sp. from *C. salviniae*.

In summary, based on the findings of this study, the high temperature treatment showed the potential to reduce *Helicosporidium* sp. in *C. salviniae*. Therefore, the present study advocates that a long period of exposure of the diseased *C. salviniae* to the high temperature treatment could significantly reduce loads of *Helicosporidium* sp. in *C. salviniae*. However, the success in reducing loads of *Helicosporidium* sp. in *C. salviniae* does not imply that *C. salviniae* will be very effective in controlling *S. molesta* at low temperature sites. But high and rapid load build-up of *Helicosporidium* sp. at the lower temperatures could have had an effect on the impact and reproductive output of *C. salviniae* on *S. molesta*. Similarly, under field conditions, Maseko *et al.* (2018) reported that shaded plants of *S. molesta* were less damaged compared to those exposed to full sun by *C. salviniae* in South Africa. As shaded areas are cooler it could be that the disease builds up in the shaded areas and is broken down in the warmer, full sun areas of infected sites.

CHAPTER 8

General discussion and conclusion

8.1. INTRODUCTION

Biocontrol using the agent, *C. salviniae,* has made a significant contribution to the control of *S. molesta* both here in South Africa and globally (Julien *et al*., 2009; Winston *et al*., 2014; Coetzee *et al*., 2011; Hill & Coetzee, 2017; Martin *et al*., 2018). Although *C. salviniae* is a very effective agent in South Africa, it was shown to be infected by *Helicosporidium* sp. (White *et al*., 2007; Julien *et al.,* 2009) but the impact of this disease was not understood.

The main focus of this study was to determine the morphological features of this disease, use molecular characterization to determine its taxonomic entity, to determine its distribution and infection rate, and to investigate the factors that have influenced the distribution of *Helicosporidium* sp. and those that could limit the build-up and spread of this disease in *C. salviniae*. The results of the studies conducted in this thesis are contextualized below.

8.2. Identification of *Helicosporidium* sp.

The molecular analyses conducted in Chapter 2 confirmed the presence of *Helicosporidium* sp. in *C. salviniae* in South Africa, supporting the work of White et al (2007). Confirmation of the disease in *C. salvinae* through morphological studies using light microcopy laid a foundation for the experimental procedures presented in the other chapters in this thesis. The propagation methods established in this study are simple and very easy to adopt, and could be used to check the presence of *Helicosporidium* sp. in other susceptible insect species.

8.3. Distribution, transmission and effect of *Helicosporidium* sp. in *Cyrtobagous salviniae*

Helicosporidium sp. was widespread throughout the field populations of *C. salvinae* in South

Africa. This was not surprising because this weevil has been repeatedly released across South Africa from mass-rearing facilities that tested positive for *Helicosporidium* sp.. The disease could be transmitted vertically through the maternal pathway in *C. salviniae* in mass-rearing facilities. It is concerning that *Helicosporidium* sp. infection is so widespread, but when quantified, loads of this disease varied across the country (Chapter 3).

In South Africa, *C. salviniae* is regarded as a very effective agent (Martin et al., 2018), although it performs better at sites that are exposed to full sun, in comparison to shaded sites of *S. molesta* (Maseko *et al*., 2018). In the current study, populations of *C. salviniae* that had high loads of *Helicosporidium* sp. were recorded from more temperate sites of *S. molesta* infestation in South Africa, while populations which had very low loads of *Helicosporidium* sp. were distributed in the hotter sites. The findings of Chapter 7 showed that *Helicosporidium* sp. was limited by high temperatures and thus this could explain the better performance of the biological control agent at sites with full sun.

8.4. Control of *Helicosporidium* sp. in *Cyrtobagous salviniae*

Although no programme has ever focused on the treatment of diseases in weed biological control agents, there are studies which have acknowledged that diseases have significant effects on the performance of these agents, and they should be treated (White *et al*., 2007; Table 8.1). The successful control of diseases in insect colonies that are bred for commercial purposes suggests that diseases in biological control agents could also be minimized in their mass-rearing facilities. Although the use of ketoconazole to treat *Helicosporidium* sp. in *C. salviniae* was not very successful, as it only resulted in a reduction of some cells of this disease in *C. salviniae* (Chapter 6), this was very much an exploratory trial that could be expanded to include multi-generational trials and possibly other antibiotics.

