The biological control of *Egeria densa* Planch. (Hydrocharitaceae) in South Africa.

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

By

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"No organism is an ecological island"

(Huey *et al.* 2012)

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: Rosali Smith

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Abstract

Over the last thirty years, biological control, the use of host-specific natural enemies, has been a huge asset in the management exotic aquatic macrophytes such as *Pistia stratiotes* L. (Araceae), *Pontederia crassipes* Mart. (Solms) (Pontederiaceae), *Azolla filiculoides* Lam. (Azollaceae), *Salvinia molesta* D.S. Mitch (Salviniaceae) and *Myriophyllum aquaticum* (Vell.) Verdc. (Haloragaceae), also known as the "Big Bad Five" in South Africa. Despite these successes, freshwater ecosystems in South Africa have been harder to restore to an invasive macrophyte-free space, due to chronic disturbances such eutrophication, propagule dispersal and hydrological alterations. In the Anthropocene, where human activities have profound effects on their environment, these disturbances weakens ecological resilience and drive aquatic plant invasions. Due to long periods of invasions and the presence of a new suite of exotic aquatic plant propagules, native vegetation recolonization has been slow or even absent. Instead, the release of resources, such as sunlight, nutrient and space through aquatic weed management acts as a catalyst for secondary biological invasion.

New invasive aquatic weeds include submerged and rooted emergent growth types, with *Egeria densa* Planch. (Hydrocharitaceae) the most widely distributed submerged aquatic weed in South Africa. It can quickly form dense monoculture stands that have ecological, economic and social impacts. Because of its ability to regenerate from plant fragments with double nodes, mechanical control is inappropriate. Additionally, mechanical and chemical control not only affects *E. densa* but have significant non-target effects. In response to its rapid spread over the last 20 years, especially following floating invasive aquatic management, a biological control programme was initiated, and in 2018, the leaf-mining fly, *Hydrellia egeriae* Rodrigues (Diptera: Ephydridae) was released. This was the first release of a biological control agent against *E. densa* in the world, and the first agent released against a submerged aquatic weed in South Africa.

This thesis comprises the subsequent step of a biological control program when permission for the release of an agent have been obtained. A brief history of macrophyte invasions in South Africa's unique freshwater systems are given in the literature review. Contributing factors to secondary invasions within the context of ecological resilience are introduced. An argument for the benefit of biological control as nuisance control is given, especially because *E. densa* and its natural enemy, *H. egeriae* is the focus species of this thesis.

The main goal after permission for the release of an agent have been obtained, is to establish and build-up field populations. Research questions in this thesis aimed to investigate factors that contribute to or negate this goal. Through laboratory and field experiments we investigated the thermal physiology of the agent, and its climatic suitability to its novel range; different release strategies on field establishment and biotic resistance through the acquisition of novel parasitoids. Considering the longevity of this biological control program, we investigated the effects of elevated CO_2 on the interaction between *E. densa* and *H. egeriae* through open top chamber experiments.

Laboratory thermal physiology results showed that the agent is able to survive, develop and proliferate at all *E. densa* sites throughout the year. This is confirmed with the establishment of the agent at two release sites, the Nahoon River in the Eastern Cape Province and the Midmar Dam in KwaZulu-Natal. Post-release surveys showed that *H. egeriae* requires augmentative releases to sustain field populations. Without augmentative releases, *H. egeriae* herbivory levels were almost negligent. However, a contributing factor to low field-populations was parasitism. The biological control agent acquired three parasitoids, which have previously been described from *Hydrellia lagarosiphon* Deeming (Diptera: Ephydridae), a specific herbivore to *Lagarosiphon major* (Ridl.) Moss (Hydrocharitaceae).

These results provide information on the immediate establishment and effectiveness of the *H. egeriae*. Results from the elevated CO_2 study suggest that *E. densa* will become less nutritious through a shift in leaf C/N ratio, when ambient 800ppm is bubbled into experimental growth chambers. *Hydrellia egeriae* feeding was affected by ambient CO_2 levels and plant nutrient availability. The set levels of ambient CO_2 levels used in this experiment produced dissolved inorganic carbon levels that were lower than dissolved inorganic carbon levels in *E. densa* invaded sites. This suggests that, submerged aquatic plant-insect interactions may be harder to predict from only laboratory experiments. Further investigations are necessary to establish system-specific characteristics i.e. dissolved inorganic carbon and target plant nutritional quality.

The biological control of *E. densa* in South Africa is still in its infancy. This study presents results from post-release surveys up until two years after the agent was released. From this study, *Hydrellia egeriae* exhibits the potential to be an effective biological control agent, but release strategies should be adapted to sustain field populations and to limit field parasitism effects. Continued post-release surveys will provide a more comprehensive idea of the seasonal fluctuations of field-populations and parasitism. Surveys at multiple sites will provide information on potential site specific characteristics that contribute to or negate biological effort. Considering the high nutrient status of South African freshwater systems, a more holistic approach to *E. densa* management is necessary. This will require the strengthening of ecological resilience to prevent systems from shifting into an alternate invasive stable state. In addition, aquatic weed management needs to be addressed by a resilient social network, which ultimately calls for the strengthening of socio-ecological resilience.

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1. Literature review

Freshwater ecosystems occupy only 0.8% of the earth's surface area (Moorhouse & MacDonald 2015), and yet are highly biodiverse (containing 10% of world's species), with high levels of endemism compared to terrestrial and marine environments (Millennium Ecosystem Assessment 2005; Dudgeon *et al.* 2006; Strayer & Dudgeon 2010). These ecosystems fulfil important ecological, economic and social roles (Brauman *et al.* 2007; Pejchar & Mooney 2009; Geist 2011) that have been estimated at $6.5 trillion USD/year (Costanza *et al.* 1997). Despite this, an ever-increasing human population, pollution, overexploitation, habitat change, invasive alien species and climate change have placed freshwater ecosystems under tremendous pressure (Millennium Ecosystem Assessment 2005; Convention on Biological Diversity 2006; Brauman *et al.* 2007; Geist 2011). These disturbances affect species survival, distribution and diversity, which not only affects ecosystem functioning, but also resilience in a changing world (Chapin III *et al.* 2000). Holling (1973) defines *ecological resilience* as the ability of a system to absorb changes, maintain relationships and persist. This is in contrast to *stability*, defined as a characteristic of the system to return to a former state after a short period of disturbance (Holling 1973). The ability of freshwater systems to be easily manipulated has allowed humans to alter the flow of freshwater (Millennium Ecosystem Assessment 2005; Geist 2011). For example, the global number of large dams (>15 m high) has almost grown three-fold (~ 36 000) over the period 1900 to 2000 (International Commission on Large Dams 2005 in Strayer & Dudgeon 2010), which has also allowed an increase in the global freshwater withdrawals from ~ 1 000 to ~ 5 000km^3/y during the same period (Gleick 1993). This number continues to grow, with an estimated 58 000 large dams worldwide in 2020 (Mulligan *et al.* 2020).

1.1 Protecting freshwater resources

In the age of humankind or the Anthropocene, humans have a significant effect on the environment, which is exacerbated by an ever-increasing population. Global threats to freshwater ecosystems include overexploitation, pollution, land-use change, invasive alien species, flow regulation and climate change (Dudgeon 2019). Since the dawn of the 20th century, freshwater conservation has received increasing attention through a number of conventions, legislations and research (Strayer & Dudgeon 2010). For example, the initiation of the IUCN Red List, Clean Water Act (USA), and International Commission on Large Dams (Strayer & Dudgeon 2010).

In 1998, Carpenter and colleagues suggested that researchers broaden the scale of their freshwater studies to landscape level. Freshwater conservation can be challenging, because disturbances upstream and in surrounding areas must be managed (Dudgeon *et al.* 2006). For example, protected areas are terrestrial focussed, and Azevedo-Santos *et al.* (2019) suggested that freshwater protected areas must be created in Brazil, which not only include sections of rivers, but whole rivers and even basins. Not only is it necessary to protect key areas, but it is important to reconcile protection with social use. Such actions require communication between scientists, stakeholders and policy makers (Dudgeon *et al.* 2006).

Improved conservation and management of freshwater systems will require a better understanding of the factors that weaken ecosystem functioning and resilience (Brauman *et al.* 2007; Pelletier *et al.* 2020). For example, Angeler *et al.* (2014) proposed a model for freshwater management that is built from firstly measuring the vulnerabilities of the particular system. One of the factor that contribute to freshwater ecosystem vulnerability is pollution, as it changes freshwaters at the most fundamental level e.g. chemical properties, primary production, biogeochemical cycling and community structure (Strayer 2010; Phillips *et al.* 2016). Freshwater systems have been degraded by nitrogen (NH_3 and NO_3) and phosphorous

(P) pollution, i.e. eutrophication, due to industrialisation, intense agricultural activities and higher urban runoff in response (non-point and point inputs) to an ever-increasing human population (Carpenter *et al.* 1998, Millennium Ecosystem Assessment, 2005). The estimated accumulation of phosphorous in freshwater sediment was between 1.0 and 1.2 Tg/y^{-1} (1 teragram is equivalent to 1 million metric tons) pre-industrial and 1.0 and 3.1 Tg/y^{-1} at the beginning of the 21st century (Bennett *et al.* 2001). Between 1960 and 2005, inorganic nitrogen pollution increased by two to over tenfold, depending on anthropogenic activities in the area (Millennium Ecosystem Assessment 2005). Due to various feedbacks, this change in stoichiometry alters the organism composition and system functioning (Glibert *et al.* 2012, Maberly *et al.* 2020), and these interactions can either strengthen an attraction towards an alternate state, cause a regime shift or recurrent shifts between alternate states (Scheffer 2009; Phillips *et al.* 2016). For example, increases in nutrients benefit phytoplankton, acidification, and more frequent algal blooms that may create a toxic and anoxic environment (Anderson *et al.* 2002; Millennium Ecosystem Assessment 2005). Cyanobacterial toxins may reduce grazing zooplankton; e.g. *Daphnia* spp. Müller (Cladocera: Daphniidae), which has cascading effects in the food web, or may even result in fish kills (Paerl *et al.* 2001; Anderson 2002). Glibert *et al.* (2011) also found that anthropogenic mediated changes in the nitrogen to phosphorous stoichiometry in the San Francisco Bay Delta, USA favoured invasive species such as the zooplankton species *Limnoithona tetraspina* Zhang & Li (Cyclopoida: Cyclopettidae), the clam, *Corbula amurensis* Schrenck (Corbulidae), and a submerged aquatic plant, *Egeria densa* Planch. (Hydrocharitaceae). This shift in species organization caused a decline in more favourable native species, such as the zooplankton species, *Eurytemora affinis* Nordquist (Calanoida: Temoridae) and water column algae.

1.2 Ecological resilience

The idea of ecological resilience was introduced by Charles S. Elton in 1958, and although it focusses on colonization of invasive species, it also explains why systems shift into alternate states. Ecological resilience was defined as: "the natural processes or ecosystem properties that negatively influence invasion success." This meant that communities could either be susceptible to biological invasions or be resilient through its abiotic and biotic factors, which is a deterministic perspective (Elton 1958). Later, however, D'Antonio *et al.* (2001) introduced a more probabilistic perspective (Figure 1-1) whereby a successful shift to an alternate state is rather placed on a continuum of ecological resilience, and underlying processes and/or factors affect this process. Systems with low resilience can easily be invaded, and therefore propagule pressure is low. System resilience that are governed by abiotic factors, will require higher rates of propagule pressure for invaders to establish. However, propagule pressure will decrease if the invader is able to modify the abiotic conditions within the system. Similarly, when system resilience is governed by biotic factors, higher rates of propagule pressure is necessary to overcome resilience. Generally, biotic factors change over space and time, and windows of opportunity is created for propagule establishment (D' Antonio *et al.* 2001).

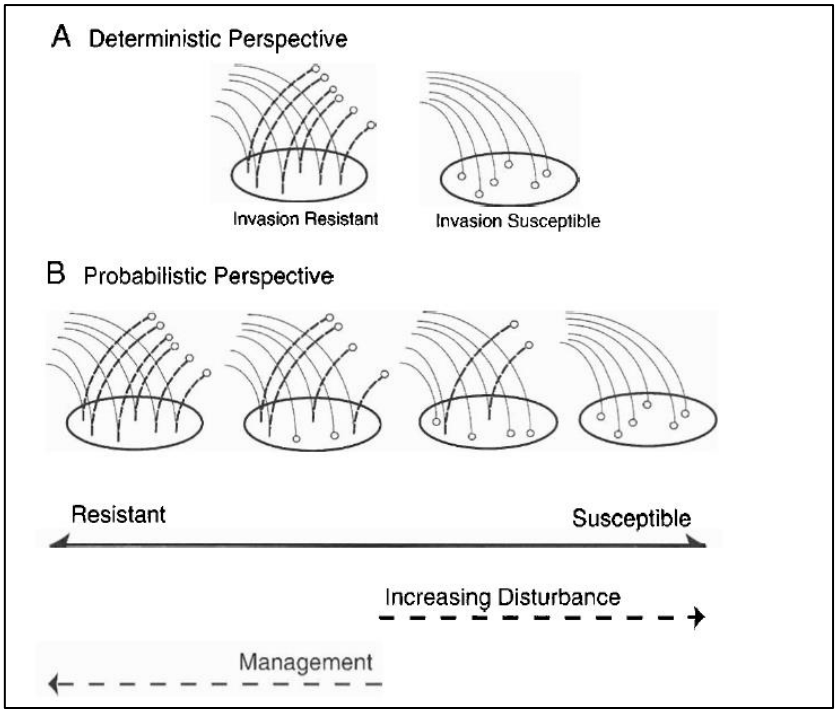


Figure 1-1: Deterministic (A) and probabilistic (B) perspective on biotic resistance (Taken from D'Antonio *et al.* 2001)

Thus, the resilience of a system will determine the amount of energy required to cause a change or regime shift. Regime shifts are non-linear shifts between stable states (i.e. system properties and processes) (Scheffer 2009). Five major threats to ecosystem processes and properties are acknowledged globally; overexploitation, pollution, habitat destruction, climate change and invasive species (Convention on Biological Diversity 2006). For the purpose of this study, I will only elaborate on two of these threats; pollution and biological invasions.

1.3 Freshwater biological invasions

Freshwater systems are particularly vulnerable to biological invasions and when invaded, experience severe negative effects compared to terrestrial and marine ecosystems (Moorhouse & Macdonald 2015). There are exceptions, where exotic species have had positive effects on systems, but these cases are in the minority, and positive only because initially the system was polluted, had low biodiversity and were dominated by algae (Hussner *et al.* 2017). Aquatic biological invasions can be attributed to numerous, sometimes unregulated primary

propagule vectors, secondary spread between water bodies and the difficulty in detecting the presence of exotic species before they become problematic (Maki & Galatowitsch 2004; Strayer 2010; Moorhouse & Macdonald 2015). For example, Nunes *et al.* (2015) found that the number of new alien species introductions in Europe increased almost tenfold between the years 1850 to 2009. Main introduction pathways include aquaculture, pet/aquarium trade, fishery, shipping, inland canals, ornamental planting and contaminated commodities. Due to overexploitation, pollution, flow modification and habitat disturbance, the resistance or resilience of freshwater systems is weakened, which increases their vulnerability to biological invasion (Moorhouse & Macdonald 2015). In such disturbed systems, the invasive species may either be a passenger (i.e. species will disappear when the disturbance is removed) or a back-seat driver (i.e. species needs a disturbance to establish, but will persist if the disturbance is removed) (MacDougall & Turkington 2005). Due to the easy spread of invasive species between and within continents, ecosystems lose their biological uniqueness, resulting biotic homogenization. Over time systems attain genetic, taxonomic and functional similarity (Lindholm *et al.* 2020).

1.4 Preventing biological invasions: Can systems defend themselves?

Naturally, ecosystems can resist biological invasions through abiotic (e.g. temperature, chemistry) and biotic properties and processes. Biotic resistance/resilience includes competition, predation, herbivory or disease (Levine *et al.* 2004). For example, in a field-based experiment, Carlsson *et al.* (2011) showed that over time, predation by the native *Callinectes sapidus* Rathbun (Decapoda: Portunidae) or blue crab, suppressed invasive zebra mussel populations in the Hudson River, USA. In a greenhouse experiment, Petruzzella *et al.* (2020) showed that macrophytes native to the Netherlands, are able to competitively reduce the establishment of the invasive species *Lagarosiphon major* (Ridl.) Moss. (Hydrocharitaceae),

and that resistance was enhanced by native community richness and functional similarity between native and invader species.

In a meta-analysis, Alofs & Jackson (2014) found freshwater communities were driven by herbivory/predation, rather than competition, and that consumptive resistance/resilience were strongest in lentic systems. Up until the 20th century, a large stream of aquatic ecologists dismissed the idea that herbivory on freshwater macrophytes was able to affect community composition (Lodge 1991). Since then, increasing research has been conducted in this field (e.g. Gross *et al.* 2001; Bakker *et al.* 2016; Grutters *et al.* 2016), indicating that herbivores significantly affect aquatic vegetation, including invasive alien plants. For example, in the USA, the specialist weevil, *Euhrychiopsis lecontei* Dietz (Coleoptera: Curculionidae) and the aquatic moth, *Acentria ephemerella* Denis and Schiffermuller (Lepidoptera: Crambidae) effectively reduce the biomass of the exotic submerged macrophyte, *Myriophyllum spicatum* L. (Haloragaceae) (Johnson *et al.* 2000).

1.5 Counting the cost of biological invasions

The invasion process is divided into four stages; introduction, establishment, spread and impact (Levine *et al.* 2004; Blackburn *et al.* 2011). When invasive species establish and spread in freshwater systems, the ecological and economic costs are high (Pimentel *et al.* 2005), and often indirect (Moorhouse & Macdonald 2015). For example, recreational losses due to one invasive aquatic species, *H. verticillata,* amounted to $10 million per year in only two lakes in Florida, USA. Consequently, management costs of invasive species are extremely high (Pimentel *et al.* 2005). Controlling *H. verticillata* in Florida cost $14.5 million per year (Center *et al.* 1997). In South Africa, the economic loss to evapotranspiration caused by *Pontederia crassipes* Mart. (Solms) (Pontederiaceae), hereafter water hyacinth, in an agricultural community was calculated by Arp *et al.* (2017). At an ET: EW ratio (evapotranspiration

relative to “normal”, open water evaporation) of 1.38, the annual net opportunity cost loss was estimated at \$3.7 million/m^3, \$7.5 million/m^3 and \$15 million/m^3 (exchange rate January 2020) at weed coverages of 25%, 50% and 100%, respectively. Furthermore, controlling water hyacinth cost between \$22 to \$226 per hectare between 1977 and 2001 (van Wyk & van Wilgen 2002).

1.6 Freshwater herbivory: a tool for nuisance control

In addition to system resilience, human intervention is also necessary to prevent invasive species establishment or to eradicate small populations before they spread and become problematic (Figure 1-2; Hussner *et al.* 2017). Although campaigns have been successful in eradicating invasive species, some freshwater invasions are often only observed when they have already become problematic, especially for submerged species. In such cases, nuisance control is necessary.

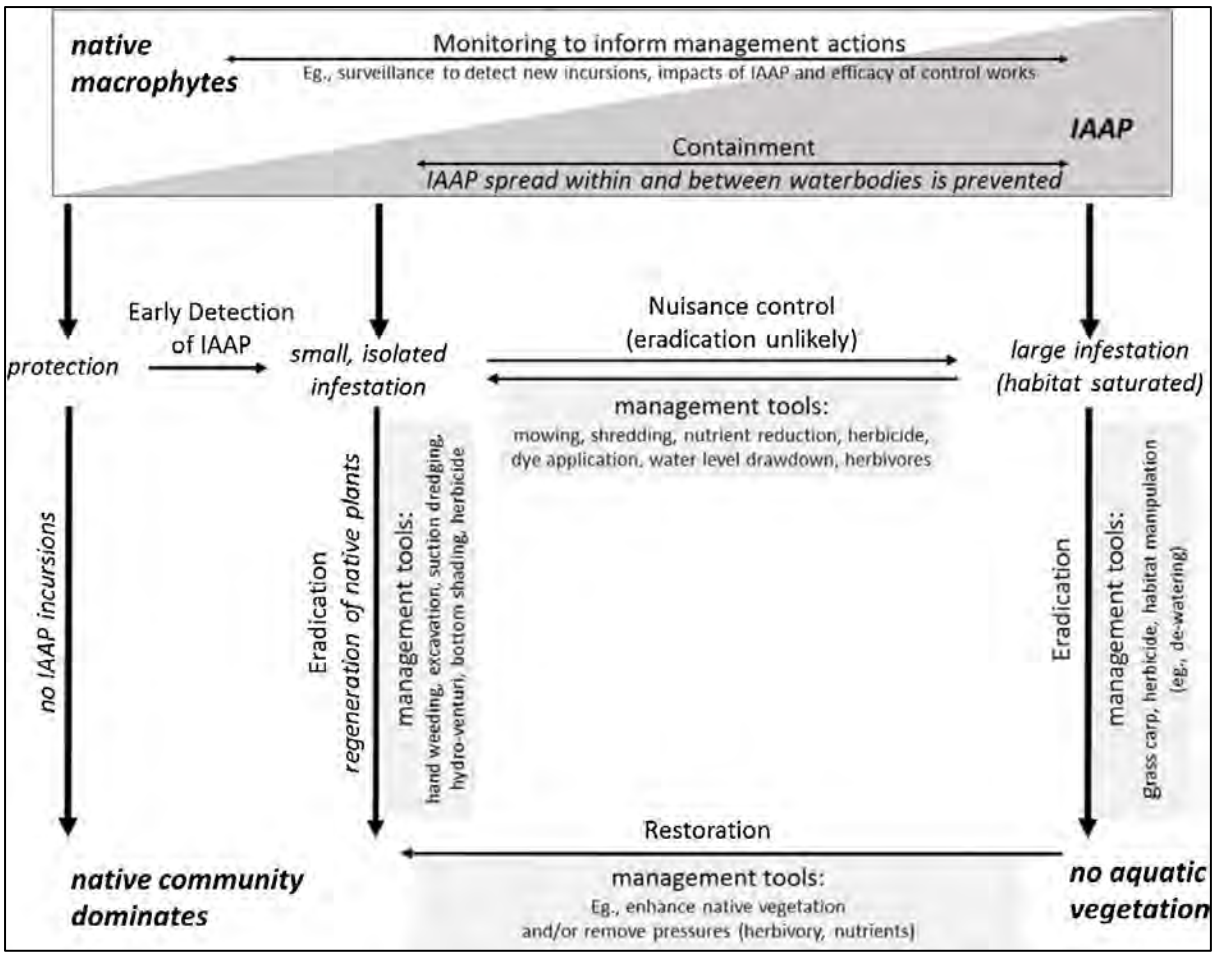


Figure 1-2. Invasive aquatic weed management framework (taken from Hussner et al. 2017).

Non-native plants often escape their natural enemies, a hypothesis that was coined the enemy-release hypothesis (ERH), which allow exotic species to be more competitive in their introduced range (Schulz *et al.* 2019). This is supported by studies that have found higher plant damage in native regions (Halbritter *et al.* 2012), and proportionally more specialist herbivores that feed on reproductive structures (Liu & Stiling 2006). The ERH is the premise for the practise of biological control, which aims to restore top-down pressure, using these specialist herbivores (Julien & White 1997; Schulz *et al.* 2019). Specialists are favourable because they express host use conservatism, which reduces non-target risk in the introduced range (Bernays & Graham 1988).

1.7 Biological control: is it safe?

Classical biological control, the intentional introduction of exotic organisms to manage invasive plant species (Julien & White 1997) has actively been practised for less than 200 years (Hinz *et al.* 2019). There are several steps that lead up to the release of a biological control agent. Initially it is important to determine the identity (genetic, taxonomic, phylogenetic) and native range of the target weed. Subsequently, native range surveys are conducted to look for potential biological control agents (Moran *et al.* 2013). During these surveys, agents are prioritized according to their host range and herbivory mode. Promising agents are then imported into the country of interest, and quarantine host-specificity testing is conducted with a number of phylogenetically related non-target native species (Moran *et al.* 2013) An application for the release of the agent is prepared, if the potential agent has exhibited host-specificity (Moran *et al.* 2013).

Due to safety concerns, specifically attack to non-target species, host-specificity methodology is continually refined in accordance with new information and technology (Hinz *et al.* 2019; Paynter *et al.* 2020). Up until 2016, some 1 555 intentional releases of 493 biological control agents have been made (Winston *et al.* 2014; Hinz *et al.* 2019). From the biological control agents released between 1991 to 2016, only two caused sustained non-target attack, meaning that the agent feed and develop on a non-target species despite the availability of the target weed (Hinz *et al.* 2020). Pre-release assessments can accurately predict the possibility of direct non-target effects if the non-target species is included in the test plant list (Hinz *et al.* 2019).

1.8 Biological control of invasive aquatic weeds

Native range surveys for specialist herbivores (Keane & Crawley 2002) against aquatic invasive weeds such as *Alternanthera philoxeroides* (von Martinus) Grisebach (Amaranthaceae) (alligator weed) and water hyacinth started in 1959 (Vail *et al.* 2001) and for

the submerged aquatic weed, *Hydrilla verticillata* L.f (Royle) (Hydrocharitaceae) in the 1970s (Purcell *et al.* 2019). Since then, 74 biological control agents have been released against 12 aquatic invasive plants globally (Winston *et al.* 2014; Hill *et al.* 2020). One of these biological control programmes has become an iconic example of the effectiveness of biological control. *Stenopelmus rufinasus* Gyllenhal (Coleoptera: Curculionidae), a frond feeding weevil, was intentionally released in South Africa and Zimbabwe against *Azolla filiculoides* Lam. (Azollaceae) or red water fern (Winston *et al.* 2014). The weevil heavily impacts its host plant and has shown the ability to clear hectares of covered water bodies within three years to such an extent that the plant is no longer regarded as problematic (McConnachie *et al.* 2003; Hill & Coetzee 2017).

1.9 **Invasive treadmill**

Although biological control, and other management options, has been successful in controlling invasive species (Hussner *et al.* 2017; Hinz *et al.* 2019), some biological invasions are a symptom of initial ecosystem degradation rather than a cause (MacDougall & Turkington 2005). In such cases, where invasion is a passenger or back-seat driver, ecosystem rehabilitation is more complex, despite the mode of invasive species management (Scheffer 2009). Before biological control of aquatic weeds was practised in the USA, water hyacinth and alligator weed, were initially managed with herbicides. However, alligator weed became resistant to herbicides, and the flea beetle, *Agasicles hydrophila* Selman and Vogt. (Coleoptera: Chrysomelidae) was introduced in 1965 as a biological control agent. Following high levels of host plant damage and diminished alligator weed populations, clear waters were replaced with water hyacinth (Sailer 1972). The threat of secondary invasions and position thereof in ecosystem rehabilitation was explained in this statement in 1972 by the late Professor Reece Sailer, a pioneering biological control practitioner and entomologist in the USA:

> "Rather, the significant progress is in the evidence that the aquatic ecosystem containing alligator weed has been modified in the direction of increased diversity and improved balance between the producer and the consumer organisms. The fact that this gain has to some extent been offset by an increased water hyacinth problem should not be regarded as a setback. In a very real sense the research on biological control of aquatic weeds is a problem in environmental engineering at the ecosystem level. While we cannot expect quick results, we can, with some confidence, look forward to the day when both alligator weed and water hyacinth will be normal, even desirable elements of the aquatic plant community."

1.10 **The practice of biological control**

The goal of biological control is to suppress the invasive weed, and reduce its ecological, economic and social impact (Cruttwell McFayden 1998; Morin *et al.* 2009; Hill & Coetzee 2017; Schwarzländer *et al.* 2018; Hoffmann *et al.* 2019). Carson *et al.* (2008) stated that: "the emphasis of biological control assessment is usually at the individual or population level, but community and ecosystem level responses arguably provide the ultimate measure of biological control success in most natural systems." The anticipation is that a reduction in the target weed population, will allow an increase in native species growth, species diversity and an improvement of ecosystem integrity (Denslow & D'Antonio 2005; Carson *et al.* 2008). However, a review of biological control post-release evaluations showed that more than half of the plants that grew after successful biological control suppression were exotic species (Clewley *et al.* 2012), and in some cases, no recolonization of any species occurred (Reid *et al.* 2009).

To establish if biological control is reducing the target weed at a population-level, and to monitor any response of the recipient community, post-release evaluations are required (Schroeder 1983; Cruttwell McFayden 1998; Blossey & Skinner 2000; Fowler *et al.* 2000; Morin *et al.* 2009). Information from post-release evaluations is particularly important to elucidate biological control success or failures, improve biological control agent predictability

and effectiveness and motivate future funding (Syrett *et al.* 2000; Paynter *et al.* 2018). Conservation "cause" and "effect" monitoring is also important for adaptive management (Strayer & Dudgeon 2010; Jones *et al.* 2018).

Post-release evaluations are often a neglected component of biological control programs, due to limited resources (e.g. finance, personnel) (Cruttwell McFayden 1998; Blossey & Skinner 2000; Madsen & Wersal 2017), and although some post-release evaluations have been conducted, biological control practitioners have been criticised for the quality of information obtained, which is often observational and qualitative (Cruttwell McFayden 1998; Morin *et al.* 2009; Havens *et al.* 2019; Hinz *et al.* 2019). This has led to an increase in empirical post-release evaluations over the last decade (Syrett *et al.* 2000; Schaffner *et al.* 2020).

1.11 Post-release evaluations: A general framework

1.11.1 Context-specific goals

The general consensus is that biological control agents should affect the target weed at a population-level and ultimately push the trajectory of ecosystem rehabilitation into a positive direction (Hoffmann *et al.* 2019). Systematic post-release evaluations will require biological control practitioners to set context-dependent goals *a priori*, which will guide fine-tuned methodology based on the life history of the biological control agent and the target weed (Blossey & Skinner 2000; Fowler *et al.* 2000). Post-release evaluations require years of investment (Martin *et al.* 2018; Hinz *et al.* 2019) and setting *a priori* goals will not only provide programme benchmarks, but will prevent confusion between agent success rates and success as a whole (Cruttwell McFayden 1998).

1.11.2 Field-based post-release surveys

Post-release evaluation focusses on 1) agent establishment and population build-up, 2) the target weed population and 3) the response of the recipient community. Therefore, agent

establishment is a prerequisite for a biological control programme to be successful. By 2012, 1 555 biological control agents had been released against 175 invasive alien plants, of which 63.2% have established (Schwarzländer *et al.* 2018). Of these releases, impact analyses were conducted for only 60.4% of the agents (Schwarzländer *et al.* 2018). Factors that affect the successful establishment of a biological control agent into its new environment include 1) propagule pressure (i.e. release strategy), 2) climate compatibility and, 3) parasitism and/or predation (Stiling 1993; Lockwood *et al.* 2005; Fauvergue *et al.* 2012)

The effect of a biological control agent is generally categorized into direct and indirect effects (Denslow & D'Antonio 2005). Direct effects measure the impact of the biological control agent on the target weed at an individual or population level (Denslow & D'Antonio 2005) which is also the focus of the success levels proposed by Hoffmann *et al.* (2019). Plant density, biomass, seeds (reproductive structures) and percent area cover are used as parameters of population size (Hoffmann *et al.* 2019). To sufficiently identify and quantify population-level effects will require some baseline information on the invaded habitat (Morin *et al.* 2009). Examples of empirical post-release evaluations include exclusion experiments, where the target weed population growth is measured with and without its biological control agent (Tipping & Center 2002; Jones *et al.* 2018) or comparing release and control sites (Morin *et al.* 2009; Havens *et al.* 2019). However, control sites may be difficult to maintain if the biological control agent is effectively reducing the target weed population and disperses to new sites (Morin *et al.* 2009).

Another direct effect includes non-target effects, whereby biological control agents feed on non-target species. This is not an active part of post-release surveys (Crutwell McFayden 1998), and is in most cases evaluated after apparent non-target attack (Havens *et al.* 2018). Biological control practitioners invest a lot of time into risk assessments, and establishing if biological control agents use any non-target species will improve agent

predictability and strengthen the reliability of the science (Hinz *et al.* 2019). For example, post-release experiments on *Mogulones crucifer* Pallas (Coleoptera: Curculionidae), a biological control agent for *Cynoglossum officinale* L. (Boraginaceae), showed that non-target attack on *Hackelia micrantha* (Eastw.) J.L. Gentry (Boraginaceae) in Canada was predicted from host-specificity testing (Catton *et al.* 2015).

Indirect effects measure the response of the surrounding biotic community to the presence of the biological control agent or a reduction of the target weed (Morin *et al.* 2009). These include the growth of native or exotic species (Denslow and D'Antonio 2005), biotic interference, i.e. parasitism or predation (Goeden & Louda, 1976; Stiling & Cornelisson 1993) or changes in food web structures (Reid *et al.* 2009; Hinz *et al.* 2019). For example, Butler and Wacker (2010) found that biological control reduced leafy spurge, *Euphorbia esula* L. (Euphorbiaceae), by 80 to 90% compared to control plots over a nine year period in Montana, USA. During this time, resident plants increased their coverage by 72 to 88%, however, comparisons between treatment and control plots showed no difference in functional groups.

1.12 Successful biological control of aquatic weeds in South Africa

South Africa's freshwater systems have been threatened by invasive aquatic alien plants since the early 1900s. Up until the early 2000s, five aquatic alien plants, also referred to as the "Big Bad Five" have had major impacts on the functioning, integrity, and ecosystem services of freshwater systems across South Africa. These include the world's worst aquatic weed, water hyacinth, as well as *Pistia stratiotes* L. (Araceae), *Salvinia molesta* D.S. Mitch (Salviniaceae), *A. filiculoides* and the emergent *Myriophyllum aquaticum* (Vell.) Verdc. (Haloragaceae) (Coetzee *et al.* 2011a). The first biological control agent released against an aquatic weed in South Africa was in 1974 (Moran *et al.* 2013), and since then biological control has drastically reduced the density of aquatic weeds across the country. These successes have also translated

into major environmental and socio-economic impact reductions (Hill & Coetzee 2017). For example, the total impact score (generic impact scoring system according to Nentwig *et al.* 2016) of water hyacinth has almost been halved from 43 to 23, *P. stratiotes* has been reduced from 38 to 6, *A. filiculoides* from 40 to 0 and *M. aquaticum* from 38 to 15 (Hill & Coetzee 2017). Furthermore, post-release surveys for *S. molesta* over a ten-year period showed that its biological control agent, *Cyrtobagous salviniae* Calder and Sands (Coleoptera: Curculionidae) has established at all sites, of which a third of the sites are no longer threatened by the weed. Overall, *C. salviniae* has reduced the mean *S. molesta* cover from between 51 and 100% to 0 and 5% (Martin *et al.* 2018).

1.13 Shifting between invasive stable states

Most of South Africa's freshwater systems are eutrophic (Coetzee & Hill 2012), with nutrient input from farming activities, herbicides, failing infrastructure and a high phosphate standard (1 mg l^{-1}) for water treatments (van Ginkel 2011). Despite the management of the "Big Bad Five" aquatic weeds, eutrophication has been counteractive in ecosystem rehabilitation (Coetzee *et al.* 2011a; Hill & Coetzee 2017). For example, Strange *et al.* (2018) found that biological control acts as a catalyst in a shift between invasive stable states. The theory of alternative stable states refers to the properties and processes of an ecosystem that constitute a certain "stable state", for example a clear lake (Scheffer 2003). However, systems may experience internal feedback or external pertubations that push a system over a threshold, resulting in a regime shift (Scheffer 2003). Thus, invasive stable state refers to a system that has been invaded, its properties and processes are altered due to an invasion and it is in a state of long-term invasion (Strange *et al*. 2019). In South Africa, top-down control of floating invasive plants increases sunlight penetration and nutrient availability, either through plant decomposition or less competition. This allows other exotic species to quickly fill these open niches, i.e. secondary invasion. This is seen by the invasion of a new suite of exotic aquatic

weeds, which includes *Nymphaea mexicana* Zucc. (Nymphaeaceae), *Iris pseudacorus* L. (Iridaceae), *Sagittaria platyphylla* (Engelm.) J.G. Sm. (Alismataceae) and *Egeria densa* (Hill & Coetzee 2017). In some systems, a regime shift from a floating aquatic weed stable state to a submerged aquatic weed stable state has occurred (Strange *et al.* 2018; Strange *et al.* 2019).

1.14 ***Egeria densa***

One of these new invader species, and one of the worst submerged aquatic weeds in South Africa, is *Egeria densa*, commonly known as Brazilian waterweed or Brazilian elodea (Cook & Urmi-König 1984). It is a rooted submerged macrophyte that prefers slow-moving water (Yarrow *et al.* 2009), and although nutrient-rich waters can increase growth, inorganic carbon limits growth and distribution (de Freitas & Thomaz 2011).

1.14.1 Distribution

Egeria densa is native to the subtropical regions of Brazil, and temperate and subtropical regions of Argentina and Uruguay (Cabrera Walsh *et al.* 2013). Winter and summer minimum temperatures for freshwater systems in the Buenos Aires are 0°C and 18°C, respectively (Cabrera Walsh pers. comm.). From its native range, *E. densa* was distributed around the world in the 1800's and 1900s as an "oxygenator" plant for rearing fish, as biological control agents for mosquito larvae (Cook & Urmi-König 1984). It was also introduced into South Africa for this reason between 1827 and 1915 (Cook & Urmi-König 1984), and was first recorded as established in the KwaZulu-Natal province in 1963. Genetic analysis shows that *E. densa* was introduced from both Malaysia and the USA (Mangan, unpubl. data). Its current distribution is along the south-eastern coast and invaded freshwater bodies have been recorded from the temperate Western Cape and subtropical KwaZulu-Natal (Figure 1-3).

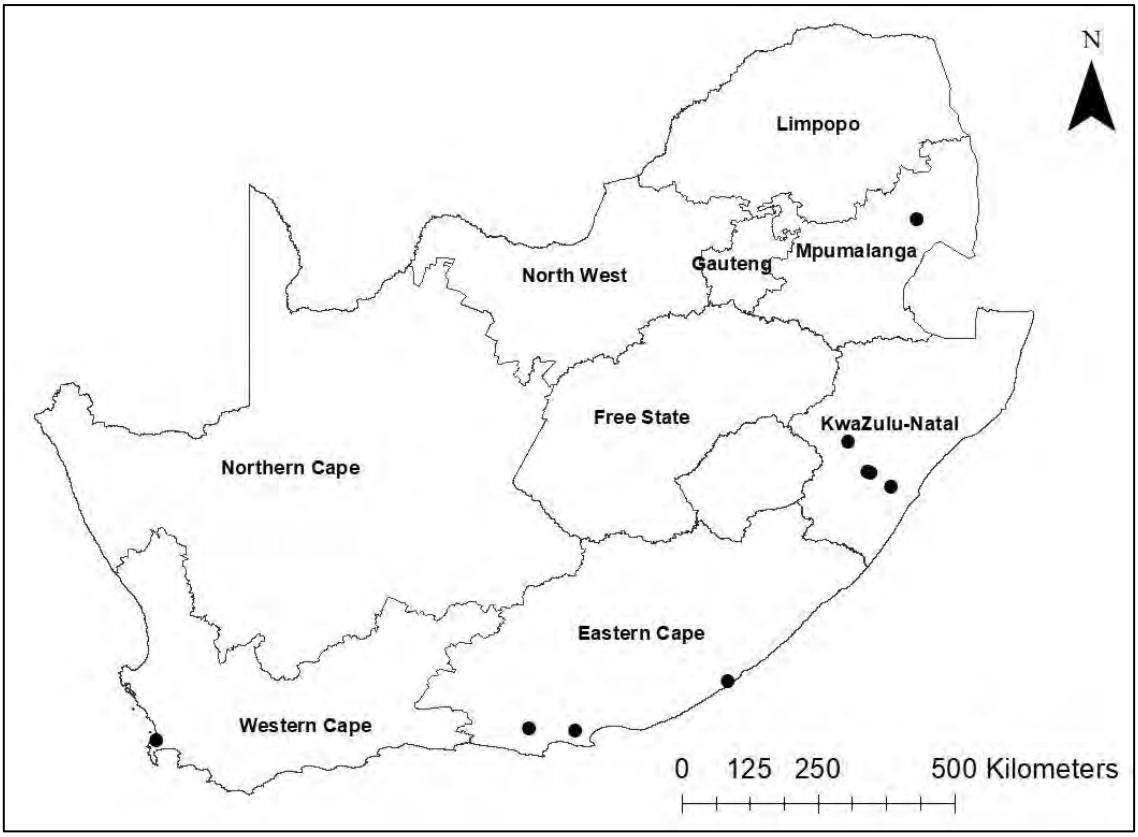


Figure 1-3: Distribution of *Egeria densa* (black dots) in South Africa.

1.14.2 Description

Stems are elongated, between 1 to 3mm in diameter, irregularly branched (Cook & Urmi-König 1984) and can grow until the water surface is reached (Yarrow *et al.* 2009). Leaves are 10 to 44mm in length and 1.5 to 4.5mm, generally grouped in four leaves per whorl (Figure 1-4) (Cook & Urmi-König 1984). Leaves form a crown at the shoot tip and can be densely packed around the stem (Gettys *et al.* 2014). Double nodes generally have eight to ten leaves, can either be reproductive, bearing flower, lateral branch or root buds or it can be sterile (Yarrow *et al.* 2009). *Egeria* spp. leaves contain idioblasts (colourless cells) (Cook & Urmi-König 1984), but the content of these idioblasts (e.g. phenolic compounds, alkaloids, fatty acids and lipids) has yet to be confirmed (Hara *et al.* 2015). Only male plants occur in South Africa. Spathes (borne on double internodes) bear 2 to 5 flowers (Yarrow *et al.* 2009). The white flowers have 3 petals, are 18 to 25mm in size and borne up to 30mm above the water surface on a pedicle (Yarrow *et al.* 2009). Flowers open above the water surface, and close if

submersed, locking an air bubble to keep the stamens and sepals dry (Yarrow *et al.* 2009). Stamens are yellow and usually carried in whorls of three (Cook & Urmi-König 1984). Flowers are developed continually in warm conditions (15 to 25°C) when light and nutrients are not limited (Cook & Urmi-König 1984).

Compared to *H. verticillata*, a highly invasive submerged aquatic weed elsewhere (Hussner *et al.* 2017) and recorded from one site in South Africa (Coetzee *et al.* 2011b), *E. densa* does not produce overwintering organs such as tubers or turions. Root crowns and double nodes that survive winter initiate growth in warmer temperatures (Getsinger & Dillon 1984). In warmer regions, winter temperatures reduce *E. densa* growth rather than producing dormancy (Getsinger & Dillon 1984).

1.14.3 Environments

Egeria densa grows optimally in temperatures ranging from 16°C to 25°C (Cook & Urmi-König 1984). Temperatures above 30°C place the plant under stress. Similarly, *E. densa* prefers attenuated light and light intensities above 30 000 μW cm^{-2} also place the plant under stress (Getsinger & Dillon 1984). The species has been used as a model plant to assess water quality, heavy metal accumulation and toxicity, and metabolism of pesticides in plants (Malec *et al.* 2009). *Egeria densa* has a high capacity to absorb nutrients such as ammonium and phosphorus from water, (Feijoó *et al.* 2002) but decomposes quickly because of its low fibre content, which mobilizes sediment phosphorous in water (Barko & Smart 1980).

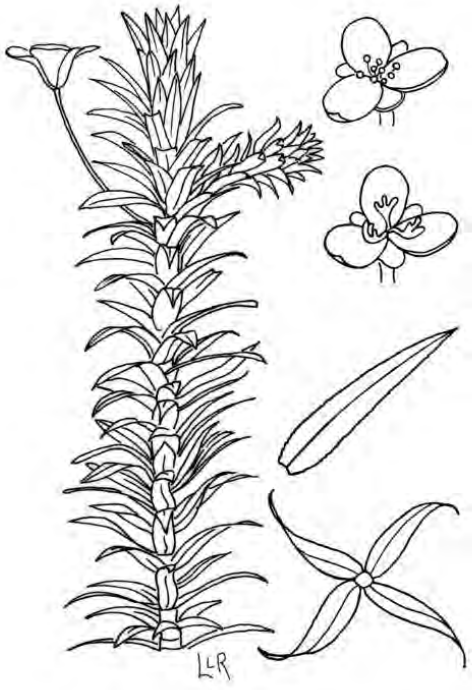


Figure 1-4: Line drawing of *Egeria densa* (Line drawing provided by University of Florida/IFAS Centre for Aquatic and Invasive Plants. Used with permission).

1.14.4 Useful attributes

Egeria densa is an oxygenator that is widely used as an aquarium plant (Martin & Coetzee 2011). Due to its simple anatomy, it is also regularly used as a study plant in biology classes (Yarrow *et al.* 2009). As an "ecosystem engineer", *E. densa* may be used to stabilize suspended particles and reduce water turbidity (Yarrow *et al.* 2009; Flood *et al.* 2020). It has the ability to absorb ammonium and phosphorous from the water column, which may reduce nutrient loading (Feijoó *et al.* 2002), and thus have value in aquaculture (Corpron & Armstrong 1983) and sewage treatments (Zhen *et al.* 2015). *Egeria densa* also has allelopathic effects on the growth of the blue-green algae *Anabaena flos-aquae* (Lyngb.) Breb. (Nostocaceae) and *Mycrocystis aeruginosa* (Kutzing) Lemm (Mycrocystacceae) and may reduce cyanobacteria blooms (Janse van Vuuren *et al.* 2006). Species that alter resource availability and ecosystem processes and functioning are seen as ecosystem engineers. This excludes species that either consume or provide food resources. (Emery Butcher *et al.* 2020).

1.14.5 Impact

Many of the systems invaded by *E. densa* in South Africa are of economic and social importance. Because of its reproductive mode and fast growth rate, estimated at 1.2cm per day (Gettys *et al.* 2014), *E. densa* can quickly crowd out surrounding aquatic plants. Experiments conducted by Strange (2017) showed that *E. densa* has a relative growth rate that is three times higher than indigenous *L. major* in South Africa, irrespective of initial planting densities. Recreational properties of waterbodies are also lost in its presence, as dense stands prohibit swimming and boating (DiTomaso *et al.* 2013). For example, the Midmar Dam, KwaZulu-Natal Province, provides water for nearly half of the Province's population. Additionally, the world's largest open water swimming tournament is hosted at this dam every year (Centre for Water Resources Research 2019). Vundla *et al.* (2017) projected that, if left unmanaged, *E. densa* could occupy approximately 46% of the Midmar Dam by 2041 (surface area 1 880ha). If only 15% of the weed is cleared every year, the predicted infestation will decrease to 12% of the water body. However, *E. densa* management in the Midmar reservoir should not exceed $55.4 million (January 2017) over a period of 30 years, otherwise any economic or recreational benefits will be lost (Vundla *et al.* 2017).

Egeria densa can reduce water flow and alter nutrient availability, light penetration (Dillon *et al.* 1988). Reduced wind induced water mixing increases surface temperatures, which affect other freshwater species (Gettys *et al.* 2014). Hill & Coetzee (2017) found that, when comparing the environmental and socio-economic impact of eight water weeds in South Africa, *E. densa* had the third highest impact score, next to water hyacinth and *H. verticillata.* According to the National Environmental Management of Biodiversity Act (NEMBA) (Act 10 of 2004, amended 2014) in South Africa, *E. densa* is a declared Category 1b weed.

1.14.6 Biological control

Only a few potential biological control agents are available for *E. densa* management. Grass carp, *Ctenopharyngodon idella* Val. (Cyprinidae) have been used to control *E. densa* in the USA, but effectiveness may be limited in colder regions (Gettys *et al.* 2014). Grass carp feed on submerged plants, but due to their ability to remove native species and whole macrophyte communities, it is not seen as a safe control option (Cuda *et al.* 2008). A bioherbicide was also developed using *Fusarium graminearum* (Shwein.) Petch (Hypocreales: Nectriaceae) in South America but has not been used on larger scales yet (Borges *et al.* 2004, Cabrera Walsh *et al.* 2013). During native range surveys in Argentina, Cabrera Walsh *et al.* (2013) found seven herbivores feeding on *E. densa*, of which the leaf-mining fly, *Hydrellia egeriae* Rodrigues (Diptera: Ephydridae) is the only specialist herbivore. Two other herbivores, *Hydrellia* sp. and *Cornops* sp. (Orthoptera: Acrididae) had unknown host ranges, and four species were polyphagous.

Hydrellia egeriae was imported into South Africa in 2014 under permit (P0063110) from the Exotic and Invasive Weeds Research (EIW) facility of the Agricultural Research Service in California, USA to the Rhodes University Quarantine Facility in South Africa. The founder culture was initiated in May 2013 from one shipment that contained individuals from four different populations in Argentina (John Herr, pers. comm.; Guillermo Cabrera Walsh pers. comm.). Quarantine host-specificity testing in showed that the leaf-mining fly is specific and damaging and it was first released in South Africa in late 2018 (Smith *et al.* 2019). The fly has a similar biology to *Hydrellia pakistanae* Deonier and *H. balciunasi* Bock (Diptera: Ephydridae), which were released as biological control agents for *H. verticillata* in the USA. Female *H. egeriae* oviposit eggs on protruding leaves and after eclosion, the fully aquatic larvae mine the photosynthetic tissue of the leaves (Figure 1-5). Larvae undergo three feeding instars, during which they mine approximately 24.5 leaves (Figure 1-6), followed by a fourth

non-feeding pre-pupa stage and pupation within the last leaf mined (Figure 1-7). Adults emerge and float the water surface in an air bubble (Figure 1-8). They feed on dead or trapped insects on the water surface, pollen and sugary substances (Deonier 1971). *Hydrellia egeriae* is the first biological control agent released against a submerged aquatic weed in South Africa, and the first invertebrate released against *E. densa* in the world (Hussner *et al.* 2017; Smith *et al.* 2019).

Figure 1-5: *Hydrellia egeriae* eggs oviposited on an *Egeria densa* leaf and neonate instars mining the photosynthetic tissue of the leaf (Source: Rosali Smith)

Figure 1-6: Second instar of *Hydrellia egeriae* within an *Egeria densa* leaf (Source: Rosali Smith)

Figure 1-7: Pupating *Hydrellia egeriae* within a pupariate (Source: Rosali Smith)

Figure 1-8: *Hydrellia egeriae* adult resting on an *Egeria densa* leaf (Photograph: Esther Mostert)

1.15 Thesis outlines and aims

The aim of this thesis is to further develop the biocontrol programme against *E. densa* in South Africa following permission to release *H. egeriae*. The first objective of this study was to develop a release strategy to optimize *H. egeriae* establishment in the field. To optimize and coordinate releases, we explored the thermal tolerance of the agent and calculated its accumulated degree-days as a predictor of the number of generations that *H. egeriae* will be able to complete at invaded sites in South Africa (Chapter 2; Coetzee *et al.* 2007). We expect that *H. egeriae* will be able to complete multiple generations annually at all sites, with slow development in colder seasons (Cabrera Walsh *et al.* 2013).

To establishthe direct impact of *H. egeriae* on its target weed at population-level, we conducted monthly and seasonal post-release surveys (Chapter 3). The response of the surrounding plant community to *H. egeriae* mining was also monitored and compared to pre-

release plant communities. We anticipate that target weed suppression will depend on field agent population size, and that herbivory will have to accumulate before noticeable effects are seen on *E. densa* populations.

Target weed suppression may be hindered by parasitism. Native *Hydrellia* spp. have their own suite of parasitoids (Martin *et al.* 2013), and thus the host range expansion of native parasitoids to include *H. egeriae* was explored (Chapter 4). Considering previous experience with *Hydrellia* spp. biological control agents and parasitism (Coon *et al.* 2014), as well as the presence of native parasitoids, we hypothesize that *H. egeriae* will acquire parasitoids.

Increasing CO_2 levels and temperatures have a direct effect on plant-herbivore interactions (Robinson *et al.* 2012). A general observation of elevated CO_2 is an increase in the C/N ratio of the plant, and a reduction in the host plant nutritional quality (Stiling & Cornelissen 2007), although Ryan *et al.* (2010) showed that this is context dependent. We specifically explored if increasing CO_2 levels would reduce the nutritional quality of *E. densa* for its biological control agent (Chapter 5).