Table 8.1.: A list of some diseased insects treated with antibiotics

Insect species	Disease	Antibiotic	Treatment outcome	References
Blattella germanica	Blattabacterium	Rifampicin	Once off treatment caused significant reduction but, the remained cells revived the disease colony.	Rosas *et al*., 2018
Blissus insularis	Burkholderia	Oxytetracycline and kanamycin	Positively reduced the disease load but affected the fitness of the host insect.	Xu *et al*., 2016
Plutella xylostella and *Galleria mellonella*	Gut bacteria	Ciprofloxacin or oxytetracycline	Diverse impact on the disease load	Katarzyna Bronislawa Ignasiak's PhD thesis, 2016
Apis sp. (Honeybee)	Gut microbiota	Tetracycline	Affected size and composition of the host insect, increased succeptibility of the insect to other pathogens.	Raymann *et al*., 2017
Apis sp. (Honeybee)	Gut microbiota	Penicillin-streptomycin	Reduced gut bacteria load but, this increased the susceptibility of the host insect to *Nosema ceranae* infection.	Li *et al*., 2017
Spodoptera litura	Gut microbial	Streptomycin sulphate	Altered the diversity of microbial without affecting health of host insect.	Thakur *et al*., 2016
Bactrocera oleae	Gut microbiota (mostly Candidatus Erwinia dacicola Capuzzo et al.)	Streptomycin	Failed to significantly alter the gut microbiota community.	Koskinioti *et al*., 2019

Nonetheless, it is always important to evaluate the potential of other factors that could be used in conjunction with antibiotics that eliminate diseases in insects. Such factors could be adopted the performance of such diseases under field or natural conditions with multiple factors like temperature and humidity. In some cases, high temperatures have been used to reduce disease loads (Blanford *et al*., 1998; Elliot *et al*., 2002). For example, heat stress has also been successfully applied to eliminate some life stages of symbionts in aphids (Montllor *et al*., 2002; Russell & Moran, 2006). In the present study, both the antibiotic (Chapter 6) and high temperature (Chapter 7) reduced the infection of *Helicosporidium* sp. in *C. salviniae*, when tested separately. Although these treatments were not tested on *Helicosporidium* sp. together, the combination of these treatments could be synergistic and reduce *Helicosporidium* sp. loads further. Understanding the interrelations between *Helicosporidium* sp., and the efficacy of ketoconazole in *C. salviniae*, could also lead to the formulation of more effective doses of the antibiotic and this will remain useful for integrated pest management of *C. salviniae* in the future.

Most importantly, the high temperature applied to *Helicosporidium* sp. did not affect insect feeding and reproductive output but only affected the disease. Likewise, the application of ketoconazole antibiotic in *C. salviniae* did not repel this insect from feeding and its survival was not significantly affected. These are indications that the combined application of this high temperature and ketoconazole may not affect the biology of *C. salviniae*. This kind of treatment would be expected to significantly reduce the rate of transmission of the mature cyst in *C. salviniae* and this will minimise the distribution of *Helicosporidium* sp. in *C. salviniae* in South Africa.

The high-temperature treatment of the diseased *C. salviniae* results show that *C. salviniae* will remain an effective biological control solution for *S. molesta* in South Africa. Treatment of the diseased *C. salviniae* could only improve the efficacy of *C. salviniae* under cold or shaded sites of *S. molesta* in the country. Therefore, populations of *C. salviniae* maintained and released into hot sites of *S. molesta* may not be important to subject them to the antibiotic treatment.

8.5. Control of *Salvinia molesta*

The number of different species of agent that should be released on a target weed to effect control have always been a challenge for biocontrol practitioners (Myers 1984). However, in South Africa, and globally, the biocontrol of *S. molesta* has relied on a single agent.