Taken together, this study provides recommendations for effective *H. egeriae* release strategies. Biotic and abiotic factors that strengthen or weaken the effective control of *E. densa* in South Africa are highlighted. The long-term success of this programme is discussed and recommendations made.

2. Thermal physiology of *Hydrellia egeriae*

2.1 Introduction

Biological control programmes comprise of various stages (Moran *et al.* 2013). Each stage is carefully executed (Marohasy 1998; van Driesche & Murray 2004), individually adding to the knowledge basis of the programme, which allows for informative decision-making and, ultimately *biological control success* (Moran *et al.* 2013; defined in chapter 1). Of the 1 555 biological control agents that were given permission for release by 2012, 63.2% successfully established in their introduced range (Schwarzländer *et al.* 2018). When an agent is released, it is subjected to the same processes or filters as invasive species (Schulz *et al.* 2019). It must be able to establish a self-sustaining population (Robertson *et al.* 2008) that reaches sufficient densities to inflict target weed population-level damage (Schwarzländer *et al.* 2018, Hoffmann *et al.* 2019).

One of these filters that introduced populations have to overcome is different local climate, such as temperature, humidity and precipitation extremes, which is also a primary driver of organism development and survival (Campbell *et al.* 1974; Angiletta *et al.* 2002). Climatic mismatches have occurred in some biological control programmes, where the distribution, i.e. climate envelopes, between host plant and the biological control agent do no completely overlap (Harms *et al.* 2021). This has been especially true for biological control agents that have been sourced from tropical environments and introduced into temperature and subtropical environments. In such cases the biological control agent lack lower climate thresholds (Harms *et al.* 2021). To increase the overlap of host plant and biological control agent climate envelopes in the invaded range and prevent resource waste (van Wilgen *et al.* 2004; Paynter *et al.* 2015), biological control practitioners use climatic factors to guide native range surveys (Byrne *et al.* 2004; Sutton 2019). This is done using ecological niche modelling,

which is a correlative statistical approach, using available species occurrence and climate data (Figure 2-1; Kearney & Porter 2009), where a target weed's known distribution in its native and introduced range can be used to narrow down survey hotspots for potential agents (Sutton 2019). Biological control agent establishment is often limited, not by mean site climatic conditions, but by climatic extremes. Thus, native range surveys often focus on collecting biological control agents from sites that have, for example lower temperature extremes (Harms *et al.* 2021).

An agent's distribution in its native and/or introduced range, can be used to predict potential establishment at new sites (Julien *et al.* 1995; Byrne *et al.* 2004; Hoelmer & Kirk 2005; Robertson *et al.* 2008, Stratman *et al.* 2014). For example, Day *et al.* (2016) predicted the climate suitability in Australia for the gall fly *Cecidochares connexa* (Diptera: Tephritidae), a biological control of *Chromolaena odorata*. Using CLIMEX and the known distributions of the agent in its native and introduced range, Day and colleagues concluded that areas invaded by *C. odorata* along the coast close to Cairns, northern Queensland, were climatically suitable, but climatic suitability decreased further inland.

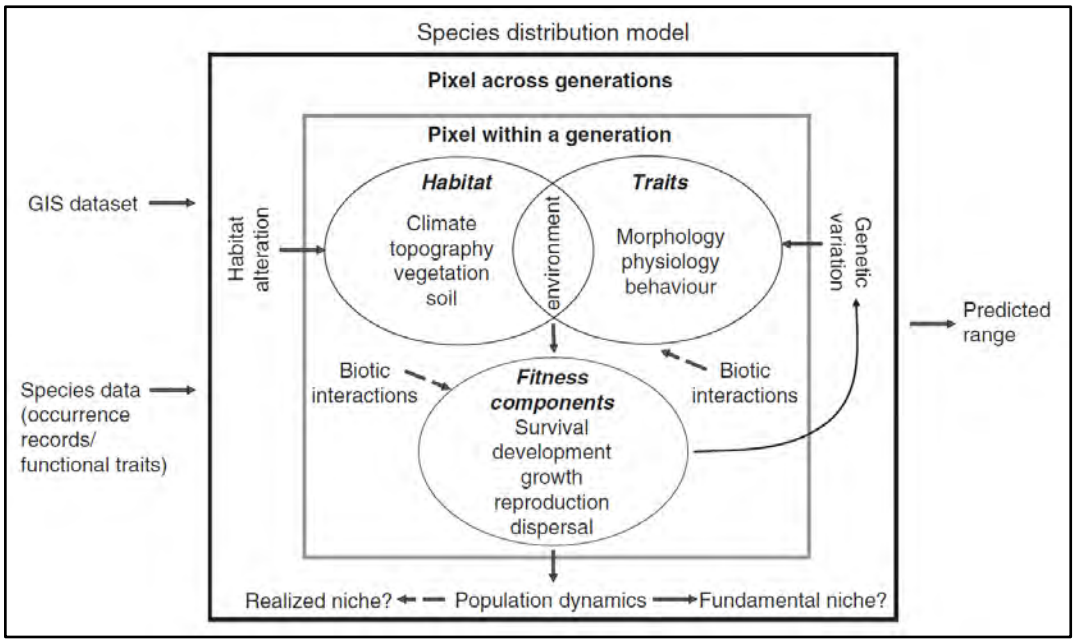


Figure 2-1: Factors incorporated into species distribution models (SDMs) (Taken from Kearney & Porter 2009)

However, to gain the fundamental niche of the organism, physiological traits should be incorporated into models (Kearney & Porter 2009). Various laboratory based methods are available to explore an organism's sensitivity to natural climatic conditions (Rezende *et al.* 2011; Sinclair *et al.* 2015). These include temperature-dependent development data that can be used to develop degree-day models, and when combined with meteorological weather station data (i.e. mechanistic approach; Kearney & Porter 2009), can assist in making predictions on the number of generations that an agent can complete within a time period at an invaded site (Byrne *et al.* 2004; Coetzee *et al.* 2007). In addition, critical thresholds experiments explore the mean temperatures where an insect loses locomotory function and lethal thresholds, temperatures from which an insect cannot recover (Angiletta *et al.* 2002; Stratman *et al.* 2014; Sinclair *et al.* 2015). For example, the tortoise beetle *Gratiana spadicea* Klug (Coleoptera: Chrysomelidae), a biological control agent for *Solanum sisymbriifolium* Lamarck (Solanaceae) in South Africa proved to have a wide thermal niche, but establishment at high altitudes was

limited due to relative humidity that was below the insect's lower lethal humidity (56.6%) (Byrne *et al.* 2002).

Insect life cycles often occur within more than one environment, for example biological control agents that feed above the surface but pupate in the soil (Winston *et al.* 2014). To gain a comprehensive understanding of the fundamental niche of a biological control agent, it is important to include agent stages that occur in different environments into testing procedures. This is especially true for freshwater systems. Ambient and water temperature do not always exhibit a linear relationship (Morill *et al.* 2005, Talling 2010), and temperature is often increased in dense stands of aquatic weeds. In a shallow, eutrophic lake in Uruguay, Mazzeo *et al.* (2003) found that water temperature in *Egeria densa* beds was on average 0.3°C warmer than open water (April 2000 to March 2001). Wheeler & Center (2001) also found that temperatures in *H. verticillata* weed mats often reached lethal temperatures (45°C) of the biological control agent *Hydrellia pakistanae* Deonier (Diptera: Ephdyridae), which has contributed to the agent's poor performance in the USA.

Establishing the thermal limits of freshwater invertebrates is also crucial, considering the current and predicted pressure on freshwater systems due to overpopulation and climate change. Elevated CO_2 is affiliated with increases in temperature and one major effect of global climate change will be changes in aquatic thermal regimes (Kernan 2015), changes in rainfall patterns (e.g. intensified El Nino events), flow regimes and the physio-chemical properties of freshwater systems (Whitehead *et al.* 2009) that may expose organisms to extreme events. It is because of this constant threat that Dallas and Rivers-Moore (2012) studied the thermal critical maximum (CT_{max}) of 27 insect families with aquatic life stages which allowed them to use certain thermally sensitive families as bio-indicators of thermal alterations. Thus, establishing the physiological range of aquatic biological control agents will not only allow practitioners to predict future agent performance, but also help to fill the knowledge gap on invertebrate

survival in a changing world. Thermal physiology studies on the biological control agents of submerged aquatic weeds are also rare. Considering the worldwide distributionof *E. densa*, this study will provide valuable information for other countries that might consider biological control as a management option.

The founder population of *Hydrellia egeriae* was collected between latitudes 27 to 34 S, and longitudes 55 to 59 W in its native range (Cabrera Walsh *et al*. 2013). The mean annual temperature of these sites are between 15°C and 25°C (Figure 2-2) (Cabrera Walsh pers. comm.). *Egeria densa* grows actively in temperatures between 10°C and 30°C (Getsinger & Dillon 1984), and can tolerate temperatures of up to 40°C (Haramoto & Ikusima 1988). Throughout its lifespan, *H. egeriae* is exposed to two types of environments: aquatic during its immature life stage and aerial for adults. The aquatic life stage is damaging to *E. densa*.

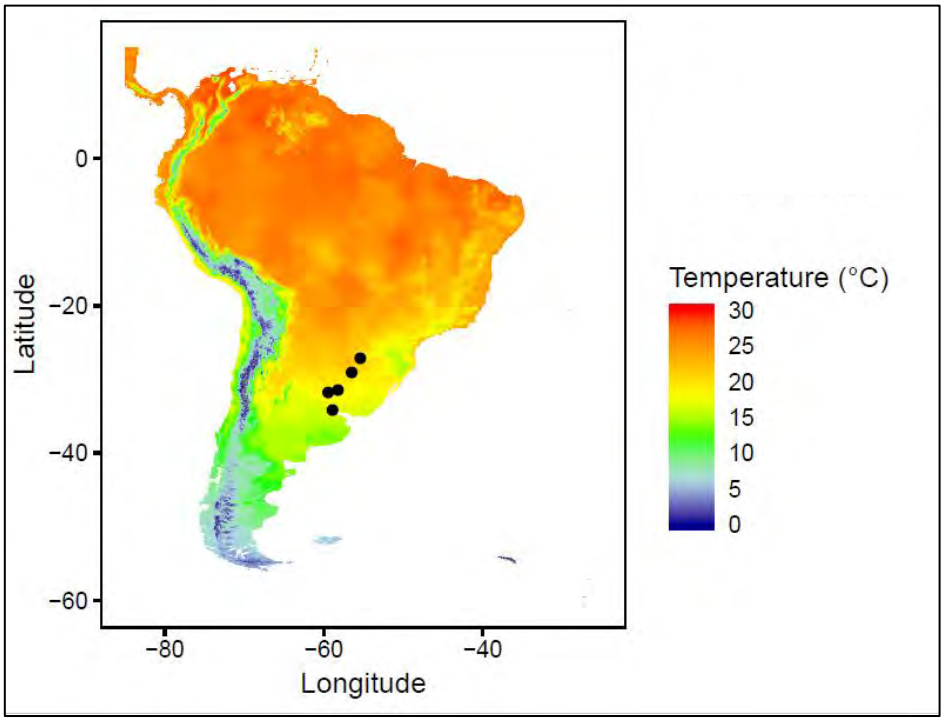


Figure 2-2: Mean annual temperature of sites where *Hydrellia egeriae* (black dots) was collected from within its native range (Cabrera Walsh, pers. comm.).

The objectives of this study were thus to establish the thermal physiology of both adult and larval stages of *H. egeriae*. We also aimed to establish the number of generations that the

agent will be able to complete during cold months and altogether annually at invaded sites in South Africa.

2.2 Material and Methods

2.2.1 Insects for testing

Hydrellia egeriae was reared in a polytunnel at the Waainek Research Facility, Rhodes University, allowing insects to develop under "almost natural" conditions. Twenty five to thirty five *E. densa* shoots, approximately 20cm in length, were individually planted in round tubs (41cm x 24cm, 13.5 litre) containing pond sediment with the slow release fertilizer Multicote^{TM} (Haifa) (ratio 0.7g per 1kg sediment). Afterwards, a 1cm silica sand layer was placed over the sediment to prevent algal growth and water clouding. Food for adults was provided on a floating petri-dish with a yeast hydrolysate/sugar mixture (4 g BactoTM TC yeastolate/7 g sugar/10 ml H2O). *Hydrellia egeriae* individuals from these populations were used for experiments in this study.

2.2.2 Development rates and degree day accumulation

Individuals for testing

The developmental rate of *H. egeriae* from egg to adult was determined by placing two to ten pairs of adults collected from the laboratory culture in a transparent box (41cm x 17cm x 29cm), half-filled with distilled water, supplemented with a nutrient stock solution according to Smart and Barko (1985) and containing 25 to 35 *E. densa* shoots, 15cm in length. Adults were given a yeast hydrolysate/sugar mixture in a petri-dish, as food. Adults were allowed to mate and females to oviposit for 24 hours, after which eggs were collected and placed in a petri-dish with water.

Experimental setup

Fifteen replicates per temperature treatment were set up. Upon hatching, an individual first instar was collected and placed into in a transparent 1.5 litre container, half-filled with

water and containing two *E. densa* shoots 10cm in length. The leaf containing the first instar was pinned to an *E. densa* shoot using minuten pins. Experiments were conducted in controlled environment chambers set at constant temperatures of 14, 18, 21, 25, 29°C. Containers were checked daily for survival and development between life stages, until all the adults emerged. The effect of temperature on the total development time (days taken from egg to adult emergence) of *H. egeriae* was determined by means of a one-way ANOVA, using the aov() function in the R environment. Significant differences between temperature treatments were determined using the emmeans tukey test from the emmeans package. The degree-day requirement were calculated from these data. This was done using the reduced major axis regression method (Ikemoto & Takai 2000), since the relationship between temperature and developmental rate is not linear.

Hourly air temperature data for *E. densa* sites in South Africa were obtained from the South African Weather Bureau for the period 2016-2018. The number of generations that *H. egeriae* can complete at each site per year iscalculated from the accumulated degree-days according to Equation 2.1 using the thermal constant K and developmental threshold t.

Equation 2.1: $K=\Sigma\ (T_{max}+T_{min})/2-t$

The mean accumulated degree-days of development for each site were calculated in the R environment (R Core Team 2019). The reduced major axis regression (RMA) was conducted using the lmodel2 package. Maps containing the number of generations that *H. egeriae* can complete at *E. densa* sites in South Africa were drawn in the R environment. This was done by overlaying the site coordinates and its corresponding number of *H. egeriae* generations over a South Africa shape file, with environmental layers containing the annual mean temperature (bio1) and minimum temperature of coldest month temperature (bio2). Maps were made with the use of the ggplot2 package.

2.2.3 Critical thermal limits

The critical thermal minima (CT_{min}) and maxima (CT_{max}) are the mean temperature at which insects lose their motor control (Mitchell *et al.* 1993). In this experiment, this was only determined for adults. The critical thermal trials were conducted by collecting 10 adults from the polytunnel culture with an aspirator into separate Eppendorf tubes each containing a slightly moistened piece of cotton wool placed at the base. The Eppendorf tubes were placed into a high density foam floating tray, allowing only the tube openings to stay above the water surface. The tray was placed into a refrigerated water bath connected to a programmable temperature controller (Grant© R4 GP200).

To determine the CT_{max}, the ten individuals were given an acclimation period by heating the water temperature from 22 to 30°C. The water temperature was held at 30°C for five minutes, after which the temperature was increased at a 0.25ºCmin^{-1} ramp rate. To determine the CT_{min}, the temperature was cooled from 20 to 10°C during an acclimation period of two minutes. Subsequently the temperature was reduced at a 0.25ºC.min^{-1} ramp rate. This rate of temperature change was sufficient to prevent insects from acclimatizing to increases or decreases in temperature; a form of short-term phenotypic plasticity that could confound the results (Chidawanyika & Terblanche 2011; Terblanche *et al*., 2017). At each 1.0°C interval, adults were checked for loss of coordinated muscle function and the corresponding temperature was recorded. This procedure was replicated three times with a new set of individuals each time. Thus, 30 individuals were used in total for the CT_{min} and CT_{max} trials, respectively. The temperatures at which individuals lost motor control was used to calculate the mean CT_{min} and CT_{max} (±SE).

2.2.4 Lethal temperatures

The lower lethal (LLT_{50}) and upper lethal (ULT_{50}) temperatures were determined for both immatures and adults. These limits describe the lower and upper temperatures at which

50% of the sample population dies, and is therefore a physiological limit (Mitchell *et al.* 1993). Ten or eleven adults or immatures (a combination of 2nd and 3rd instars) were collected from the polytunnel culture. Larvae were placed in water-filled Eppendorf tubes, and adults were collected similarly to the critical thermal limit experiments.

For the LLT_{50}, a mixture of distilled water and propanol at a ratio of 50: 50 was added to the water bath, as propanol has a lower freezing point than water. Insects were initially held at room temperature for 25 minutes, before placing them in the water bath at a temperature of 12°C. The temperature was subsequently reduced to 4°C for the LLT_{50} trials and increased to 25°C for the ULT_{50} trials at a 0.25ºC.min^{-1} ramp rate. Individuals were kept at this temperature for two hours, after which individuals were individually placed in 1.5L containers, half-filled with water and containing one *E. densa* shoots 8 to 15cm in length. The containers were kept at room temperature and recovery to normal behaviour was checked 1 and 24 hours after the experiment. A temperature range of 4°C to -7°C was used for the LLT_{50}, and 25°C and 48°C for the ULT_{50}.

Data obtained from the lethal temperature trials are binomial, and thus a generalized model with a logit function was fitted in the R environment (R Core Team 2019). Subsequently, the dose.p() function from the MASS package was used to extract the temperature at which 50% of the population dies. Results

2.2.5 Development rates and degree day accumulation

The development rate of *H. egeriae* from egg to adults is shown in Table 2-1. For the selected temperature range used in this study, the longest duration of development was 94.8 ± 1.1 days at 14°C, with a nearly 50% reduction in development time at 18°C. The total development time from egg to adult was significantly affected by temperature ($F_4 = 2135.7$; $P < 0.001$). Total development time at 25°C was almost a week less (6 days) compared to 21°C,

however larval development took the same time with 16.4 and 16.5 days. Total development time was less than a month at 25°C and 29°C.

Table 2-1: Development rates (days) of *Hydrellia egeriae* from egg to adult at five different temperatures. Letters indicate significant differences between total development time.

Life stage	14°C	18°C	21°C	25°C	29°C
Egg	12.8 ± 0.2	7 ± 0	5.7 ± 0.1	3 ± 0	4 ± 0
Larvae	51.3 ± 0.8	23.1 ± 0.3	16.5 ± 0.3	16.4 ± 0.5	13.7 ± 0.6
Adult	30.7 ± 1.1	18.7 ± 0.4	12.3 ± 0.2	9.4 ± 0.2	7.3 ± 0.2
Total	94.8 ± 1.1[a]	48.8 ± 0.4[b]	34.4 ± 0.2[c]	28.4 ± 0.6[d]	25.0 ± 0.5[e]

The reduced major axis regression equation (Equation 2.2) and plot (Figure 2-3) shows that at a lower threshold temperature (t) of 9.2°C, *H. egeriae* development (K) from egg to adult requires 448.7 degree-days.

Equation 2.2: $DT = 9.20x+448$ ($R^2 = 0.99$, $P < 0.001$)

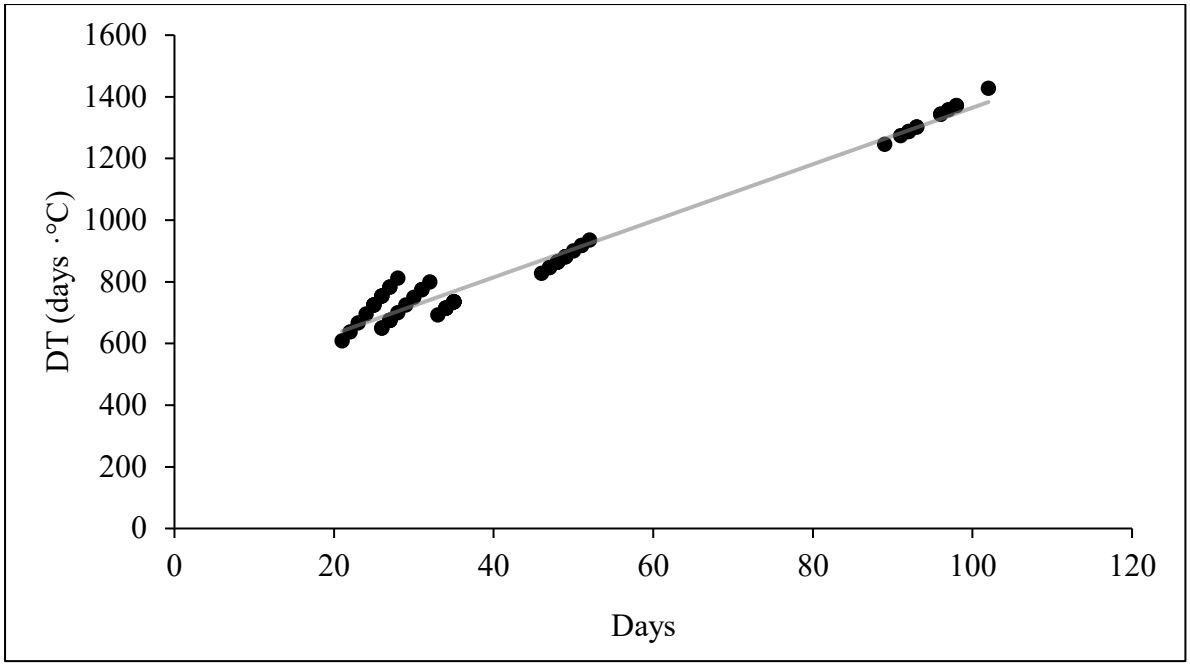


Figure 2-3: Temperature-dependent egg to adult development. DT is the product of developmental time (D) and temperature (T) of *Hydrellia egeriae* using the reduced major axis regression method (Ikemoto & Takai, 2000).

The number of generations that *H. egeriae* was predicted to complete annually within South Africa ranged from 5.9 to 8.3 (Figure 2-4). During winter months (June to August), the biological control agent was predicted to only be able to complete one generation (Figure 2-5). One of the largest *E. densa* infestations is in the Midmar Dam, KwaZulu-Natal Province, and the estimated number of generations per year is 6.9. This may be attributed to prolonged low winter temperatures (3.9 ± 0.2°C for 2016 to 2018) at this higher altitude site. The highest annual turnover of *H. egeriae* generations are within the Eastern Cape Province with 8.3 and 8.1 generations in the Kouga River and Nahoon River, respectively.

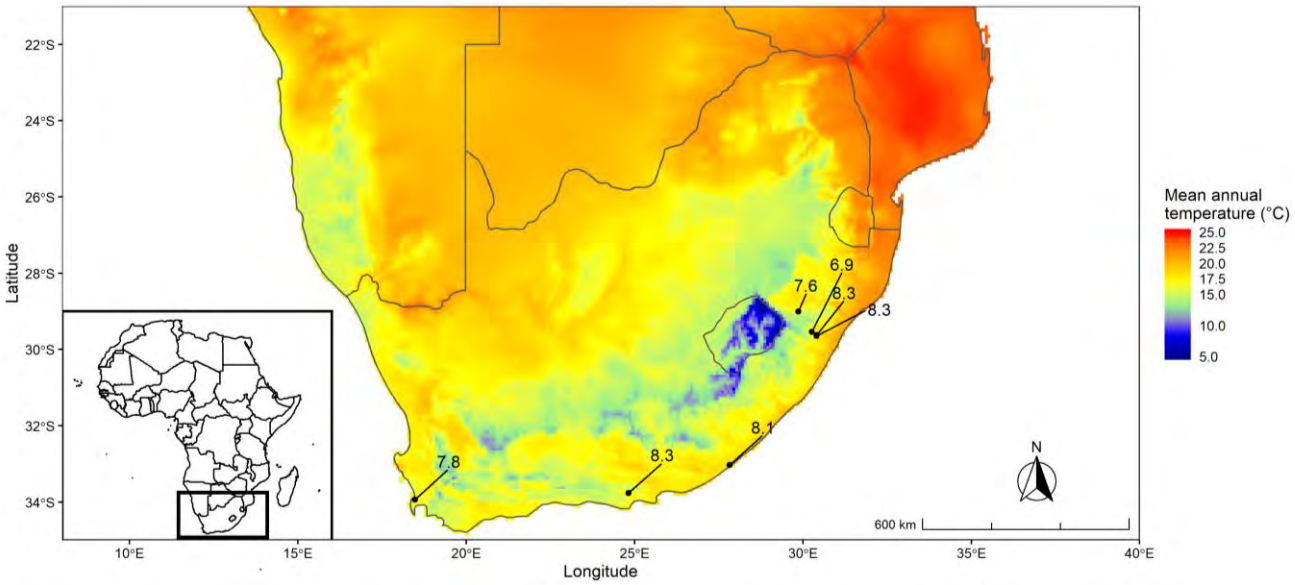


Figure 2-4: Number of generations that *Hydrellia egeriae* can complete annually at *Egeria densa* sites (black dots). Estimations from the reduced major axis regression and 3 years of daily temperature data (2016-2018).

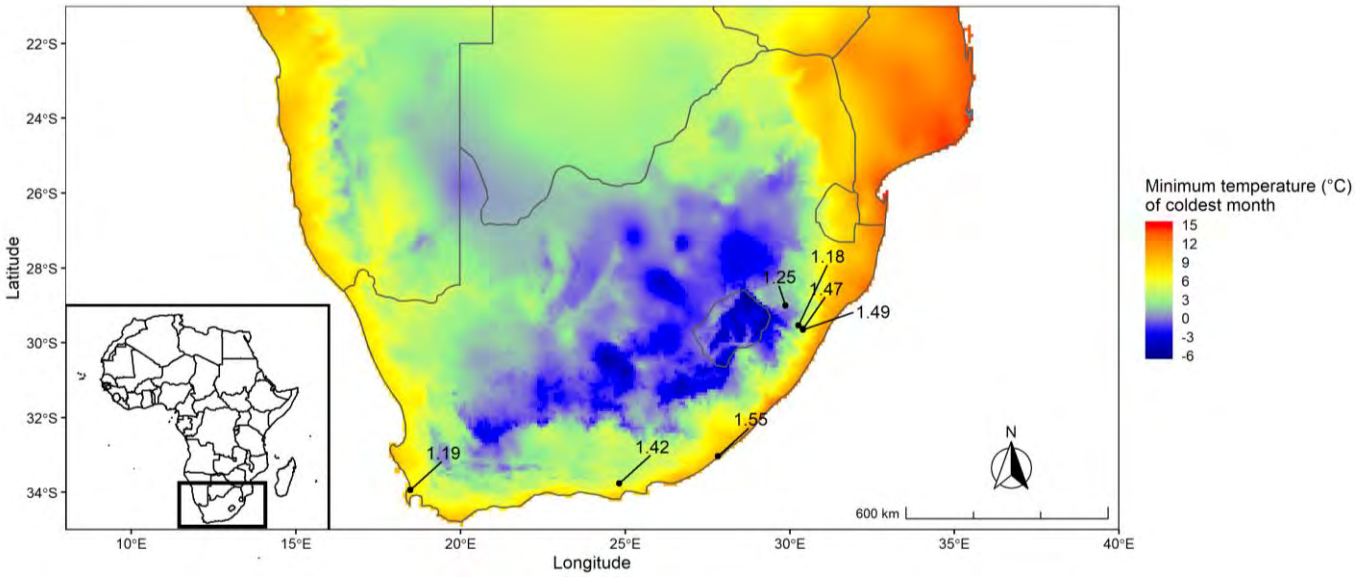


Figure 2-5: Number of generations that *Hydrellia egeriae* is predicted to complete during winter (June to August) at *Egeria densa* sites. Estimations from the reduced major axis regression and 3 years of daily temperature data (2016-2018).

2.2.6 Critical thermal limits and lethal temperatures

The temperature range at which *H. egeriae* adults do not lose locomotory function lies between 2.6°C and 47°C (Table 2-2). The upper lethal temperature (ULT_{50}) for larvae was slightly higher, but not statistically different ($t_{7.7} = -0.79$; $P = 0.45$)) compared to adults (40.5 ± 0.89°C). Interestingly, adults only lost locomotary control at 47°C, whereas 50% of the

population died at a lower temperature (40.5°C). This difference can be explained by the experimental methodology. In the LT_{50}experiments, specimens are held at a temperature for two hours, whereas in the CT experiments, temperatures are only ramped. Thus, *H.egeriae* adults will be negatively affected, locomotary and lethal, if it experiences extreme temperatures for prolonged periods of time. *Hydrellia egeriae* larvae and adults had a lower lethal temperature (LLT_{50}) below freezing point, of -6.3°C and -5.9°C respectively. The LLT_{50} of larvae was not statistically different ($t_{19.3} = -0.33$; $P = 0.75$) from the LLT_{50} of adults.

Table 2-2: The critical thermal limits (±SE °C) of *Hydrellia egeriae* adults and the lethal temperatures (±SE °C) of immatures and adults. Letters indicate stastical differences between larvae and adults.

Thermal parameter	Larvae ($2^{nd}/3^{rd}$ instars)	Adults
CTmin	-	2.6 ± 0.15°C
CTmax	-	47.0 ± 0.22°C
LLT50	-6.3 ± 0.24°C [a]	-5.9 ± 0.00°C [a]
ULT50	41.3 ± 0.75°C [a]	40.5 ± 0.89°C [a]

2.3 Discussion

An organism's vulnerability to environmental change depends on its: 1) sensitivity; 2) exposure time; 3) resilience to disturbances; and 4) adaptive capacity (Huey *et al.* 2012). This study illustrated that immatures and adult stages of *H. egeriae* have a wide thermal range under laboratory conditions. This thermal range encompasses its host plant's optimal temperature range of 10 to 35°C (Getsinger & Dillon 1984, Haramoto & Ikusima 1988), and thus predicts that South Africa's climate will not inhibit the establishment of the agent. The generated maps also predict that *H. egeriae* is capable of completing multiple generations within its introduced range, despite cold winter temperatures at *E. densa* sites.

Insects often have complex life cycles, which are composed of sessile and mobile life stages occurring within different habitats (Huey *et al.* 2012). *Hydrellia egeriae* is exposed to different thermal conditions during its development; atmospheric and aquatic. Water temperature is affected by topography, seasonal and temporal variations, water depth, atmospheric conditions, thermal pollution and climate change (Caissie 2006, Talling 2010). *Hydrellia egeriae* larvae mostly feed in the top 15 to 25cm of *E. densa* stands, where the leaves are more nutritious (Cabrera Walsh *et al.* 2013). It is also in this top layer of *E. densa*, especially in dense canopies, that water temperature is higher compared to open water (Mazzeo *et al* 2003). In Florida, USA, naturally moving water was slowed down by *H. verticillata* stands, and with intense sun exposure, increased water surface temperature (45°C) and larval mortality (ULT_{100} 36°C) of *H. pakistanae* (Wheeler & Center 2001). Although, *H. egeriae* can tolerate warm conditions for short periods of time of up to 47°C, the agent's tolerance to extended periods of high temperatures are lower (40.5°C and 41.3°C). Thus, extreme temperatures in *E. densa* stands may be lethal.

Hydrellia egeriae responded to constant cold temperature through prolonged development, which explains the low population turnover predictions for winter. A similar trend was also seen from temperature development studies in the agent's native range, where development time doubled at 15°C from 20°C with 74 days compared to 35 days (Cabrera Walsh *et al.* 2012). Adult flies had a critical minimum of 2.6°C, however lethal temperatures (for both larvae and adults) were much lower at -6°C. From this result, it is evident that *H. egeriae* is able to survive sub-zero temperatures for short periods. Ectotherms overcome sub-zero temperatures, through either freeze avoidance or freeze tolerance. For some organisms, freezing is lethal, and thus antifreeze proteins (AFPs) depress the point of freezing (Duman 2015). To avoid freezing, insects will either accumulate AFPs during winter or eliminate gut microbial ice nucleators through feeding behaviour in autumn (Lee 2010). Organisms can also

use AFPs to tolerate freezing, which can be done through extracellular freezing; ice-binding proteins that control recrystallization; and cell membrane ice-binding proteins that control the freezing temperature, rate and location (Duman 2015). Previous studies have shown that Dipteran species express freeze tolerance (Toxopeus 2018). For example, Koštál *et al.* (2012) was able to lower the freeze tolerance of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) to -5°C when as much as 50% of their body fluid was frozen. To achieve this, larvae were exposed to temperatures that induce quiescence and secondly, larvae were given a proline cryoprotectant diet (AFP). Many other Dipteran families are known to be freeze tolerant (Chironomidae, Heleomyzidae, Mycetophilidae, Tephritidae and Tipulidae) (Toxopeus 2018). It is therefore likely that *H. egeriae* expressed freeze tolerance during the lower lethal temperature experiments in this study. Further investigations are necessary to identify the specific AFPs involved.

An organism's sensitivity to its environment is not only made up of its physiological traits, but also its behavioural (Huey *et al.* 2012), which allows it to thermoregulate within a spatial and temporal thermal heterogeneous habitat (Angiletta *et al.* 2002). For example, adults of the leafhopper *Megamelus scutellaris* (Hemiptera: Delphacidae), a biological control agent for water hyacinth, expresess wing dimorphism, allowing it to disperse if conditions become unfavourable (Tipping *et al.* 2014). *Hydrellia egeriae* adults use short, hopping flights (Cabrera Walsh *et al.* 2012), which allow them to move to cooler or shaded areas when temperatures are too high. Although larvae mine within leaves, they can also exit leaves and drop deeper into the water column (Cabrera Walsh *et al.* 2013), where temperatures may be cooler (Stratman *et al.* 2014). However, larvae move slowly, and sudden temperature spikes may be lethal. Although both ambient and aquatic life stages have their own constraints, it is likely that the overlapping life stages will favour population survival, especially during highly fluctuating conditions or extreme events (Fauvergue *et al.* 2012, Goode *et al.* 2019).

Investigating the thermal physiology of an agent allows for prediction on the potential establishment and population turn-over rates at invaded sites (Fauvergue *et al.* 2012; May & Coetzee 2013), which is important for target weed suppression. From this study we can predict that the agent can be released at all *E. densa* invaded sites, even at high altitude sites. Biological control agents often undergo bottlenecks during importation and long periods of laboratory rearing, and may loose important climatic alleles. When introduced into a novel environment, populations may undergo climatic niche shifts due to selective pressures (Harms *et al.* 2021). It is important to ground-truth these predictions and measure the realized establishment of the agent (Muskett *et al.* 2020), which will allow foradaptive management through fine-tuned release strategies and maximum biological control effort.

3. Post-release evaluation of *Hydrellia egeriae*

3.1 Introduction

Biological control success, as defined in chapter 1, is dependent on the successful establishment and population build-up of an agent when released. Ultimately, the biological control programme should reduce the ecological, economic and social impact of the target weed (Cruttwell McFayden 1998; Morin *et al.* 2009; Hill & Coetzee 2017; Schwarzländer *et al.* 2018; Schaffner *et al.* 2020). It is important to ground-truth these predictions and measure the realized establishment of the agent (Muskett *et al.* 2020), which allows for adaptive management through fine-tuned release strategies and maximum biological control effort.

Post-release evaluations are integral in measuring the effectiveness of the biological control programme, firstly by measuring agent establishment and dynamics and its impact on the target weed population (Hoffmann *et al.* 2019; Schaffner *et al.* 2020). Secondly, post-release evaluations should measure the larger effect of the reduction of the target weed, either from an ecological, economic or social perspective, on a local or regional scale (Schroeder 1983; Cruttwell McFayden 1998; Blossey & Skinner 2000; Morin *et al.* 2009). For example, in South Africa, yearly nationwide quantitative surveys of *A. filiculoides* have been conducted from 1997 to 2017 to monitor the effect of its biological control agent, *S. rufinasus* (Martin *et al.* 2018; Schaffner *et al.* 2020). These post-release surveys revealed that 100 of 105 infested sites have been eradicated by the weevils and the weed is under complete control at the other five.

Systematic post-release evaluations require context-dependent goals or hypotheses *a priori*, which will guide evaluation methodology (Blossey & Skinner 2000; Fowler *et al.* 2000). This may also require pre-release assessments or knowledge of the invaded system before the agent is released, and will form a basis against which the progress of the biological control program can be measured. For example, *Prosopis* spp. (Fabaceae), was introduced to South

Africa for fodder, fuelwood and soil stabilization. In the Northern Cape Province, it has increased its distribution from 128 000 hectares in 1974 to 1.5 million hectares by 2007 (Shackleton *et al.* 2014). Interviews with farmers revealed that an average of US$ 1 914 yr^{-1} (range US$10 to over US$ 500) was spent per farm to manage prosopis through manual, mechanical or chemical means. Another example includes the management of *A. filiculoides*, where mechanical and chemical control were valued at US$ 1 005 and US$136 per ha/year. However, due to the rapid regeneration of the weed, these control methods delivered no long-term relief of the weed (McConnachie *et al.*, 2003). Comparatively, biological control were valued US$7 976 per ha/year in the first year of the programme and US$276 per year from 1996 to 2000. With this type of information, it would be easy to set management goals, for example, biological control should reduce the amount of money farmers spend on clearing invasive plants.

3.1.1 Managing *Egeria densa* in South Africa: an ecological approach

Egeria densa has ecological, economic and social impacts on the systems where it becomes invasive. Pre-management assessments have found that, for example the cumulative net present value (NPV) of the Midmar Dam is nearly $46 million, but if no *E. densa* management is done, its value will drop to -$1 million (Vundla *et al.* 2017). Using a generic impact scoring system (GISS) to prioritize invasive species impact according to Nentwig *et al.* (2016), Hill & Coetzee (2017) found that *E. densa* has an impact score of 42 of 60 maximum, making it the third most impactful aquatic species in South Africa, next to water hyacinth and *H. verticillata*, with impact scores of 43 each. Considering the high impact score of *E. densa*, any management option against *E. densa* should reduce its reproduction, stand density and spread in the systems it has invaded.

In a review on ecosystem recovery after invasive plant management, Reid *et al.* (2009) found that from three invasive aquatic plant management programmes in Australia (*A.*

philoxeroides, *Cabomba caroliniana* A. Gray (Cabombaceae) and *S. molesta*), only one study measured the response of the recipient community and its ecosystem processes to management. Measuring non-target species or community response to a reduction in a target weed population is seldom evaluated in biological control programmes. This may be due to the complexity of the system, resource shortages or limited time available to do post-release surveys (Schaffner *et al.* 2020). This type of information is important, not only as a measure of ecological recovery, but can serve as lessons upon which the practice of classical biological control can be improved (Schaffner *et al.* 2020). This would be particularly useful for biological control of aquatic weeds in South African systems. Invasive aquatic weeds (floating, emergent and submerged) often co-occur, and systems may shift between trophic states i.e. floating to submerged aquatic plant weed dominance. Long-term data of aquatic weed dynamics in South Africa can elucidate stable state cycles and their catalysts (Scheffer 2009), and assist aquatic weed management.

3.1.2 Aquatic plant surveys

There are several ways to survey aquatic plants, depending on the desired outcomes of the survey. Some survey examples include: non-destructive surveys through hydro-acoustics or satellite imagery, point-intercept surveys that measure plant absence or presence and plant biomass surveys with rakes or rotating plant samplers (Howard-Williams & Longman 1976; Titus 1993; Madsen & Wersal 2017). Sampling method may produce different types of data, require different levels of effort, accommodate different plant growth types and have different applications, i.e. whole system surveys vs. long-term site specific surveys (Madsen & Wersal 2017). Ultimately, a well-designed sampling method must be objective and quantifiable for statistical analyses (Madsen & Wersal 2017, Madsen pers. comm. 2020).

3.1.3 *Egeria densa* post-release evaluation: finding middle ground

Because South African freshwater systems have different growth types of aquatic species, i.e. submerged, emergent, floating, with particular emphasis on shifts between stable

states, it is important to find a method that could potentially accommodate both *E. densa* parameters and record surrounding non-target species. Measuring submerged, emergent and floating aquatic plants adds an additional dimension to aquatic plant sampling. However, *E. densa* grows in the littoral zone, and sites in South Africa where it has become invasive are generally shallow enough to wade through (maximum depth approximately 1 meter). The water surface level offers a middle ground between the different plant growth types (Figure 3-1).

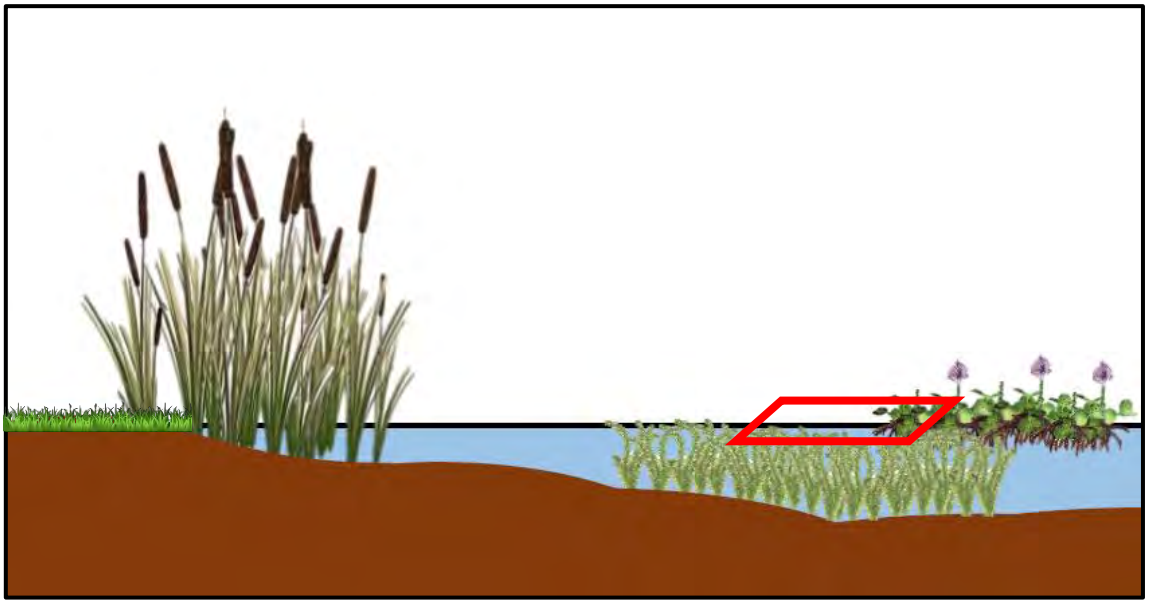


Figure 3-1: Schematic diagram of different aquatic plant growth types found within the littoral zone of South African freshwater systems. Red square: water surface as a middle ground for aquatic plant surveys.

This study is ecologically focused, i.e. *E. densa* cover, biomass and response of non-target species. An ecological focused approach is important not only to measure biological control parameters, but also because any economic and social benefit of *E. densa* biological control depends on the reduction of the invasive aquatic plant's population size and cover at invaded sites. This also includes knowledge on the response of the recipient community, i.e. open water, native species recruitment, secondary invasion or shift in stable state.

The aim of this study was to determine the establishment of *H. egeriae* at released sites. Secondly, the aim was to measure the level of leaf-mining, and its effect on *E. densa* shoot

biomass.. Subsequently, we aimed to map the change in *E. densa* population density and that of non-target species (native or exotic) in the presence of the biological control agent. For this study the effectiveness of the biological control agent was measured using a before-after design without control. Therefore, *E. densa* and the aquatic community within a site was compared before and after the release of *H. egeriae*.

3.2 Material and Methods

3.2.1 Study sites

Two sites were selected for this study: the Nahoon River in the Eastern Cape Province and the Midmar Dam located in KwaZulu-Natal Province. The Nahoon River was selected because of its close proximity (183km) to the Centre for Biological Control. This allowed for monthly post-release surveys. The Midmar Dam is approximately 773km away from the Centre for Biological Control, and was selected due to its economic and ecological importance. Post-release surveys were done every three months at the Midmar Dam. To explore different release strategies and its effect on the establishment of the biological control agent, the Nahoon Rver was selected for one release event, while the Midmar Dam was selected for augmentative releases. The number of individuals per release event was not predetermined, and generally the largest number of individuals available at the mass-rearing facility was collected.

3.2.1.a *Nahoon River*

The release and post-release evaluation site in the Nahoon River was situated approximately 22.7 km downstream from the gravity type Nahoon Dam, established in 1966, and approximately 500 meters before a weir that leads the freshwater river into an estuary (Figure 3-2). The river flows through various land-use types from the dam wall to the *E. densa* invaded site, including agricultural lands (cattle and crops), natural, rural and urban areas. This system forms part of the Mzimvubu to Keiskama water management area, and receives rain throughout the year, with peaks in summer. Dry months are in June and July. The river is home

to the endangered *Sandelia bainsii* Castelnan (Anabantidae), a genus of climbing gouramis fish. The Nahoon River, nearing the weir, is heavily polluted due to the various land uses around it and failing sewage treatment plants that spill sewage into the river. Not only has this contributed to aquatic biological invasions downstream, but also fish kills (observed during the study period). Aquatic invasive species present in the river include water hyacinth, *P. stratiotes*, *A. filiculoides* and *E. densa*. Biological control agents were found on the floating aquatic weeds, and due to the exponential growth of water hyacinth, augmentative releases of *M. scutellaris* were made by the Centre for Biological Control, and members of the community as part of an eduction project.

Figure 3-2: Aerial view of the Nahoon River showing the 58 meter transect (red line) used during monthly surveys (Source: Google Earth).

Hydrellia egeriae was released for the first time in South Africa at the Nahoon River in October 2018. Approximately 10 000 immatures of *H. egeriae* were released during this event

(Figure 3-3). To explore the potential of the biological control agent to establish from one release, this was also the only release for this site.

Figure 3-3: First release of *Hydrellia egeriae* against *Egeria densa* in the Nahoon River, October 2018 (Source: Kim Weaver).

3.2.1.b *Midmar Dam*

The Midmar Dam is situated 1 044 meters above sea level within the uMngeni catchment, KwaZulu-Natal Province, storing water from the uMngeni, Gqishi and Nguklu rivers. The dam's surface covers 1 880 hectares, and is surrounded by critically endangered Midland Misbelt Grassland and endangered Southern KwaZulu-Natal Moist Grassland (Ezemvelo KZN Wildlife 2009). The climate in the region is temperature with summer rain (November to January) and cool, dry winters (May to July) (Ezemvelo KZN Wildlife 2009). The dam serves as a water source for more than half of the province's population (Vundla *et*

al. 2017). The Midmar Dam offers several tourism and sporting activities, in particular the annual Midmar Mile swimming competition (http://www.midmarmile.co.za). Land use around the reserve includes commercial farming, an informal settlement (Mpophomeni Township) and the town, Howick (Ezemvelo KZN Wildlife 2009).

Egeria densa distribution is mostly localized in the bays of the dam (Figure 3-5). A sonar survey for submerged vegetation was done in 2014 (Figure 3-1), and although it presents submerged vegetation presence and distribution, it cannot distinguish between species, for example *E. densa* and *Lagarosiphon muscoides* Harv. (Hydrocharitaceae). However, a pre-release assessment was conducted in 2018 to investigate invasiveness of *E. densa* in the dam. This was done by boat, focussing on the littoral zone, and areas with plants or bays were investigated using replicated (9 to 10) quadrat (0.5cm x 0.5cm) sampling. This pre-release survey (Figure 3-5) confirmed the distribution results from the sonar survey (Figure 3-4). Other invasive species in the dam include *M. aquaticum* and water hyacinth, but populations are patchy and small due to effective management, either through manual removal or biological control. Native plant diversity in the dam is high (Figure 3-5).

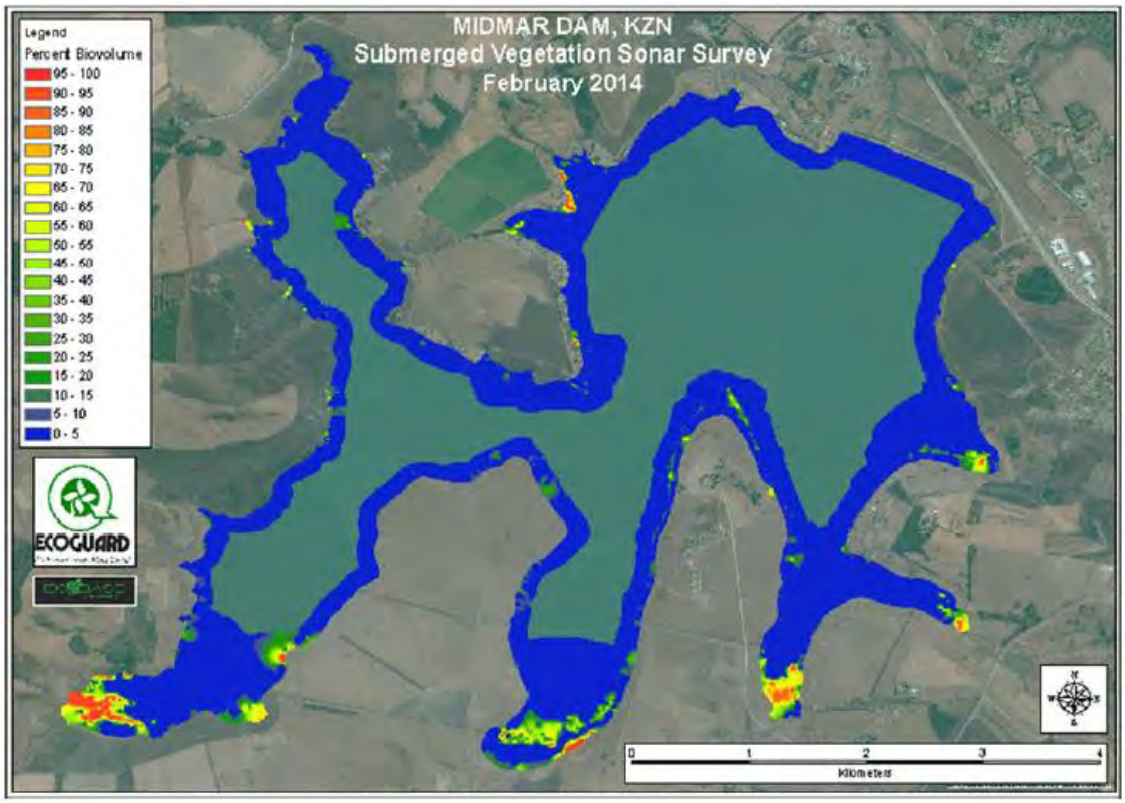


Figure 3-4: *Egeria densa* distribution in the Midmar Dam (Source: Vundla et al. 2017).