Although *C. salviniae* has contracted *Helicosporidium* sp., this weevil has shown considerable damage to *S. molesta* (Chapters 5, 6 & 7). Further, the present study has proven that *Helicosporidium* sp. could be reduced or eliminated in *C. salviniae* and this is expected to improve the efficacy of *C. salviniae* on *S. molesta* (Chapters 6 & 7). Suppose *Helicosporidium* sp. was strongly affecting *C. salviniae* with no factor that could be used to reduce loads of this disease, the release of another agent would be triggered to supplement the diseased *C. salviniae*. This is strongly supported by the results of Chapters 6 and 7, which showed that *Helicosporidium* sp. can be controlled in *C. salviniae*.

8.6. Importance of disease screening, identification and assessment methods in biocontrol

The presence of *Helicosporidium* sp. in *C. salviniae* has shed light on the possibility that insects, including biological control agents, contract infectious diseases. However, screening for and treatment of diseases in weed biological control agents is not routinely practiced. Insect agents could be screened during both the pre-release and post-release assessment studies, because the unknown health status of insect agents may have unintended effects on their effectiveness.

Screening can be quick, simple and relatively inexpensive; thus, a large insect population can quickly be screened. In the current study, homogenizing techniques have successfully detected the presence of *Helicosporidium* sp. and quantified its loads in *C. salviniae*. The disease testing solutions used in this study have also had the ability to culture cells of *Helicosporidium* sp. Thus, culturing of *Helicosporidium* sp. has provided more information on how they differentiate, replicate and transmit in populations of *C. salviniae*. Most importantly, this technique is effective in the study of diseases that infect mostly hemolymph of insects.

A number of insects have been bled in tubes or petri dishes to collect their haemolymph to test for the presence of the suspected infectious species (Table 8.1). Diagnosing infections in insects is also done on their gut contents (Table 8.1). Screening and treatment of diseases in insects is an approach

that is gaining more interest with commercial insect breeders (Table 8.1). For example, the "Insect Doctors" programme has been established in Europe to equip entomologists and pathologists with skills to manage diseases in commercial insects. Outcomes of this programme could benefit biocontrol researchers with new knowledge to monitor and manage diseases in field and inhouse insect colonies. However, disease management in insect cultures requires an integration of many research disciplines including, molecular biology, entomology, microbiology, pathology, chemistry and others. The present study has focused on finding possible ways of enhancing the effectiveness of a biocontrol agent, *C. salviniae* infected with the infection, *Helicosporidium* sp., the results of this study have contributed new knowledge, shedding light on the possibility of disease management in insect cultures.

Based on the track record of the side effects of antibiotics and treatment failures on insects, some scientists are skeptical about the use of antibiotics to manage diseases in insect cultures (Table 8.1). Failures of insect disease treatment are mostly reported from insect colonies that have been given a single antibiotic treatment (Table 8.1). Whilst multiple treatments have been successful in treating diseased insect colonies (Table 8.1). Comparing the successful and failed antibiotic treatment programmes (Table 8.1), it becomes apparent that, some insects could be sensitive to certain antibiotic doses, The sensitivity of such insects could also be due to the strong doses of the antibiotics. In the present study, it was discovered that mild doses of antibiotics could gradually reduce load of the infection *Helicosporidium* sp. while promoting feeding and survival of the adults in the treatment. Nonetheless, disease screening, identification and antibiotic treatment need more explorations to shed light on insect disease management by insect breeders.

8.7. Conclusion

Therefore, the exposure of the diseased adults of *C. salviniae* to high temperatures first will not only reduce loads of *Helicosporidium* sp. but the costs of sourcing more ketoconazole antibiotic. High temperatures will reduce the replication of *Helicosporidium* sp. in *C. salviniae* and ketoconazole could be used as the supplementary treatment to eliminate cells of *Helicosporidium* sp. This sequence could ensure a reduction in the rate of transmission and spread of *Helicosporidium* sp. through the mature

cyst in both the mass rearing facilities and field sites of *S. molesta*. However, these interventions may only be beneficial to the colonies of *C. salviniae* occurring under shaded or low-temperature sites of *S. molesta*.

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