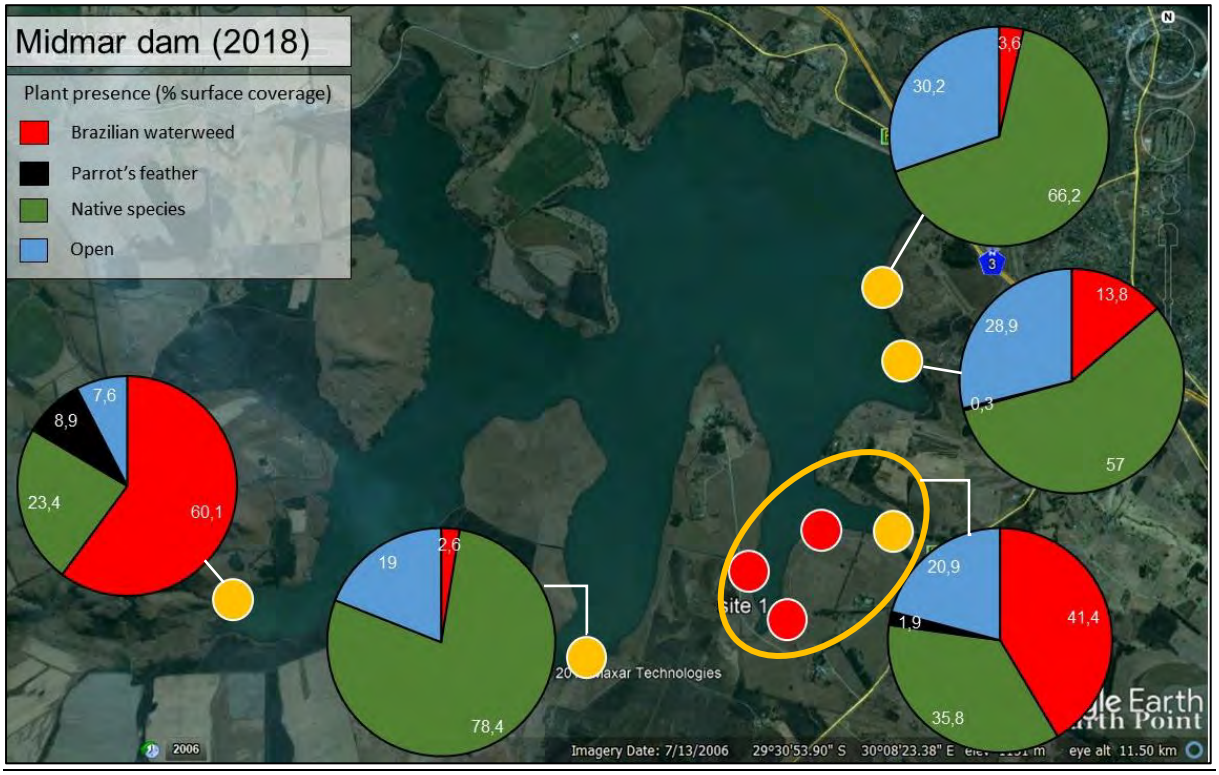


Figure 3-5: Community composition, expressed as percentage surface cover, of *Egeria densa* sites in the Midmar Dam, August 2018. Orange circles indicate survey sites. Red circles indicate biological control agent release sites.

This site was selected for augmentative releases to explore if an increase in release events affect establishment success (Table 3-1). A subset of the pre-release survey sites was selected, mainly because of easy accessibility, limited resources (biological control agents) and limited time to achieve study aims (2018 to 2020). *Hydrellia egeriae* was released for the first time in March 2019.

Table 3-1: Date and number of *Hydrellia egeriae* immatures (eggs, larvae and pupae) released at Pilon Bay, Midmar dam for the period 2019 to 2020.

Date	Number of insects released (immatures)
March 2019	6 726
June 2019	8 800
October 2019	30 000
March 2020	14 400
Total	85 526

3.2.2 Post-release evaluation

3.2.2.a *Aquatic macrophyte mapping*

For this post-release evaluation, the line transect method was selected, as it requires moderate effort, includes submerged, emergent and floating aquatic plant types and collects data on aquatic plant presence/absence, cover and abundance (Madsen & Wersal 2017). Surveys were conducted at each site along a pre-determined, fixed transect, for example a 58m transect in the Nahoon River (Figure 3-2). The transect in the Midmar Dam consists of two smaller transects due to areas in the sites that are too deep to wade through. A quadrat (0.5m x 0.5m) was randomly thrown at the start of the transect, and the percentage surface cover presented by aquatic species in the quadrat, including open water, was recorded. The quadrat was then thrown forward, along the transect, to record the same measurements. This was repeated, until 10 to 15 quadrats along the transect have been sampled. Thus, quadrats were

not fixed, and changed during sampling events. Water chemistry measurements were also taken with a handheld multi-meter (Eutech PCSTestr 35) approximately 5 – 10cm deep every three to five quadrats. This included temperature, pH, electrical conductivity (EC) and total dissolved solids (TDS). The Nahoon River was sampled on a monthly basis, and the Midmar Dam, every three weeks.

3.2.2.b *Impact of* Hydrellia egeriae

To establish the abundance of *H. egeriae* at the specific site, one to two apical *E. densa* shoots (± 15 – 25cm) per quadrat were collected along the transect and placed in a Ziploc® bag. In the laboratory, the bag was kept in a fridge until further inspection within three days. Shoots were cut to 15cm in length, gently dried with paper towel, and weighed to obtain fresh mass (g) using an analytical balance (RADWAG® AS 110.R2). The number of whorls per 15cm shoot was recorded. Subsequently, whorls were inspected under a stereo microscope (Olympus SZ61) and the number of mined leaves was recorded. The number of whorls were multiplied by four to give a total number of leaves per shoot. Four was selected as a proxy, since *E. densa* generally have four leaves per whorls (Cook and Urmi-König 1984; Yarrow *et al.* 2009). The number of mined leaves was divided by the total number of shoot to determine the percentage mining per individual shoot.

3.2.2.c *Abundance of* Hydrellia egeriae

In addition, the number of *H. egeriae* eggs, larvae and pupae was also recorded. Individual fresh shoot mass and the recorded number of *H. egeriae* individuals was used to calculate the number of *H. egeriae* immatures kg^{-1} fresh mass of *E. densa* (Equation 3.). Subsequently, -the mean number of immatures.$kg^{-1}$of *E. densa* was calculated and used to describe the population size of the site per sampling event.

Equation 3.1:

$$\chi = \frac{Number\ of\ immatures\ per\ shoot}{fresh\ shoot\ mass\ (g)} \times 1000$$

3.2.3 Statistical analyses

Normality of data (number of immatures kg-1 fresh mass of *Egeria densa* and percentage leaf-mining) was visually checked. The non-parametric Kruskal Wallis test was used to establish if biological control agent abundances and percentage leaf-mining changed over time at each site. The post hoc Kruskal Wallis Nemenyi Tukey test was used to determine differences between sampling months. A visual normality test showed that *E. densa* biomass (g) was normally distributed. A linear mixed effect model was used to establish the effect of leaf-mining and water chemistry measurements on the biomass (g) of *E. densa*. Sampling date was used as a random variable. Water pH was excluded from the predictor variables, due to high correlation with water temperature. Thus, only water electrical conductivity (EC) and temperature were ultimately used. Model selection was done using the Multi-model inference (MuMIn) package. Models with $\Delta AIC < 6$ were considered plausible models. Instead of selecting one model and obtaining coefficient estimates from it, model averages were obtained, and refers to using several models (plausible models AIC > 6) at once for making predictions. These analyses were done separately for each site due to major differences in effect sizes of measured parameters.

The change in aquatic plant community composition for each site over the study period was visualized using a non-metric multi-dimensional scaling (nMDS) plot. Non-metric multi-dimensional scaling uses Bray-Curtis similarity index, whereby values between 0 and 1 are used (0 meaning two surveys share the same species, and 1 meaning two surveys do not share any common species). Stress values are used for plot validity (< 0.05 = excellent; < 0.10 = good; < 0.20 usable; > 0.20 unacceptable) (Clarke 1993). Stress provides a measure of the

degree to which the distance between samples in reduced dimensional space (usually 2-dimensions) corresponds with the actual multivariate distance between the samples. Lower stress values indicate greater conformity and are desirable. High stress values indicate that there were no 2-dimensional arrangement of points that reflect their similarities. All analyses were conducted in the R environment (R Core Team 2019).

3.3 **Results**

3.3.1 Nahoon River

3.3.1.a *Abundance of* Hydrellia egeriae

Four months after the first release of the biological control agent, the number of immatures recorded per kilogram fresh mass of *E. densa* was 61 ± 27 (Figure 3-6*:*). After that, the population size increased substantially and reached a mean population size of 515 ± 147 immatures kg^{-1} fresh mass of *E. densa* in June 2019. However, after 2019, the population declined and no evidence of the biological control agent was found from July to September 2020, with 91 ± 91 recorded for October 2020. Over sampling periods, the *H. egeriae* population changed significantly ($H = 33.4$; $df = 11$, $P < 0.001$), but there were no significant differences between sampling months (Figure 3-6*:*).

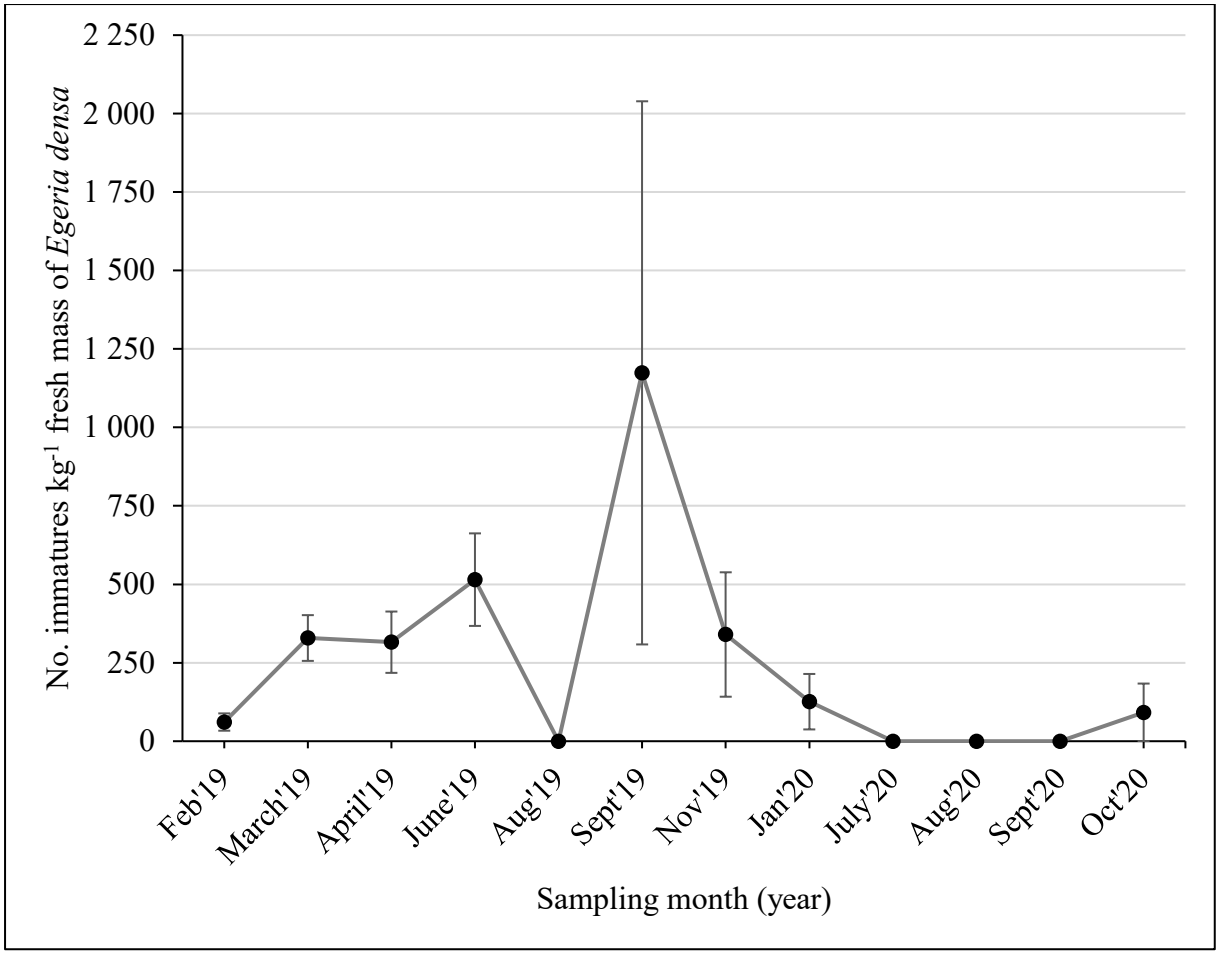


Figure 3-6: *Hydrellia egeriae* abundance, measured as number of immatures kg^{-1} fresh mass of *E. densa* in the Nahoon River for the period February 2019 to October 2020. Error bars represent standard error (±SE).

Percentage leaf-mining ranged between 0 and 1.1% between February 2019 and October 2020 (Figure 3-7*:*). Mean percentage leaf-mining for the study period was 0.38 ± 0.01%, with the highest single damage score at 1.1%, recorded October 2020.

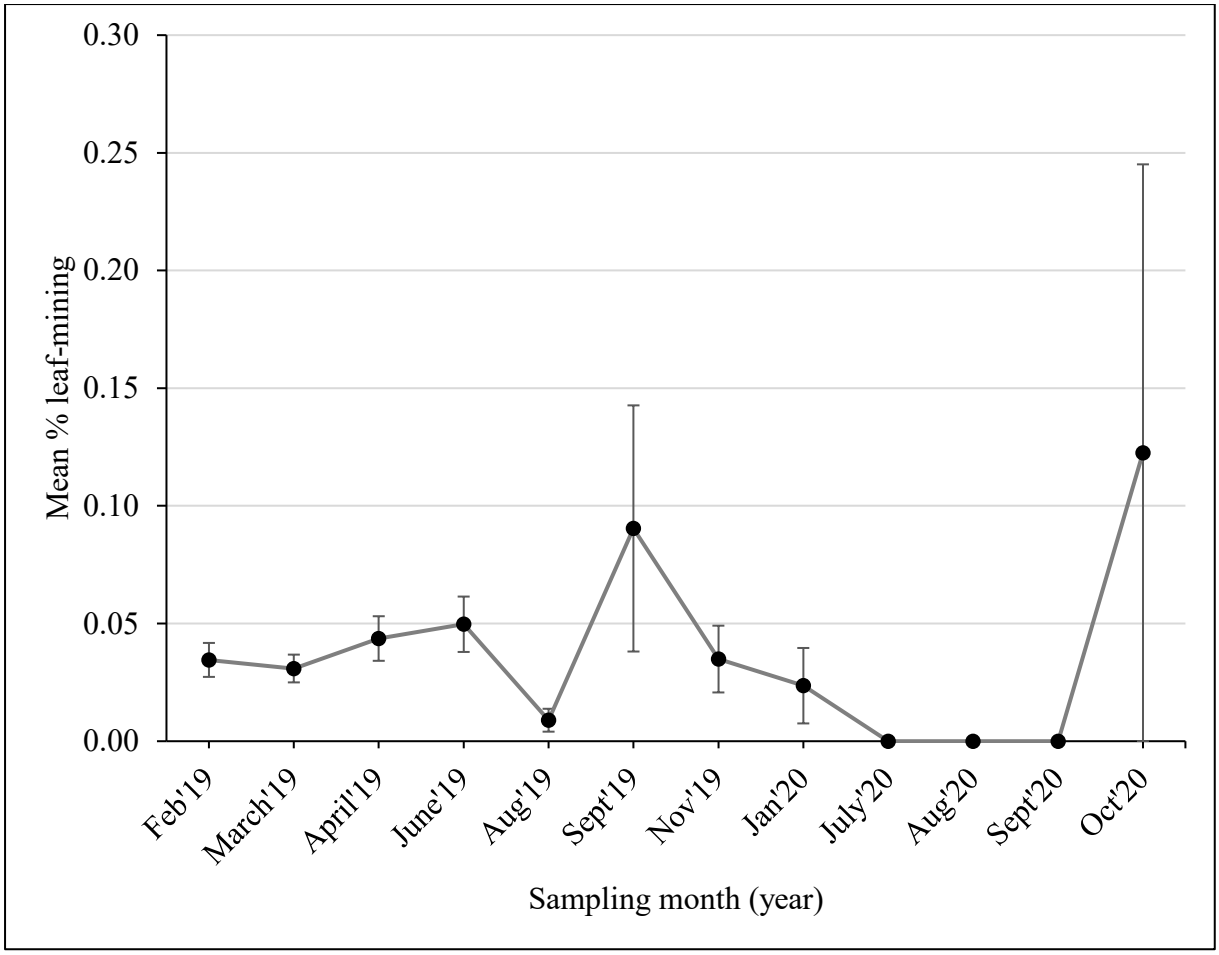


Figure 3-7: Mean percentage (%) leaf-mining recorded for *Egeria densa* (15cm shoot) in the Nahoon River for the period February 2019 to October 2020. Error bars represent standard error (±SE).

3.3.1.b *Impact of* Hydrellia egeriae *mining on* Egeria densa *biomass*

Model selection from a global model containing main effects between percentage leaf-mining, water electrical conductivity, water temperature and sampling date as random variable and their effect on *E. densa* biomass (g) in the Nahoon River is presented in Table 3-2. A model containing only water temperature (model 1) was weighted the most, and was 1.95 times (0.39/0.2 = 1.95) more likely to explain the variance in *E. densa* biomass than model 2 that contained no predictor variables (null model). Model 2 was also 1.7 times more likely to explain the variance in *E. densa* biomass compared to a model with predictor variables leaf-mining and water temperature (model 3).

Table 3-2: Model selection output for *Egeria densa* shoot biomass (g) linear mixed model (LMMs). Model selection was performed using Akaike's information criterion corrected for small sample sizes (AICc). K = number of parameters specified in the model. Only plausible models (ΔAICc < 6) are presented. EC: electrical conductivity.

Model no.	Model structure	K	LogLik	AICc	ΔAICc	Weight
1	Temperature	11	-79.22	183.8	0.00	0.39
2	Null model	10	-81.17	185.1	1.29	0.20
3	Leaf-mining + Temperature	12	-79.05	186.2	2.33	0.12
4	EC + Temperature	12	-79.17	186.4	2.56	0.11
5	Leaf-mining	11	-81.00	187.4	3.55	0.07
6	EC	11	-81.04	187.5	3.64	0.06
7	EC + Leaf-mining + Temperature	13	-79.00	188.8	4.96	0.03
8	EC + Leaf-mining	12	-80.87	189.8	5.97	0.02

Full model averages are presented in Table 3-3 and indicate that none of the predictor variables had a significant effect on *E. densa* biomass in the Nahoon River for the study period.

Table 3-3: Global model averages (subset at ΔAICc < 6) with coefficient estimates of *Egeria densa* biomass (g) affected by leaf-mining, water temperature and electrical conductivity in the Nahoon River. EC: electrical conductivity.

Predictor variable	**Estimate**	**Std Error**	***z* value**	***P* value**	**Confidence intervals**		**Range**	**Mean (S.E.)**
					2.5%	**97.5%**		
Intercept	-0.508	2.631	0.19	0.85	-5.70	4.68		
Temperature	0.118	0.110	0.98	0.33	-0.02	0.39	15.9 – 27.5°C	22.5 ± 0.5°C
Leaf-mining	0.044	0.174	0.25	0.80	-0.45	0.82	0 – 1.1%	0.04 ± 0.01%
EC	-0.0001	0.001	0.14	0.89	-0.003	0.003	529 – 1 653µs cm^{-1}	1 148.6 ± 35.0µs cm^{-1}

3.3.1.c *Aquatic macrophyte mapping*

Hydrellia egeriae was released in the Nahoon River in October 2018. A pre-release survey of the community composition was conducted in August 2018, and the first post-release survey was conducted in November 2018. The nMDS plot shows that for the year 2018, the composition of the aquatic vegetation remained similar (Figure 3-8). Although only released in October, 2018, the biological control agent was present from October to December. The aquatic vegetation composition started to change within the following two years, with some points dispersing away from this "initial community" (grey and blue symbols). This change in community composition did not occur due to the presence of the biological control agent, as communities with and without the agent showed no clustering. Therefore, for this study period, in addition to biological control efforts, other factors must have contributed to the change in aquatic vegetation composition in the Nahoon River. For example, in April 2019, a flooding event washed aquatic vegetation downstream, which opened parts of the river surface (Table 3-4).

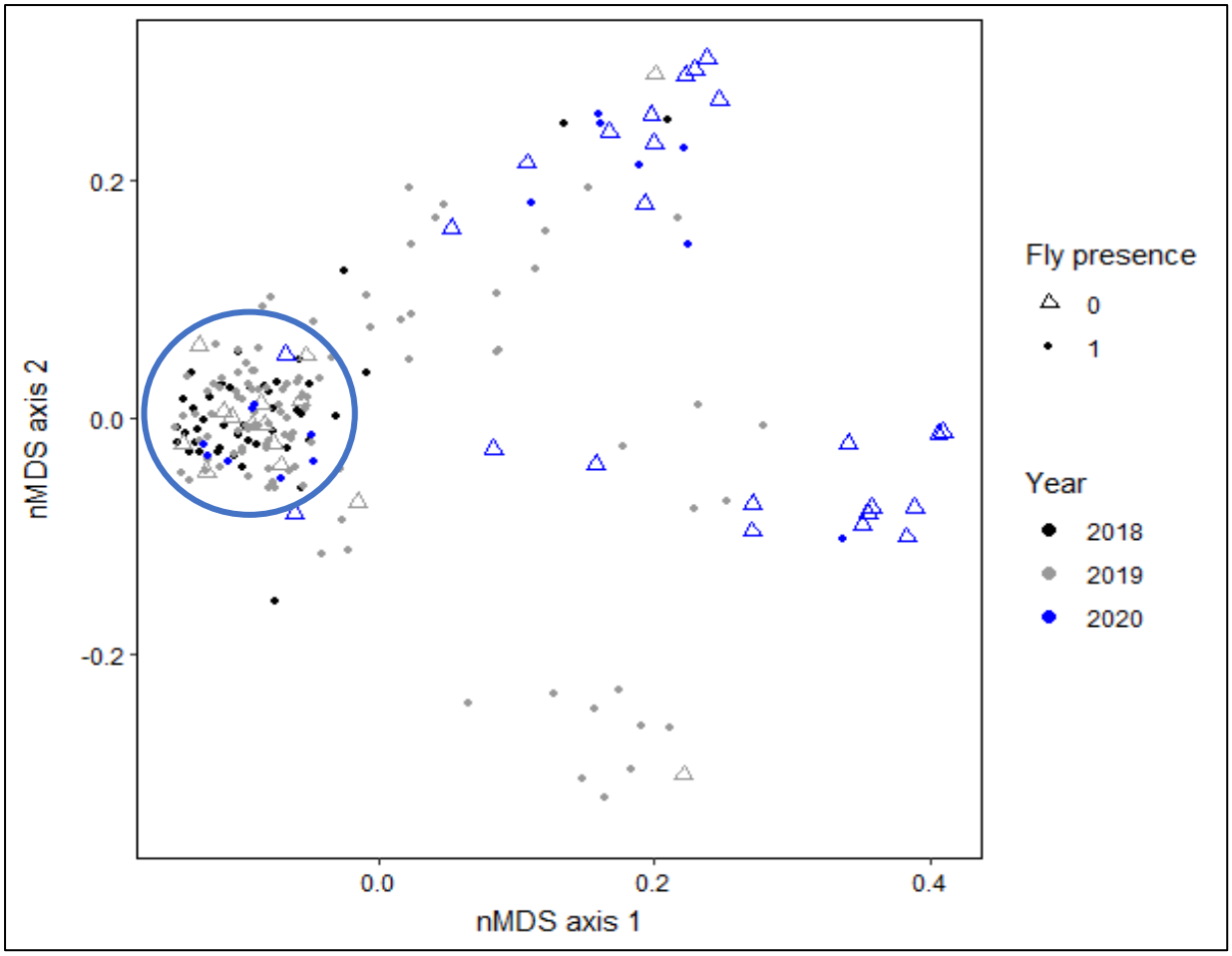


Figure 3-8: Non-metric multi-dimensional scaling plot of the aquatic vegetation community composition in the Nahoon River from 2018 to 2020. Plot stress value is 0.08. Blue circle: clustering of quadrat measurements indicating similar macrophyte community composition.

Table 3-4: Mean percentage surface cover of aquatic vegetation (±SE) recorded in the Nahoon River during pre- and post-release surveys for the period 2018 to 2020. Red shaded columns represent invasive species, and green shaded columns represent indigenous species.

Year	Month	Submerged			Floating			Emergent		Open water
		Egeria densa	*Ceratophyllum demersum*	*Potamogeton pusillus*	*Pontederia crassipes*	*Pistia stratiotes*	*Azolla filiculoides*	*Persicaria sp.*	Grass	
2018	Aug	79.6 ± 9.5	14.2 ± 9.3	0.0 ± 0.0	0.6 ± 0.6	0.6 ± 0.2	0.0 ± 0.0	0.7 ± 0.5	4.0 ± 0.0	0.0 ± 0.0
	Nov	89.7 ± 6.0	6.6 ± 4.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Dec	96.5 ± 1.6	2.4 ± 1.3	0.0 ± 0.0	1.2 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2019	Jan	85.5 ± 6.3	14.3 ± 6.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
	Feb	81.0 ± 6.9	15.7 ± 5.2	0.0 ± 0.0	3.3 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	March	77.3 ± 7.5	15.8 ± 5.2	0.0 ± 0.0	2.2 ± 1.7	0.0 ± 0.0	0.4 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	4.2 ± 4.2
	April*	69.8 ± 10.8	5.1 ± 3.6	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	25.5 ± 11.0
	June	90.9 ± 6.8	0.0 ± 0.0	0.0 ± 0.0	8.7 ± 0.0	0.0 ± 0.0	0.5 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	8.7 ± 6.8
	July	45.1 ± 12.9	1.0 ± 1.0	0.1 ± 0.1	12.3 ± 8.4	1.0 ± 1.0	0.8 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	6.7 ± 6.7
	Aug	83.2 ± 8.9	7.0 ± 6.7	0.0 ± 0.0	1.2 ± 1.2	0.0 ± 0.0	0.6 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	8.0 ± 6.7
	Sep	78.5 ± 8.7	4.0 ± 2.1	0.1 ± 0.1	2.0 ± 2.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Nov	57.9 ± 10.4	8.8 ± 6.2	0.0 ± 0.0	23.7 ± 10.2	0.9 ± 0.7	1.1 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	7.7 ± 5.2
2020	Jan	56.7 ± 12.4	32.9 ± 11.2	0.0 ± 0.0	9.3 ± 7.0	0.0 ± 0.0	0.4 ± 0.1	0.0 ± 0.0	0.7 ± 0.6	0.0 ± 0.0
	July	16.6 ± 6.7	42.7 ± 14.7	0.0 ± 0.0	38.7 ± 12.3	0.0 ± 0.0	1.3 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
	Aug	15.7 ± 9.0	31.6 ± 12.0	0.0 ± 0.0	52.0 ± 12.0	0.5 ± 0.3	1.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Sept	9.5 ± 4.3	21.1 ± 8.5	0.0 ± 0.0	46.3 ± 11.3	2.4 ± 1.3	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	20.3 ± 9.7
	Oct	18.7 ± 8.5	25.3 ± 9.8	0.0 ± 0.0	54.5 ± 11.1	0.7 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

* A flooding event occurred during this month

3.3.2 Midmar Dam

3.3.2.a *Abundance of* Hydrellia egeriae

Post-release surveys in the Midmar Dam were conducted every three months since the release of the biological control agent in March 2019. During the first post-release survey, the *H. egeriae* population count was 139 immatures kg^{-1} fresh mass of *E. densa*. A Kruskal Wallist test showed that the fly population changed significantly over sampling events (*H = 25.51; df = 4; P < 0.001*; Figure 3-8). In March 2020, the agent population significantly declined to 0 immatures kg^{-1} fresh mass of *E. densa*. However, augmentative release (Table 3-1) significantly boosted the agent population, which is seen in population counts of 2 411 and 502 immatures kg^{-1} fresh mass of *E. densa* during August and October 2020 (Figure 3-8). In addition, the number of releases significantly affected the population size of the *H. egeriae* between the two sites (*H = 4.72; df = 1; P < 0.05*).

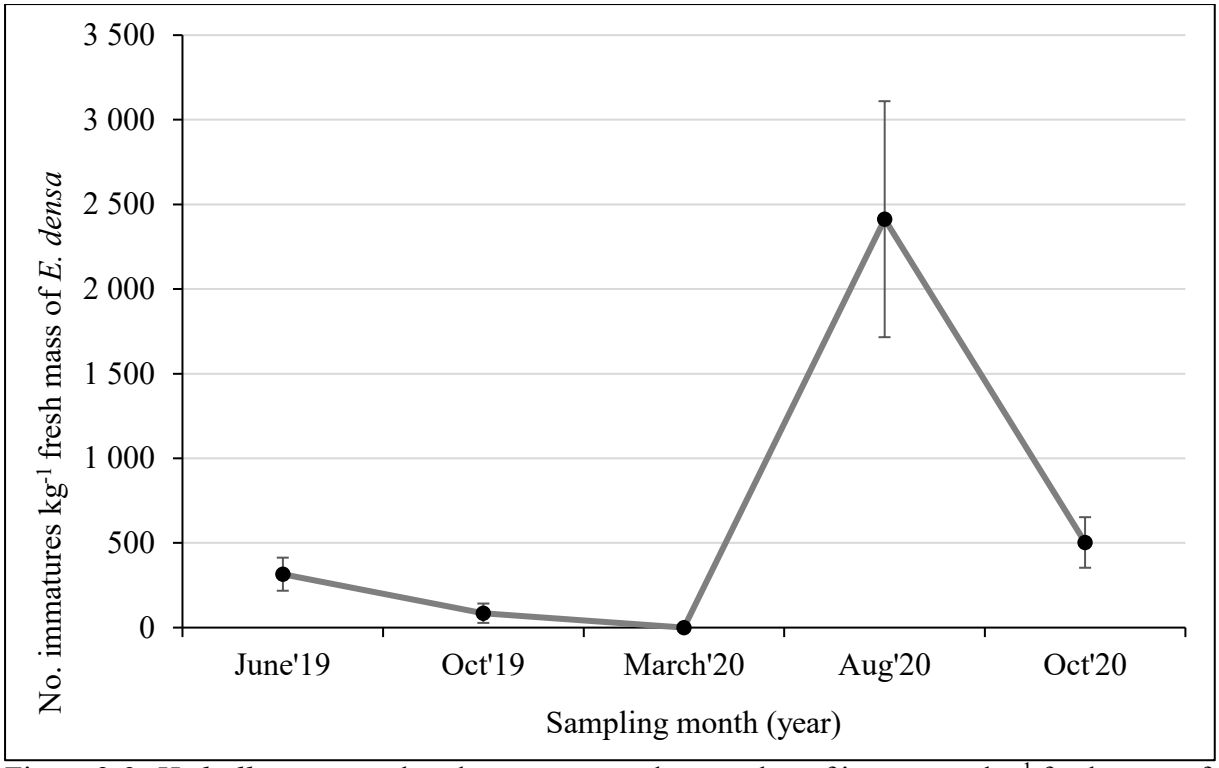


Figure 3-9: *Hydrellia egeriae* abundance, measured as number of immatures kg^{-1} fresh mass of *E. densa* in the Midmar Dam for the period June 2019 to October 2020. Error bars represent standard error (±SE).

The percentage leaf-mining recorded for *E. densa* in the Midmar Dam ranged between 0 and 73.1% between June 2019 and October 2020 (Figure 3-9). Percentage leaf-mining for the study period was 10.15 ± 2.17%, which is 27 times more than what was recorded for the Nahoon River.

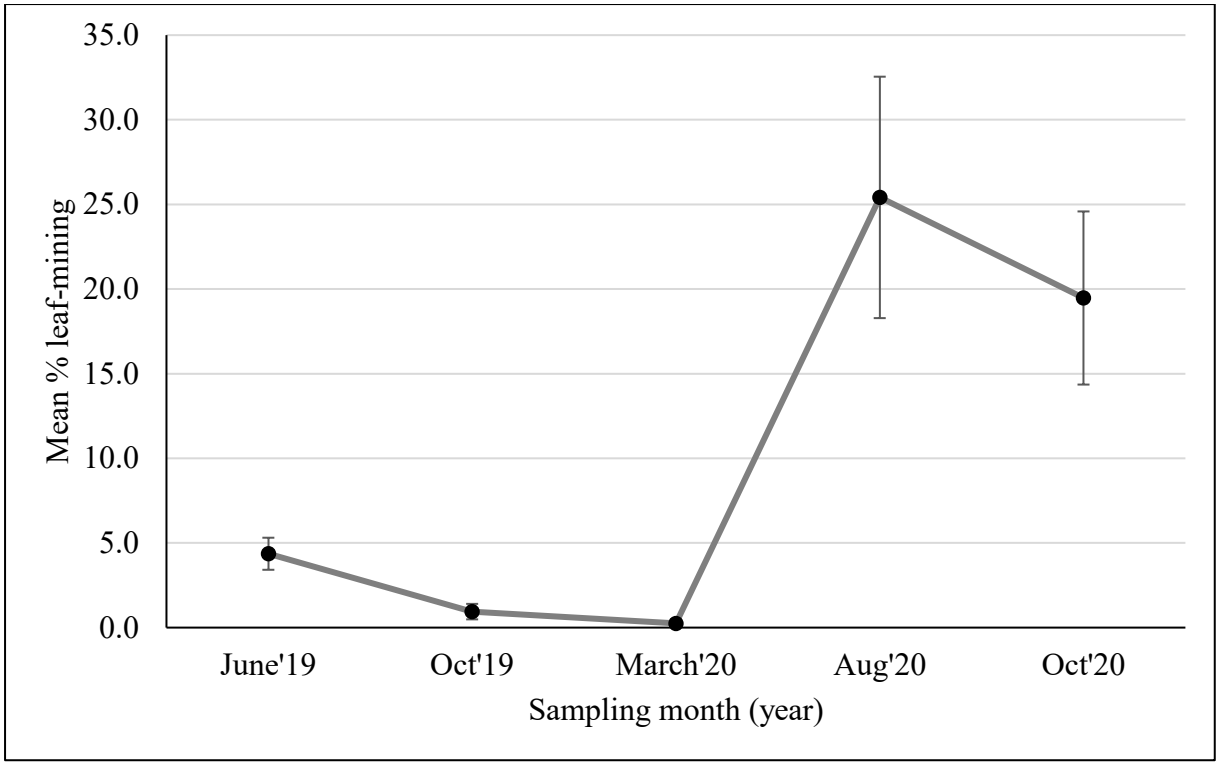


Figure 3-9: Mean percentage (%) leaf-mining recorded for *Egeria densa* (15cm shoot) in the Midmar Dam for the period February 2019 to October 2020. Error bars represent standard error (±SE).

3.3.2.b *Impact of* Hydrellia egeriae *mining on* Egeria densa *biomass*

Model selection from a global model containing main effects between percentage leaf-mining, water electrical conductivity, water temperature and sampling date as random variable are shown in Table 3-5. The weight of the model containing water electrical conductivity and temperature was 0.510, followed by a model containing water electrical conductivity, water temperature and leaf-mining at 0.46. This means that model 1 is 1.1 times more likely (0.51/0.46=1.1) to explain the variation in *E. densa* biomass (g) than model 2 and 17 times more likely than model 3, which contains only water electrical conductivity. Full model averages, indicate that none of the predictor variables statistically increased or reduced *E. densa* biomass (Table 3-6).

Table 3-5: Model selection output for *Egeria densa* shoot biomass (g) linear mixed model (LMMs). Model selection was performed using Akaike's information criterion corrected for small sample sizes (AICc). K = number of parameters specified in the model. Plausible models (ΔAICc < 6) are presented. EC: electrical conductivity.

Model no.	Model structure	K	LogLik	AICc	ΔAICc	Weight
1	EC + temperature	8	-37.9	95.5	0.00	0.51
2	EC + leaf-mining + temperature	9	-36.4	95.7	0.18	0.46
3	EC	7	-42.1	101.1	5.62	0.03

Table 3-6: Full model averages (subset at (ΔAICc < 6) with coefficient estimates of *Egeria densa* biomass (g) affected by leaf-mining, water temperature and electrical conductivity in the Midmar Dam. EC: electrical conductivity.

Predictor variable	**Estimate**	**Std Error**	***z value***	***P value***	***Confidence intervals***		**Range**	**Mean (S.E.)**
					2.5%	**97.5%**		
Intercept	**3.324**	**1.012**	**3.22**	**<0.01**	**1.300**	**5.348**		
EC	0.017	0.002	1.42	0.15	-0.006	0.041	66 – 203.4µs cm^{-1}	127 ± 28.6µs cm^{-1}
Temperature	-0.168	0.053	0.63	0.53	-0.703	0.357	15.8 – 24.1°C	20.6 ± 1.7°C
Leaf-mining	-0.005	0.007	0.71	0.48	-0.025	0.002	0 – 73.1%	11.7 ± 2.7%

3.3.2.c *Aquatic macrophyte mapping*

A pre-release survey of the community composition in the Midmar Dam was conducted in August 2018, and the first post-release survey was conducted in June 2019 (Table 3-7). The nMDS plot shows that community composition was distinct between years and the presence of the biological control agent (Figure 3-10). In 2018 (grey, open triangles), the community was more heterogeneous, i.e. more aquatic plant species present, and *E. densa* only covered 41.1% of the study site. However, in the following years, *E. densa* became more dominant, covering over 90% of the site, despite the presence of the biological control agent. From the nMDS plot, there is distinct clustering between the absence and presence of the biological control agent. However, it is clear that *E. densa* cover did not decrease in the presence of the biological control agent, but instead it increased in cover (Table 3-7). Similar to the community composition change in the Nahoon River, other factors must have contributed to the change in the community composition in the Midmar Dam.

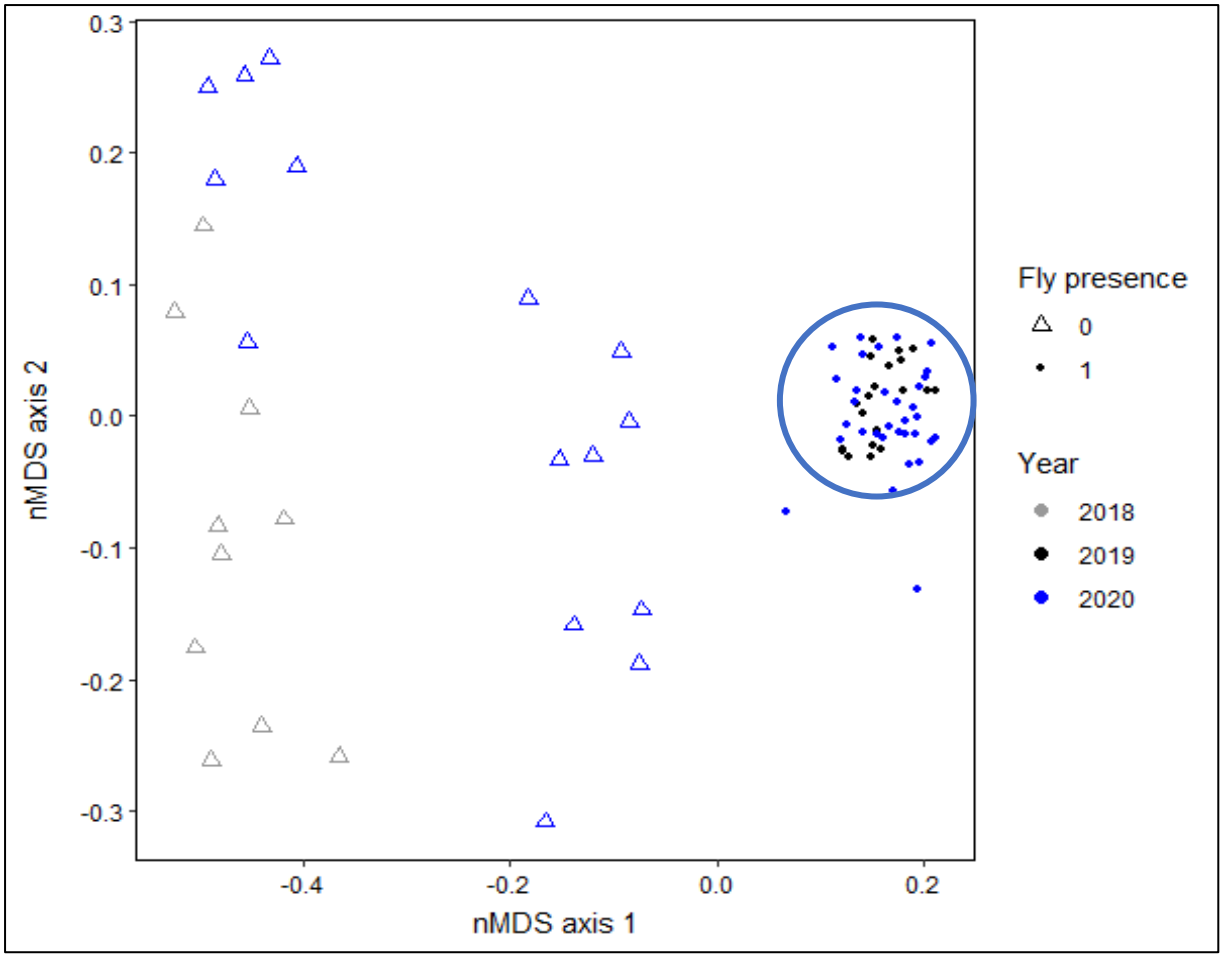


Figure 3-10: Non-metric multi-dimensional scaling plot of the aquatic vegetation community composition in the Midmar Dam for the year 2018 to 2020. Plot stress value is 0.03. Blue circle: clustering of quadrat measurements indicating similar macrophyte community composition.

Table 3-7: Mean percentage surface coverage of aquatic vegetation (±SE) recorded in the Midmar Dam during pre- and post-release surveys for the period 2018 to 2020. Red shaded columns represent invasive species, and green shaded columns represent indigenous species.

Year	Month	Submerged		Emergent			Floating	Open water
		Egeria densa	***Lagarosiphon muscoides***	***Myriophyllum aquaticum***	**Grass**	***Persicaria senegalensis***	***Spirodela/Lemna***	
2018	August	41.4 ± 1.6	0.2 ± 0.2	1.9 ± 1.0	26.0 ± 11.2	9.6 ± 6.3	0.0 ± 0.0	0.0 ± 0.0
2019	October	99.3 ± 0.5	0.1 ± 0.1	0.0 ± 0.0	0.6 ± 0.5	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
2020	March	55.1 ± 12.7	0.0 ± 0.0	0.5 ± 0.5	3.5 ± 3.3	0.5 ± 0.5	16.0 ± 7.3	36.1 ± 12.2
	August	97.5 ± 2.5	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.5	1.8 ± 0.0
	October	99.8 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2

3.4 Discussion

Results from this study indicate that *H. egeriae* has the ability to establish and proliferate at released sites.Augmentative releases significantly increased the biological control agent population in the Midmar Dam, which resulted in 27 times more agent herbivory compared to the Nahoon River, where only one release was made. During the highest population count, which was recorded in the Midmar Dam in August 2020, leaf-mining reached to 25% shoot damage, followed by 19% in October 2020. This level of damage came close to native range damage, where on average, 35% of the whorls of 15 to 25cm *E. densa* shoot was damaged by *H. egeriae* (Cabrera Walsh *et al.* 2012). However, in the Midmar Dam, these levels of leaf-mining was periodic, and during the study period, did not translate into an overall significant reduction in *E. densa* biomass.

Electrical conductivity is a measure of ion concentration, which includes dissolved inorganic carbon (DIC), chloride, sulphate and associated cations. Although electrical conductivity did not statistically affect *E. densa* biomass, it is clear that ion-rich waters provide higher submerged macrophyte productivity. In addition, the high influx of nutrients, i.e. sewage and agricultural runoff, present at both sites cannot be ignored. For example, water phosphate (PO_3^{4-}) readings of 3.43 ± 0.65 mg/L and 1.5 ± 0.3 mg/L were measured in the Nahoon River and Midmar Dam respectively during August 2020. In the USA, tank feeding trials with *H. pakistanae* indicated that the agent was able to reduce the biomass of its target plant, *H. verticillata*, at low nutrient levels (5g fertilizer / 17kg sand using Osmocote plus N: P: K 18: 6: 12). Under high nutrient levels (15g fertilizer / 17kg sand), plant biomass remained the same, despite high agent densities (Wheel & Center 2001). These results highlight what has been seen in freshwater biological control efforts, i.e. that high nutrient levels promote aquatic macrophyte growth and counteract biological control efforts (Coetzee *et al.* 2011a; Coetzee & Hill 2012; Hill & Coetzee 2017; Strange *et al.* 2018).

Freshwater systems are open, dynamic systems that are susceptible to external factors, i.e. extreme weather, anthropogenic activities, and internal factors, i.e. biochemical processes, community composition (Scheffer 2009). Both aspects affect the stable state, shifts or loops that freshwater systems undergo (Scheffer 2009). Although *E. densa* is an ecosystem engineer in the systems it invades, it is still subjected to these external and internal factors and processes. Thus, the changes in *E. densa* biomass and cover in the Nahoon River cannot be explained by the presence or absence of the biological control agent only, as it is a complex system. For example, *E. densa* had to compete with other floating aquatic weeds, which have competitive advantage for sunlight (Strange *et al.* 2018). Indeed, water hyacinth increased its dominance and spread at the site during the study period. Additionally, *C. demersum* is also present in the Nahoon River, and has also increased its dominance. In comparison, species richness was lower in the Midmar Dam, and the only other invasive aquatic weed was *M. aquaticum*, which is successfully suppressed by its biological control agent, *Lysathia* sp (Coleoptera: Chrysomelidae) (Cilliers 1999). The only other aquatic plant that increased its distribution in the Midmar dam was duckweed, which has a floating growth form.

Thus, results from this post-release evaluation suggests that *H. egeriae* has established, but in the case of the Nahoon River, where only one release was made, field populations start to diminish without augmentative releases. As is seen in the Midmar Dam, augmentative releases supported and boosted field populations. The biological control agent has also shown potential to affect its target weed, as was seen for a short period in the Midmar Dam, but it has not translated into population-level reductions in density within the time scale of the study period. Population-level impact isconstrained due to generally low population levels, as was seen in the Nahoon River. In a laboratory experiment, Mangan & Baars (2016) showed that *L. major* increased shoot and side shoot growth with damage inflicted by *H. lagarosiphon*. Shoot biomass was not significantly reduced, even after 134 days, however, there was a reduction in

establishment of damaged shoots. Considering the time lag for leaf-mining damage to translate into reduced plant fitness, it is possible that *H. egeriae* can have population-level effects on its target weed, but this result will depend on continued biological control effort and post-release surveys. Similarly, future studies should investigate side shoot production in the presence of *H. egeriae* herbivory.

This study is an initial step in using *H. egeriae* as a biological control agent, after permission for release was granted. Results from this study indicate that a release strategy will require augmentative releases in South Africa. Future work can investigate the number of individuals required per plant or area for optimal top down control. Comparatively, Wheeler and Center (2001) found that *H. pakistanae* leaf-mining reached an asymptote at 4 000 larvae/m^2, however field populations only reached 15 adults/m^2 and 15% of *H. verticillata* whorls damaged.

At this point, it would be tempting to conclude that *H. egeriae* top down control is only limited by release events, since the agent is climatically compatible with its novel range. However, factors that affect successful agent establishment include propagule pressure (i.e. release strategy), climate compatibility and parasitism/and or predation (Stiling 1993; Lockwood *et al.* 2005; Fauvergue *et al.* 2012). In its native range, *H. egeriae* parasitism rates were 10.4% from hymenopteran wasps of the families Braconidae and Figitidae (Cabrera Walsh *et al.* 2012). Considering the presence of host-parasitoid interactions in the native range of *H. egeriae* (Cabrera Walsh *et al.* 2012) and the presence of *Hydrellia* spp. parasitoids in South Africa (Martin *et al.* 2013), the acquisition of new parasitoids are highly likely, especially because of the phylogenetic relatedness of both host-parasitoid associations (Paynter *et al.* 2010). Therefore, *H. egeriae* field populations may be affected by parasitism, and if so, it would be beneficial to establish parasitism rates.

4. Parasitism of *Hydrellia egeriae*

4.1 Introduction

There are many contributing factors to successful biological invasions (McEvoy 2018), including the enemy release hypothesis (Keane & Crawley 2002). Introduced biological control agents should be able to establish, proliferate and disperse in their novel environment, because they are released, partially (enemy inversion) or completely (enemy release), from their natural enemies (Keane & Crawley 2002; Heimpel & Mills 2017; Schulz *et al.* 2019). Plant-herbivore relationship occurs within the first and second trophic level, but, ecological interactions are far more complex. In a literature review on the hypotheses that imply that antagonist effects contribute to invasion success, Schulz *et al.* (2019) found ten hypotheses that function up until the fourth trophic level, and there are eight hypotheses that apply to introduced biological control agents (second trophic level) of invasive alien plants (Figure 4-1).

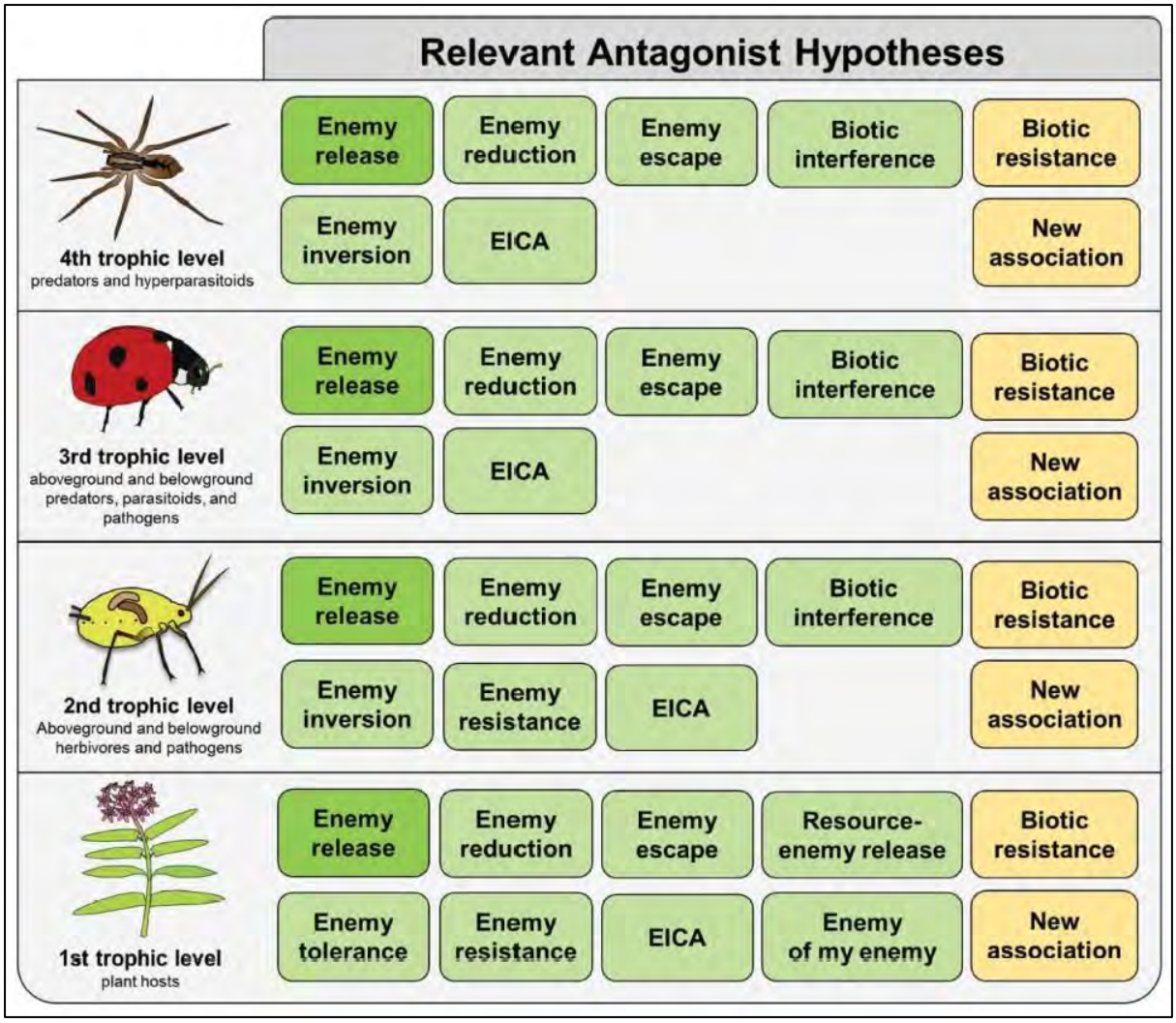


Figure 4-1: Framework of antagonist hypotheses that can function within each trophic level. EICA: evolution of increased competitive ability. Taken from Schulz *et al.* 2019.

Previous studies have shown that biological control agents often experience antagonism i.e. biotic resistance in their introduced range (Cornell & Hawkins 1993; Hill & Hulley 1995; Paynter *et al.* 2010; Martin *et al.* 2018), which reduces their capacity to exert top-down control on the target weeds, i.e. biotic interference (Schulz *et al.* 2019). Because of the impact that parasitism/predation can have on the success of a biological control programme, it is preferable to consider potential parasitism/predation before an agent is released (Goolsby *et al.* 2006; Paynter *et al.* 2010). Fortunately, over the last few decades, there has been an increase in studies that investigate these indirect non-target effects. Patterns obtained from these studies provide some prediction as to which potential biological control agents may experience antagonism in

the introduced range, with particular emphasis on parasitism. Here, it is important to distinguish between parasitoid richness and parasitism rates, because richness can be high, but overall parasitism may be low or *vice versa* (van Klinken & Burwell 2005).

4.1.1 Native vs. introduced communities

The number of parasitoids that agents acquire in their introduced range, i.e. spillback (Kelly *et al.* 2009) is often a reflection of the number of parasitoids in their native range attacking similar species or guilds; i.e. ecological analogues (Cornell & Hawkins 1993; Paynter *et al.* 2020). Parasitoids in the introduced range are often generalist species (Cornell & Hawkins 1993), but specialist parasitoids can also extend their host range if the biological control agent is in the same genus as a native host i.e. phylogenetic relatedness (Godfray *et al.* 1995).

The feeding guild of the biological control agent has also been identified as a predictor of potential parasitism. Hill and Hulley (1995) found that poorly concealed endophages was the most affected with 62.5% of introduced species in this guild being parasitized, followed by well concealed endophages (47.1%) and ectophages (20%). When biological control agents were grouped into insect orders, Hill and Hulley (1995) also concluded that insect order may be a predictor of parasitism. Biological control agents within the orders Diptera and Lepidoptera are most attacked, while other orders are less likely to acquire parasitoids. For example, Christensen *et al.* (2011) conducted post-release surveys in 1997, three to four years after the release of *Oxyops vitiosa* (Pascoe) (Coleoptera: Curculionidae), a coleopteran biological control agent released against *Melaleuca quinquenervia* (Cav.) S. T. Blake (Myrtaceae). They found no parasitoid acquisition, but only predation from arthropods and arachnids. In addition, while no formal surveys have been conducted since 2011, anecdotal evidence suggests that *O. vitiosa* continues to remain parasite free in Florida, USA (Pratt, pers. comm.). Thus, in some cases it seems that certain insect orders may have less chance of acquiring parasitoids, and so far, time since introduction has not increased its chances.

4.1.2 Natural enemies of *Hydrellia* spp.

Considering these factors, it is likely that *H. egeriae* will experience parasitism in its introduced range, South Africa. The Ephydridae family has approximately 2 000 described species that are distributed worldwide. Species within the family are generally herbivores of aquatic and semi-aquatic plants. Natural enemies of the genus *Hydrellia* are well documented (Deonier 1971; Stiling *et al.* 1984; Kula *et al.* 2009; Cabrera-Walsh *et al.* 2012; Coon *et al.* 2014; Katzenberger & Zacharias 2015). The most common parasitoid family associated with *Hydrellia* spp. is Braconidae, the second largest family within the order Hymenoptera, containing more than 40 000 known species (Goulet & Huber 1993).

Investigations have shown that *Hydrellia* spp. parasitism rates vary, but they can be high and exert significant top-down control. In a revision on Nearctic *Hydrellia*, Deonier (1971) found that, for example, parasitism of *Hydrellia ischiaca* Loew and *Hydrellia pulla* Cresson by braconids and diaprids were 38% and 63%, respectively. In South Africa, *H. lagarosiphon* has its own suite of parasitoids, which includes the parasitic wasps *Ademon lagarosiphonae* van Achterberg (Opiinae), *Chaenusa anervata* van Achterberg and *Chaenusa seminervata* van Achterberg (Alysiinae) (Martin *et al.* 2013). Parasitism by *Chaenusa anervata* (Hymenoptera: Braconidae: Alysiinae) varies from 30% to 50%, with higher parasitism rates in winter (Martin *et al.* 2013). Some of the parasitoids were also new to science.

In its native range, *H. egeriae* parasitism rates were 10.4% and included *Chaenusa aurantium* Kula and Martinez (Hymenoptera: Braconidae), *Hydrelliaeucoila egeria* Díaz and Gallardo (Hymenoptera: Figitidae), and an unidentified pupal fungus (Cabrera Walsh *et al.* 2012). Considering the presence of host-parasitoid interactions in the native range of *H. egeriae* and the presence of *Hydrellia* spp. parasitoids in South Africa, the acquisition of new parasitoids is highly likely. The probability is especially increased by the phylogenetic relatedness of both host-parasitoid associations (Paynter *et al.* 2010), and further supported by

the biological control programme against *H. verticillata* in the USA, where *Trichopria columbiana* Ashmead (Hymenoptera: Diapriidae) expanded its host range to *H. pakistanae* and *H. balciunasi* (Coon *et al.* 2014). Despite parasitism rates of between 20% to 30%, the *Hydrellia* spp. biological control agents still established, although target weed suppression was compromised (Coon *et al.* 2014). Generally, the challenge is not to predict parasitism diversity, but rather parasitism rates (van Klinken & Burwell 2005). Considering these factors, it is predicted that *H. egeriae* will experience some level of parasitism, and, post-release surveys are necessary to establish parasitism rates.

4.1.3 An indirect effect

In addition to measuring the parasitism richness, rate and effect on the biological control agent, parasitoid acquisition also has indirect effects on biological control programmes (Paynter *et al.* 2020), as it may change native food webs by for example, increasing parasitoid abundance of native herbivores (Willis & Memmott 2005; Veldtman *et al.* 2011; Todd *et al.* 2020). These indirect non-target effects are harder to predict (Paynter *et al.* 2020), simply because they may only appear after some time has lapsed, i.e. three to four years (Hill & Hulley 1995) or after evolutionary adaptation (van Klinken & Burwell 2005). Post-release surveys also offer the opportunity to measure these indirect effects *ad hoc* (Veldtman *et al.* 2011) and provide valuable information for future biological control programmes (Paynter *et al.* 2020).

The objectives of this study were to determine if *H. egeriae* has acquired parasitoids at released sites in South Africa, and if so, the second objective was to identify the parasitoid species. To optimize biological control efforts and fine-tune release strategies, it is important to establish if parasitism rates change during with seasons.

4.2 Material and methods

4.2.1 Parasitoid collection

The presence and rate of *H. egeriae* parasitism was measured quarterly at the Nahoon River and Midmar Dam sites. Three to five quadrats (0.5m x 0.5m in size) of *E. densa* along the same sampling transect used in Chapter 3 were collected. All the *E. densa* found within a quadrat was collected and placed it in a black bag, water within the bags was drained and bags were placed in travelling containers with ice packs to keep conditions cool during transportation. At the laboratory, bags were placed in a 5°C fridge until dissected. In contrast to Chapter 3, where 15cm of an *E. densa* shoot was inspected for inviduals, 20 to 30cm of a shoot was inspected for *H. egerie* pupae in this study. The pupal life stage was chosen, because previous studies have shown that *Hydrellia* sp. parasitoids generally feed on late instar and pupae (Deonier 1971; Kula *et al.* 2009; Martin *et al.* 2013; Coon *et al.* 2014). For example, in its native range, researchers collected *H. egeriae* pupae during a first inspection, and collected larvae turned pupae during a second inspection (Kula *et al.* 2009). Deonier (1971) mostly observed endoparasite larvae feeding on their host after *Hydrellia* spp. pupariation, which may be a result of physiological changes that stimulate parasitoid larval feeding.

A plant fragment containing a pupa was broken off from the *E. densa* shoot with forceps and placed in a vial containing fresh water. In some cases (e.g. where plant material had to be transported by plane) only three bags were collected. All the pupae found in the plant material per bag were collected. The number of pupae collected from all bags per sampling event was pooled. The total number of specimens collected per each sampling event are given in the results section. Vials were placed in a laboratory at room temperature and checked every second day for *H. egeriae* adult emergence or parasitoid emergence. Eclosing insects were collected and placed in an Eppendorff™ containing 96% ethanol. The percentage parasitism

were calculated by dividing the number of parasitoids that emerged by the total number of pupae that were collected per sampling event.

4.2.2 Parasitoid identification

Parasitoid specimens were sent for identification to Dr Simon van Noort at the Iziko Museum in Cape Town, South Africa. For comparison, holotype specimens of parasitoids collected from *H. lagarosiphon* were used. Images were acquired the Iziko Museum of Cape Town at SAMC with a Leica LAS 4.9 imaging system, comprising a Leica® Z16 microscope with a Leica DFC450 Camera and 0.63x video objective attached. The imaging process, using an automated Z-stepper, was managed using the Leica Application Suite V 4.9 software installed on a desktop computer. Diffused lighting was achieved using a Leica LED 5000 Dome. All images included in this chapter, and online interactive keys to species are available on WaspWeb: www.waspweb.org. Specimens used and described in this study have been deposited at the SAMC: Iziko South African Museum, Cape Town, South Africa (Curator: Simon van Noort) and SANC: National Collection of Insects, ARC, Pretoria (Curator: Vivienne Uys).

4.2.3 Statistical analysis

The distribution of percentage *H. egeriae* parasitism was visually checked for normality, and because the data were unevenly distributed, a Kruskal Wallis test was used for comparing percentage parasitism between sites (Nahoon River and Midmar Dam). In addition, potential changes in percentage parasitism over seasons was compared. Due to small sample sizes, autumn and winter were combined (cold) and spring and summer were combined (warm), which were used as the predictor variables in a Kruskal Wallis test. All analyses were conducted in the R environment (R Core Team 2019).

4.3 **Results**

4.3.1 Parasitoid richness

Three parasitoid species were reared from *H. egeriae* pupae, *Chaenusa seminervata* (Figure 4-2), *C. anervata* (Figure 4-3) and *Ademon lagarosiphonae* (Figure 4-4). Only one parasitoid species, *A. lagarosiphonae*, was recorded from the Nahoon River and all three parasitoids were recorded from the Midmar Dam site. In addition, a hyperparasitoid, *Janicharis africanus* Gumovsky & Delvare (Chalcidoidea: Eulophidae) (Figure 4-5), was collected from the Nahoon River[1].

Van Noort S., Smith R. & Coetzee J.A. 2021. Identity of parasitoid wasps (Hymenoptera: Braconidae and Eulophidae) reared from aquatic leaf-mining flies (Diptera: Ephydridae) on invasive Brazilian waterweed Egeria densa in South Africa. *African Invertebrates*. Manuscript submitted for publication

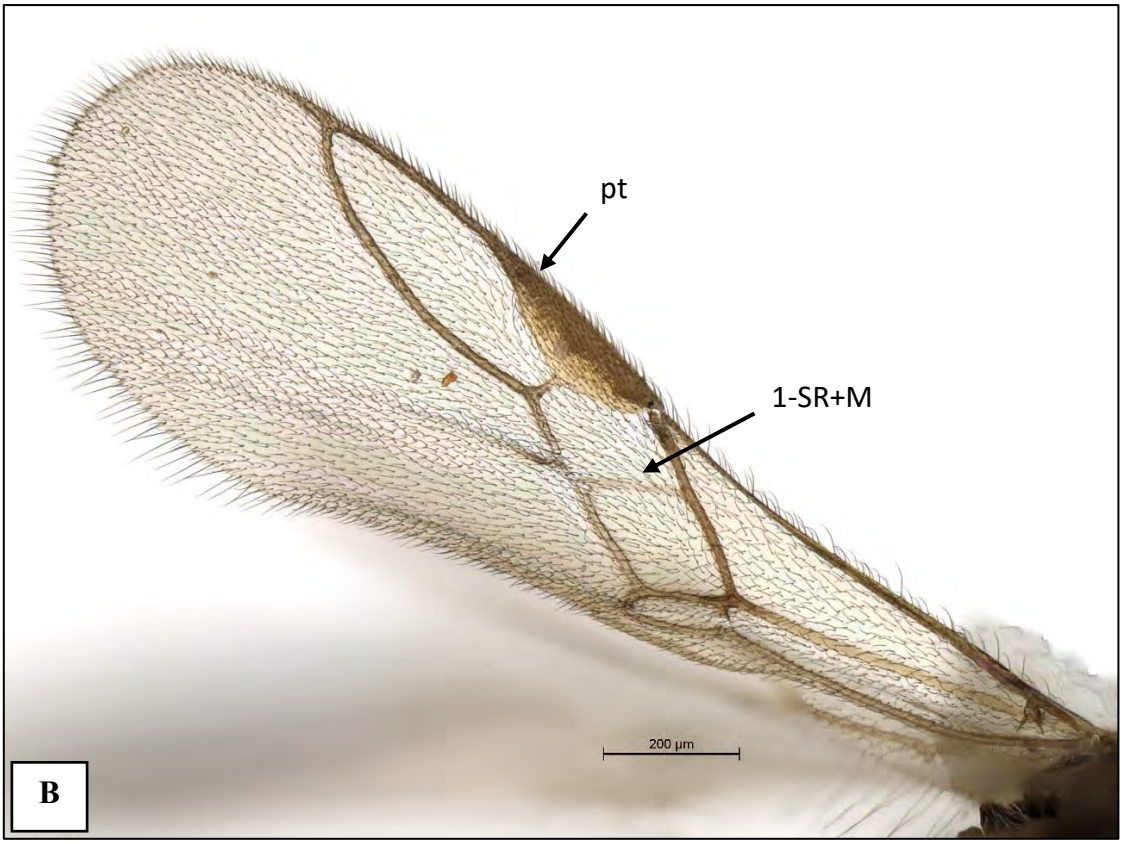


Figure 4-2: a) Full body of an adult *Chaenusa seminervata* and b) Wing characteristics specific to *C. seminervata* with Vein 1-SR+M of forewing usually pigmented, but largely unsclerotized; distal end of pterostigma (pt) comparatively elongate (Photo credit: Simon van Noort).

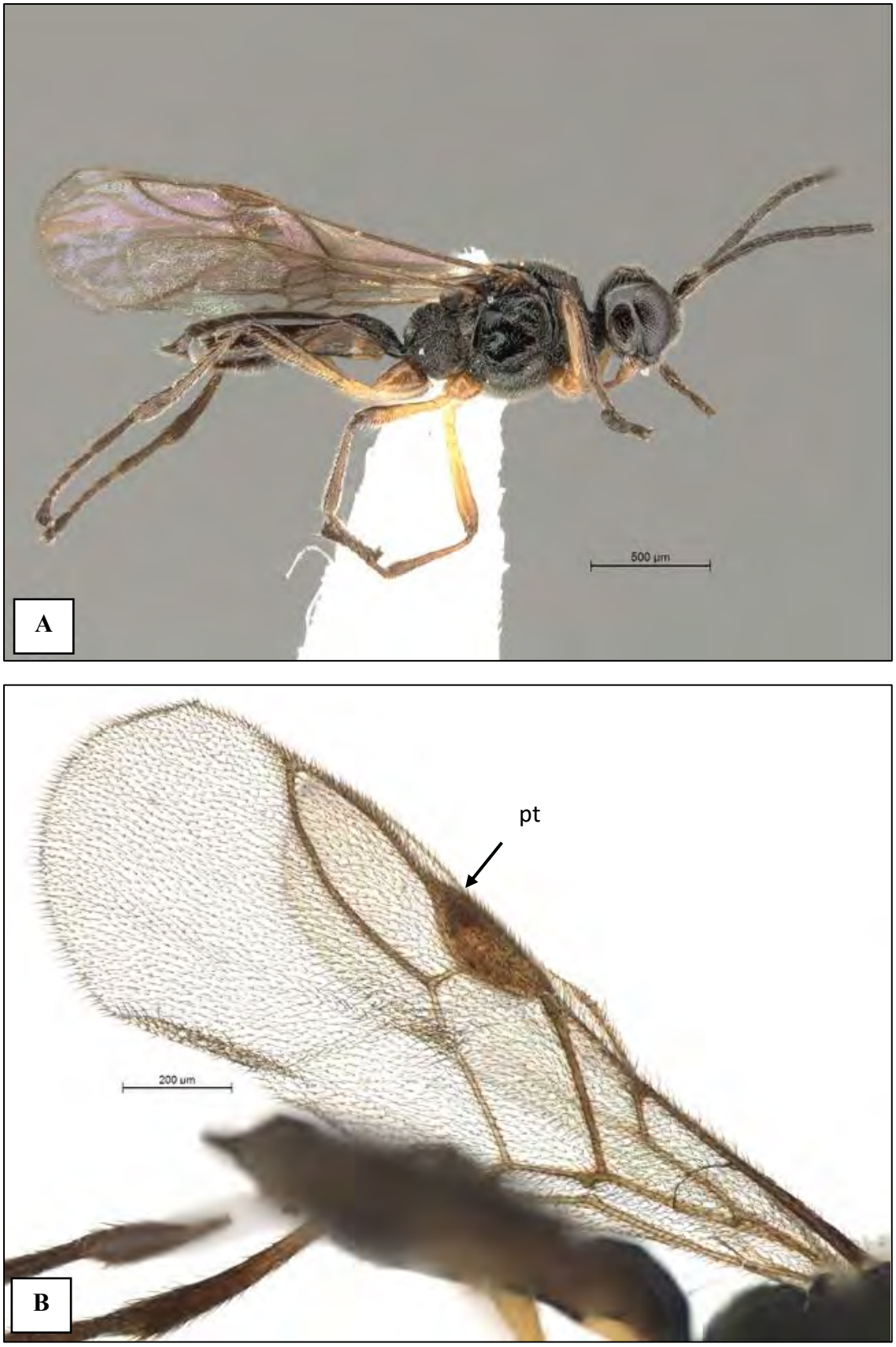
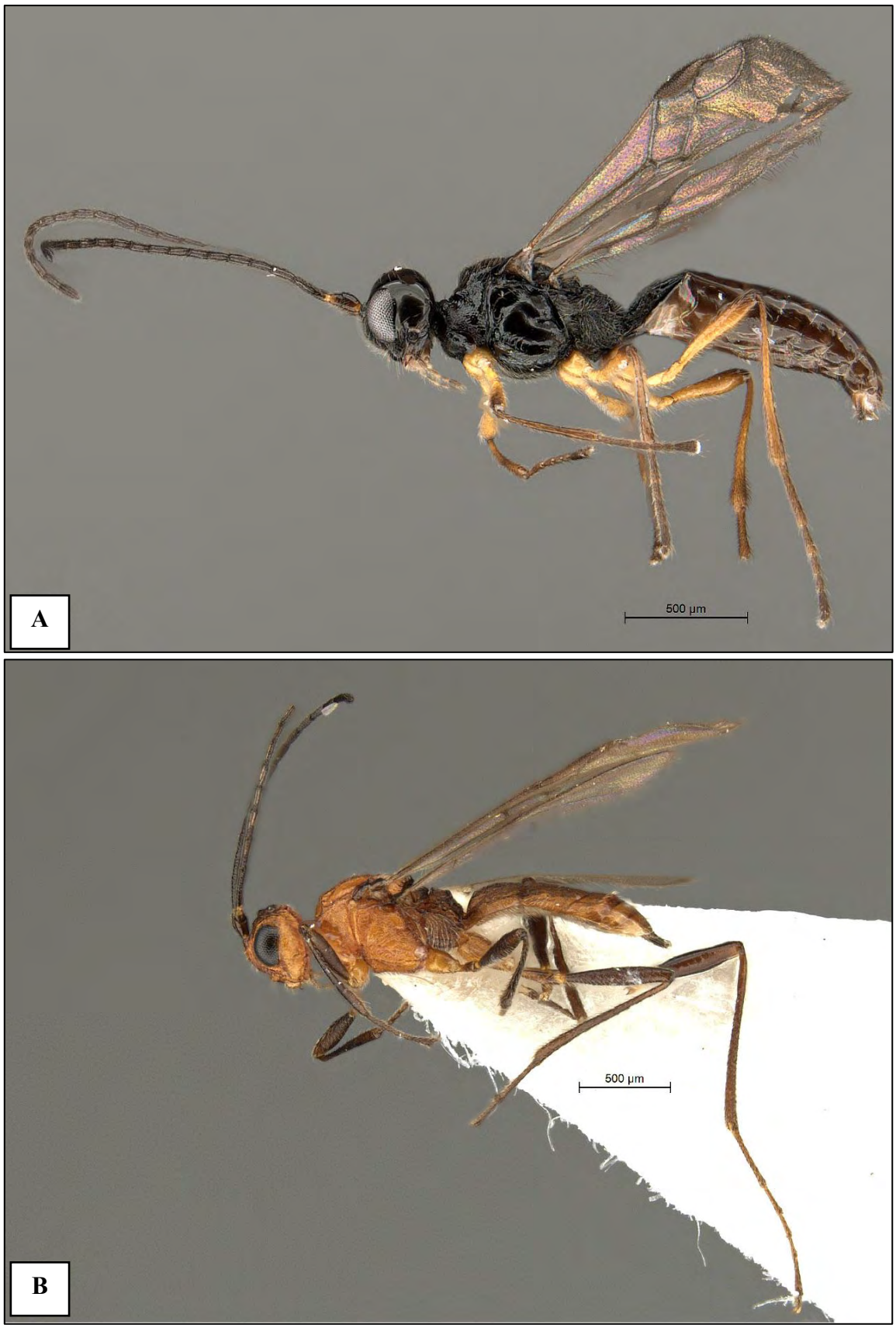


Figure 4-3: a) Full body of an adult *Chaenusa anervata* and b) Wing characteristics specific to *C. anervata* with Vein 1-SR+M of forewing absent; distal end of pterostigma (pt) comparatively shorter and more robust (Photo credit: Simon van Noort).


A
500 µm
B
500 µm

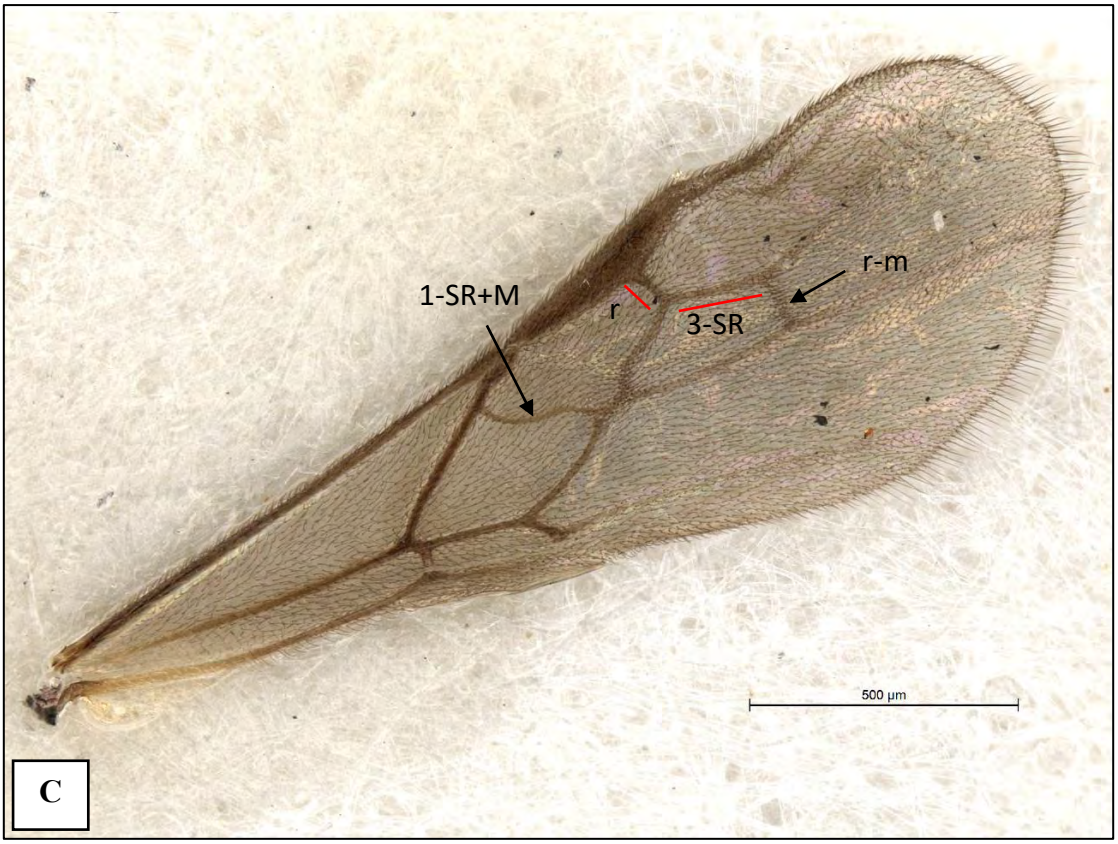


Figure 4-4: Full body image of an adult *Ademon lagarosiphonae* a) black variation and b) orange variation. c) Wing vein characteristics specific to *A. lagarosiphonae*. Vein 3-SR is 2.1–3.5 times as long as vein r; vein 1-SR+M usually strongly sinuate; and vein r-m vertical or nearly so (Photo credit: Simon van Noort).

Figure 4-5*:* a) Habitus of a female *Janicharis africanus* lateral view and b) habitus dorsal view illustrating the wing morphology. (Photo credit: Simon van Noort).

4.3.2 Parasitism rate

Percentage parasitism was not significantly different between the two sites ($H = 2.11$; $df = 1$; $P = 0.15$). The highest mean parasitism rate for 2019 to 2020, was in the Midmar Dam with 53.6 ± 14.0 % (Table 4-1). The lowest parasitism rate was in the Nahoon River with 23.3 ± 20.4%. However, the biological control agent population started to decline in the Nahoon River after November 2019. The population size for November 2019 (Chapter 3) was 340 immatures kg^{-1}, 138 immatures kg^{-1} for January 2020 and 0 immatures kg^{-1}fresh mass of *E. densa* for July to September 2020. The last population count in October 2020 recorded 91 immatures kg^{-1} fresh mass of *E. densa*.

Percentage parasitism was not significantly different between cold and warm months ($H = 0.25$; $df = 1$; $P = 0.62$). The mean parasitism rates for all sites during the cold months of 2019 and 2020 was 41.3 ± 15.5% and 30.0 ± 30.0% for warm months (Table 4-1). There were only three data points for the warm months, of which two had a value of zero, which explains the mean and standard error for this treatment.

Table 4-1: Extent of *Hydrellia egeriae* parasitism (%) recorded per season over two years (2019-2020) from two *Egeria densa* sites.

Site	Season	Year	No. pupae collected	No. flies emerged	No. parasitoids	Parasitized (not emerged)	Dead pupae	% parasitism
Nahoon River	Autumn	2019	56	51	5	-	-	8.9
	Winter	2019	70	5	48	11	6	84.3
	Summer	2019	9	0	0	0	9	0
	Winter	2020	2	1	0	0	1	0
	Spring	2020	0	0	0	0	0	0
	Mean %							**23.3 ± 20.4**
Midmar Dam	Winter	2019	51	27	17	7	0	47.1
	Spring	2019	10	1	2	4	3	60
	Autumn	2020	5	2	1	0	2	20
	Winter	2020	171	27	17			87.4
	Mean %							**53.6 ± 14.0**

4.4 Discussion

Results from this study indicate that the biological control agent, *H. egeriae* has acquired parasitoids within its introduced range, as predicted by factors such as ecological analogues, phylogenetic relatedness and feeding niche. In addition, this study corroborates studies that have found that dipteran and lepidopteran herbivores often have a higher parasitoid richness in their native and introduced range, compared to coleopteran and hymenopteran species (Cornell & Hawkins 1993; Hill & Hulley 1995).

Parasitoids collected from *H. egeriae* only a few months after its release were initially recorded from *Hydrellia lagarosiphon* (van Achterberg & Prinsloo 2012), a specialist herbivore of *L. major* in South Africa. These parasitoids have thus extended their host range to *H. egeriae* in a very short space of time (< 2 years). *Hydrellia lagarosiphon* parasitism rates ranged between 13 to 28% in summer and 0 to 52% in winter (Martin *et al.* 2013). In comparison, *H. egeriae* parasitism were higher, both in cold months and warmer months. *Chaenusa seminervata* and *C. anervata* were collected from the Midmar Dam, while *Ademon lagarosiphonae* was collected from both the Midmar Dam and the Nahoon River. Interestingly, no *Lagarosiphon* species occur in the Nahoon River, while only *L. muscoides* occurs in the Midmar Dam. *Hydrellia lagarosiphon* has also been collected from *L. muscoides* and *Potamogeton* species (Mangan *et al.* 2019). However, considering the absence of these species in the Nahoon River, it could be that *A. lagarosiphonae* is oligo- or polyphagous, rather than a specialist to *H. lagarosiphon*.

Classical biological control often requires augmentative releases to reduce weed populations. Knowledge of the antagonistic interaction between the biological control agent and parasitism in its introduced range allows for adaptive management through timed augmentative releases. For example, Harms & Grodowitz (2011) studied the overwintering biology of *Trichopria columbiana* Ashmead (Hymenoptera: Diapriidae), a generalist parasitoid

that extended its host range to *Hydrellia pakistanae*, between 2007 and 2008. From their results, they were able to fine-tune augmentative releases, by collecting *H. pakistanae* from open field rearing ponds during periods when parasitism is lowest. From this study, field parasitism rates were lowest in autumn, and thus it would be worth investigating if augmentative releases done in autumn reduce parasitism rates and increases *H. egeriae* abundance in the following seasons. However, this study is also limited, as it had more cold month sample events than warmer month sample events. It will thus be necessary to continue parasitoid focussed post-release surveys to obtain a better understanding of seasonal parasitoid patterns.

Biological control agents should not be disregarded due to possible parasitism in their introduced range (Hill & Hulley 1995; van Klinken & Burwell 2005). For example, the gall fly, *Procecidochares utilis* Stone (Diptera: Tephritidae), a biological control agent of *Eupatorium adenophorum* Spreng. (Asteraceae) in Hawaii, experienced high levels of parasitism (Bess & Haramoto 1972). The dipteran agent acquired four parasitoids in its introduced range, and surveys between 1950-1957, and 1966-1971 showed that parasitism rates ranged between 30 to 93% in winter and 7 to 72% in summer. Despite the high levels of parasitism, the agent effectively reduced the presence and spread of its target weed (Bess & Haramoto 1972). Additionally, Cornell & Hawkins (1993) have found that herbivores with a mixed life-style had the lowest parasitoid richness values. Similarly, *H. egeriae* has a mixed life style, with adults living on the water surface, and immatures in the aquatic environment. Thus, the absence of adult and egg parasitoids, reduce *H. egeriae*'s susceptibility to parasitism.

It is also important to consider the potential impact that *H. egeriae* parasitoid acquisition can have on native food webs, i.e. native dipterans (Willis & Memmott 2005; Paynter *et al.* 2020). *Hydrellia* species are poorly studied in South Africa, and *Hydrellia* species that occur in the Nahoon River and Midmar Dam have not been recorded previously. In addition, the

parasitoids recorded in this study was also discovered due to native range surveys for *Lagarosiphon major* (Martin *et al.* 2013; van Achterberg & Prinsloo 2012). Therefore, there is a knowledge gap on the native food web of these parasitoids and their hosts. This study only measured the level of parasitism on *H. egeriae*, however, it is highly likely that a larger parasitoid host pool could increase parasitism pressure on native hosts (Hinz *et al.* 2019). Changes in food web interactions are difficult to establish, and may be beyond the time and resources that biological control practitioners have for post-release surveys. However, field observations together with laboratory experiments may shed light on potential changes in food web interactions. Therefore, continued post-release surveys on *H. egeriae* should firstly identify native parasitoid hosts at released sites, and secondly monitor their presence periodically.

Taken together, results from this study and chapter 3 suggest that *H. egeriae* field populations are both limited by release effort and parasitism. Therefore, timely augmentative releases should be explored for its potential to promote biological control efficacy against *E. densa*. Thus far, this study has considered factors that affect agent establishment (Stiling 1993; Lockwood *et al.* 2005; Fauvergue *et al.* 2012). Freshwater systems are open, dynamic systems that are susceptible to both internal and external factors (Scheffer 2009). Due to human activities ecosystems are also threatened by overexploitation, pollution, habitat destruction, climate change and invasive species (Convention on Biological Diversity 2006). One of these threats, climate change, fundamentally alter plant-insect interactions (Stiling & Cornelissen 2007), and is therefore important to consider for biological control programmes.

5. Predicting the effect of elevated CO_2 and temperature on the biological control of *Egeria densa*

5.1 Introduction

Over the last 160 years, greenhouse gas (GHG) emissions, such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) have increased exponentially. This is mainly driven by the burning of fossil fuels, industrialisation, economic and population growth and increased food demand (Intergovernmental Panel on Climate Change 2014). Since the pre-industrial era, average global CO_2 concentrations have increased from 280ppm to about 410ppm (2019) (climate.nasa.gov, 2019). Similarly, methane increased from just under 800ppb (parts per billion) to 1800ppb, and nitrous oxide from 270ppb to 330ppb (IPPC, 2014). These gases trap thermal radiation from the earth's surface, and have contributed to global warming (i.e. greenhouse effect). The average land and ocean surface temperature has increased by 0.85°C from 1885 to 2012 (IPPC 2014). Due to the major effect that human activity has had on the earth's climate, and thus natural processes, this geological age is known as the Anthropocene.

5.1.1 Carbon dioxide in freshwater systems

To understand the potential threat that climate change poses to freshwater systems, it is important to understand inorganic carbon dynamics within these systems. Inorganic carbon within freshwater systems is influenced by the physical, chemical and biological properties around and within the system (Hasler *et al.* 2016). Sources of inorganic carbon include weathering of carbonate rocks and soils, and microbial organic breakdown (Jarvie *et al.* 2017). Although aerial CO_2 is abundant, its contribution to the aquatic environment is dependent on the rate of air-water surface mixture (Maberly 1996). In freshwater systems, there are three species of inorganic carbon and the dominance or quantity of each is dependent on an interplay of factors, such as the water alkalinity, pH and temperature (Talling 2010). When mixed into the water, CO_2 binds with H_2O to form carbonic acid or free CO_2^* (H_2CO_3). Carbonic acid only remains the dominant inorganic carbon species in waters with a pH below 6.0 (Talling 2010;

Hasler *et al.* 2016). In more alkaline water (pH 7.0 – 10.0), carbonic acid dissociates with a proton (H+), forming bicarbonate (HCO_3^-), a less diffuse gas. Bicarbonate can also dissociate its proton in environments with a pH greater than 10.0 to form carbonate (CO_3^{2-}), which cannot be used by phototrophs, except through back titration on plant surfaces in acid conditions (Pedersen *et al.* 2013). The composition of these three carbon species forms the total dissolved inorganic carbon (DIC) population.

5.1.2 Freshwater macrophyte photosynthesis

Similar to terrestrial C_3 and C_4 plants, some freshwater macrophytes can switch between using CO_2^* and HCO_3^-, for example when CO_2^* availability decreases (Casati *et al.*, 2000). Due to the limitation that low CO_2^* availability can cause in conditions with a pH above 8.2, the ability to use HCO_3^- is beneficial. This is seen in more than 50% of freshwater plants that are able to use HCO_3^- (Hussner *et al.* 2016). In contrast to terrestrial C_4 Kranz anatomy, C_4 photosynthesis in submerged species occurs in a single cell. The C_4 enzyme phosphoenolpyruvate carboxylase (PEPC) is located in the cytosol and NADP-malic enzyme (NADP-ME) in the chloroplast (Casati *et al.* 2000). This means that stems may also exhibit a C_4-like photosynthesis, as has been found by Gu *et al.* (2015). Although HCO_3^- is an attractive alternative, it costs metabolically more, and affinities for this carbon source are species dependent (Hussner *et al.* 2016). In some cases, aquatic species are so well adapted to using HCO_3^- that they show no preference between CO_2^* and HCO_3^- (Hussner *et al.* 2016). The ability to use carbon species is also a determining factor in freshwater community composition (Maberly *et al.* 2015; Titus & Pagano 2017), and specificially is a limiting factor for *E. densa* development (de Freitas & Thomaz 2011).

5.1.3 Projected direct effects on *Egeria densa*

Predicting the effect of elevated CO_2 on freshwater systems is difficult, considering the many factors that influence carbon species and quantity within such systems. Likewise, many

freshwater systems are already functioning at DIC levels well above ambient CO_2 (Maberly 1996; Hasler *et al.* 2016). For example, Cole *et al.* (2007) found freshwater lakes functioning at 16 times atmospheric CO_2 level. They also found a global freshwater lake CO_2 mean of 1000µatm. Past studies that focused on specific biotic and abiotic factors give some indication on how climate change could affect freshwater ecosystems.

Elevated CO_2 is likely to affect systems with low alkalinity (low buffering capacity). In such conditions, the pH will be low, allowing CO_2* to be the dominant carbon source (Talling 2010). Thus, if the alkalinity is low, CO_2 obligate users, which are carbon limited, will respond strongly to increases in CO_2 (Schippers *et al.* 2004). However, HCO3- users such as *E. densa*, *C. demersum* and *L. major*, increased their photosynthesis rates up to five times at low alkalinity (0.1 mequiv. L^{-1} pH 7.0) compared to high alkalinity (3.4 mequiv. L^{-1} pH 8.3) (Cavalli *et al.* 2012). This is because obligate CO_2 users have a lower CO_2 compensation and saturation point than plants that can use both CO_2 and HCO_3^- (Sand-Jensen *et al.* 1992)

Because elevated CO_2 is affiliated with increases in temperature, one major effect of global climate change will be changes in thermal regimes (Kernan 2015). Through distribution modelling, Gillard *et al.* (2017) showed that under all RCP scenarios, suitable environments for *E. densa* will increase in exotic ranges such as North America and Europe. The optimum and maximum growth temperature for *E. densa* is 16 °C and 25°C (Cook and Urmi-König 1984), however *E. densa* can tolerate temperatures of up to 35°C (Haramoto & Ikusima 1988). Seasonal studies have shown that *E. densa* increases its leaf area size (LAS), RGR and root biomass with increases in temperatures (Haramoto & Ikusima 1988; Silveira & Thiébaut 2017). This is supported by Hussner *et al.* (2019) who showed that the RGR of *E. densa* was significantly affected by temperature, within a given range, instead of light and carbon. This is because, increases in temperatures increases the internal processes within the plant..

5.1.4 Effects of climate change on herbivory

A general observation of the effects of elevated CO_2 on plant physiology is an increase in the C/N ratio of the plant, and a reduction in the host plant nutritional quality (Stiling & Cornelissen 2007). The Carbon-Nutrient Balance Hypothesis (CNBH) states that under elevated CO_2, plants increase carbon-based defence compounds (e.g. phenolics, terpenoids and flavonoids) whereas nitrogen based compounds (e.g. alkaloids, cyanogenic glycosides) will decrease (Bryant *et al.* 1983). For example, Erhard *et al.* (2007) found that flavonoids of *Elodea nuttallii* Planch. St. John (Hydrocharitaceae) deterred feeding by the aquatic moth *Acentria ephemerella* Denis & Schiffermüller (Lepidoptera: Pyralidae). However, a recent review has shown that the CNBH is context dependent (Ryan *et al.* 2010), and that it is necessary to take a wider approach on the effect of elevated CO_2 levels on plant-insect interactions (Robinson *et al.* 2012). For example, Du *et al.* (2019), found that a shift in C/N/P stoichiometry was more pronounced in the southern hemisphere, compared to the northern hemisphere. Other effects of elevated CO_2 include increases in leaf toughness, plant physical properties such as trichome density and leaf area size (LAS) (Robinson *et al.* 2012); LAS increases are more pronounced at higher latitudes compared to sub-tropical semi-arid areas (Liu *et al.* 2019). Changes in host plant quality will affect herbivorous insects. Studies have found increases in herbivore development time and plant consumption, but decreases in herbivore mass and fecundity with reductions in host plant nutritional quality (Stiling & Cornelissen 2007; DeLucia *et al.* 2008). Upon closer inspection, these responses are guild-dependent, for example leaf-miner abundance decreased with 70%, whereas phloem feeders and scrapers increased by 22% and 59% (Robinson *et al.* 2012).

5.1.5 Climate change and the biological control of *Egeria densa*

Egeria densa is a widely distributed submerged aquatic weed in South Africa. It is predicted to expand its distribution range with climate warming (Gillard *et al.* 2017) and RGR

with increases in CO_2^* and HCO_3^- (Cavalli *et al.* 2012: Hussner *et al.* 2016). Past studies have already shown that under elevated CO_2, *E. densa* leaf nutritional quality decreases (Hussner *et al.* 2019). Considering these factors, it is necessary to establish the feeding behaviour of the biological control agent under climate change. With this information, *E. densa* control strategies can be adjusted to optimize biological control efficacy.

The aim of this study was to establish the effect of climate change, *viz.* elevated CO_2 and temperature, on the leaf-mining behaviour and subsequent mass of the biocontrol agent *H. egeriae* on its host plant, *E. densa*. Firstly, it was important to determine the dissolved inorganic carbon at *E. densa* invaded sites; and secondly, to experimentally establish how elevated CO_2 and temperature will affect the nutritional quality of *E. densa* when grown at high and low plant densities within Open Top Chambers (OTC). Furthermore, potential changes in plant nutritional quality on *H. egeriae* herbivory and development was investigated through feeding trials.

5.2 Material and methods

This experiment was divided into two stages, firstly the growth of *E. densa* at elevated CO_2 levels and secondly, *H. egeriae* feeding trials using plants grown at elevated CO_2 levels.

5.2.1 Part 1: Determining the effect of elevated CO_2 and different planting densities on the nutritional quality of *Egeria densa*

The study was conducted at the Open Top Chamber (OTC) facility (-33.311230, 26.510240) at Rhodes University, Makhanda, South Africa. Each OTC is decagonal, 320cm in diameter, 270cm in height and made up of F-Clean plastic panels that transmit 94% visible light. Chambers were fitted with a 3-phase fans that introduce air into the chamber via a circular diffuser, 34cm in diameter and perforated with 300, 1cm holes for equal distribution. The diffuser was positioned 1 m above the ground and airspeed entering the chamber was regulated via the fan speed. Airspeed was controlled to keep the difference in temperature between ambient and the interior of the chamber within 4 °C. Temperatures were monitored via CS215 temperature/humidity sensors (Campbell Scientific) and data were processed via a CR6 data logger (Campbell Scientific) and Loggernet software (Campbell Scientific), using proportional–integral–derivative (PID) procedures, to alter fans speeds according to temperature differentials. CO2 concentrations within the chambers were measured with open-path CO_2 analysers (GMP343, Visala, Finland) via the same procedure as controlled fan speed, but using different PID equations that altered the CO2 injection into the chamber ventilation system. Six chambers were used for the experiment. Three CO_2 levels were selected for the experiment; 400ppm, 600ppm and 800ppm (Figure 5-1). Each CO_2 concentration was replicated twice.

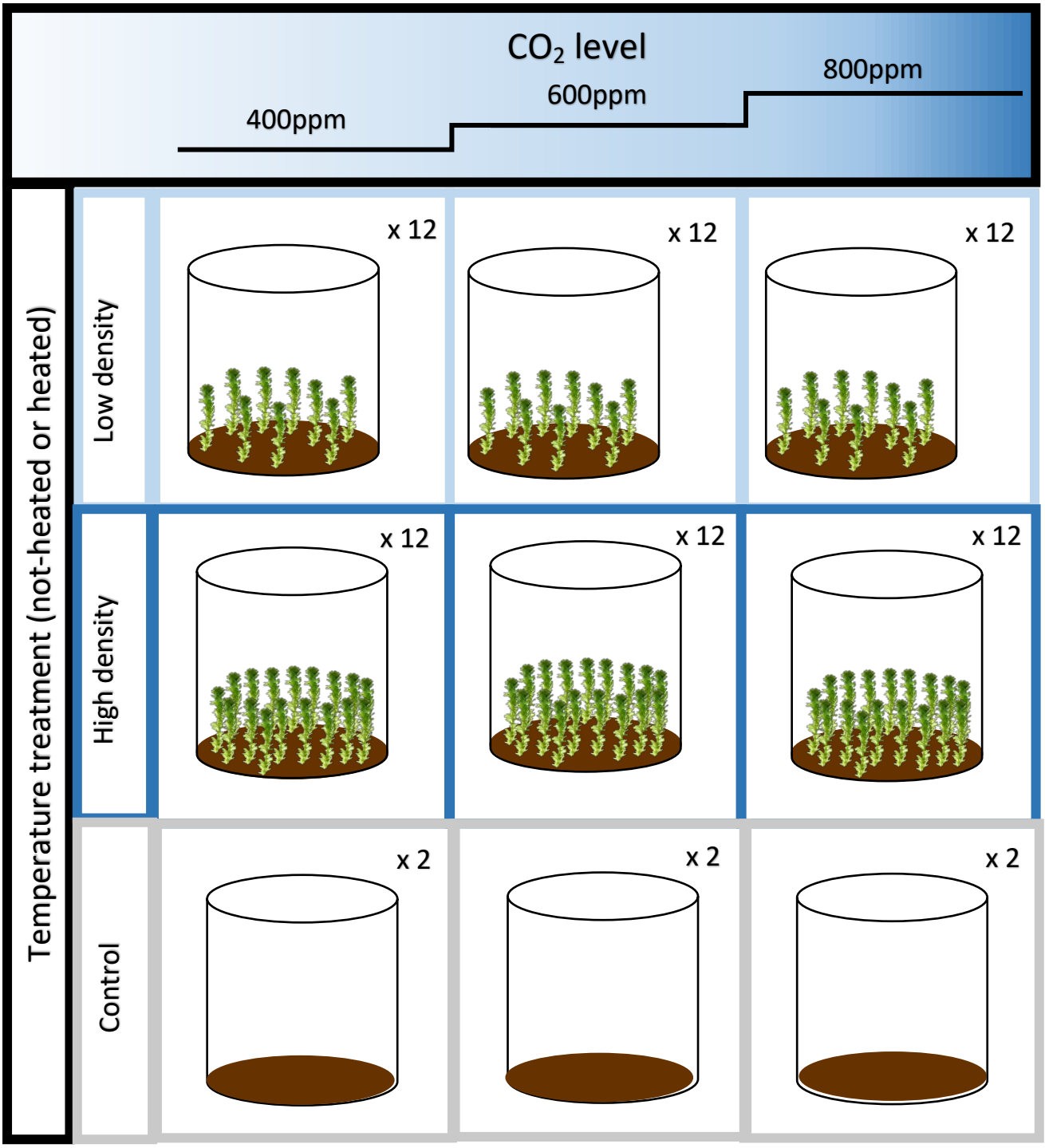


Figure 5-1: Number of replicates per combination of CO_2 concentrations, planting densities and temperature treatments.

One snapset pool, 183cm in diameter, 38cm in height and a water carrying capacity of 977 litres was placed in each chamber. To manually increase the water temperature, black damp course was placed inside pools, one replicate per CO_2 (Figure 5-1; Figure 5-2). Thirteen 25 litre buckets were randomly placed in each pool (Figure 5-3). A quarter of each 25L bucket

was filled with pond sediment collected from Jameson Dam, Eastern Cape, South Africa (33°19'07.2"S, 26°26'24.0"E) that naturally contains 11.0 mg N kg^{-1} and 11.65 mg P kg^{-1}. Pre-cultured *E. densa* shoots from the Waainek Research Facility, 10cm in length, were planted at two densities following the De Wit replacement series (De Wit and Van der Bergh 1965). Six buckets were planted at a low plant density (nine shoots), another six buckets at a high density (18 shoots) and one bucket had no shoots as a control. Different planting densities were used to elucidate if nutritional quality from plants in higher densities are different from plants with less intraspecific competition. A control was used to establish the difference in DIC and temperature between buckets with plants, and those without plants.

Sediment was covered with a thin layer of washed silica sand to minimize water clouding. Buckets were subsequently filled with tap water containing 1.5 mM DIC. Daily temperature spikes were buffered by half-filling the snapset pools with tap water (Figure 5-3). Air at each CO_2 concentration was bubbled into the water by means of a water pump placed in each bucket. Air pumps were placed next to the pool and pumped ambient air from the chamber at the set CO_2 level into the water pumps (Figure 5-3). Buckets were covered with 80% shade cloth to prevent algal growth. Buckets and snapset pools were filled up by tap water as necessary for the duration of the experiment.

Figure 5-2: Open Top Chambers (OTC's) at Rhodes University, Makhanda (photo: Brad Ripley)

Figure 5-3: Experimental setup within each OTC. The black damp course plastic used to increase water temperatures is seen on the bottom of the snapset pool (photo: Rosali Smith)

5.2.2 Carbon: Nitrogen ratios

In order to determine C/N ratios of *E. densa*, approximately 1g of shoots were collected from each treatment combination and oven-dried at 100°C. Dried leaves were collected from shoots with forceps and sent to UP Stable Isotope Laboratory, University of Pretoria, for carbon and nitrogen analysis. The δ 13C and δ 15N isotopic values were reported as ‰, normalised to internal standards (Merck and DL-Valine) calibrated to the International Atomic Energy reference materials (IAEA-CH-3 and IAEA-CH-6 for δ13C, IAEA-N1 and IAEA-N2 for δ15N). Results are expressed in standard delta notation using per mil scale, δX (‰) = $(Rsample\ Rstandard - 1) \times 1000$, where X = 15N or 13C, and R is the ratio of the heavy over the light isotope (15N/14N or 13C/12C), respectively. Average analytical precision for δ 13C, and δ15N was ± 0.03 and ± 0.02 respectively.

5.2.3 Water chemistry measurements:

The water temperature in the pool, high plant density, low plant density and control buckets were measured with iButton® (Thermochron Model: DS192IG-F5: −40 through to 85 °C, with an accuracy of 1.0 °C (between −30 to +70 °C)). One iButton per plant density treatment for each OTC was placed into a waterproof silicone capsule, and then into a sealed plastic vial containing sand as a weight, which positioned it at the bottom of the water column. Temperature readings were taken every 30 minutes for six weeks.

The total dissolved inorganic carbon within experimental buckets were determined at the end of the experiment. This was done by collecting 100ml water samples from buckets within each CO_2, temperature and planting density treatment. The pH, temperature and total dissolved solids (TDS) of the 100ml sample were measured with a handheld pH multi-meter (Eutech PCSTestr 35 multi-parameter). The water sample was titrated to a pH of 4.3 with hydrochloric acid (HCl) and the volume used was recorded in millilitres. Another water sample (100ml) from the same treatment was collected, titrated to a pH of 8.2 with sodium hydroxide

(NaOH) and the volume used was recorded (ml). If a water sample had a pH above 8.2, the water sample was first titrated with HCl to 8.2, a fresh water sample was collected and then titrated with HCL to a pH of 4.3. The volumes of HCl and NaOH, as well as the initial pH, temperature and TDS were used to calculate the CO_2, HCO_{3-}, CO_3^{2-} and total DIC in mmol according to Klee (1998). These values obtained are presented in Table 5-1, and only provide context for the experimental study.

5.2.4 Part 1: Statistical analyses

5.2.4.a *Water temperature*

All statistical analyses were conducted in the R environment (R Core Team 2019). The effect of added damp course on treatment water temperature was evaluated with a Student's *t*-test, and results are shown in Table 5-21, which provides context for the experimental conditions.

5.2.4.b *The effect of elevated CO_2 and planting density on Egeria densa nutritional value*

The leaf C/N ratio data were normally distributed linear modeling was performed. The final model contained only main effects between predictor variables CO_2 and Plant density. Initially, a linear mixed effects model was performed with chamber as a random effect. Anova comparisons between models showed that the random effect (chambers) did not significantly improve the model. Normality of the final model was visually inspected using Normal Q-Q and residual plots. The dredge() function in the MuMIn package was used to select plausible models using AIC, ΔAIC and weight scores. Models with $\Delta AIC < 6$ were seen as plausible models. Instead of selecting one model and obtaining coefficient estimates from it, the model average function was used to obtain coefficient estimates from all plausible models. If significant variables were present, the pairwise post-hoc tukey test from the emmeans package was used investigate difference between treatments.

5.2.4.c *Part 2: The effect of elevated CO_2 on Hydrellia egeriae herbivory and mass*

The feeding behaviour of *H. egeriae* larvae and subsequent mass of emerged adults when given plants grown at elevated CO_2 concentrations and temperatures was examined in the quarantine facility of the Centre for Biological Control, Rhodes University. At this point of the study, the fly had not yet been cleared for released in South Africa.

To obtain test individuals, ten pairs of newly emerged adults from a stock culture maintained in quarantine were placed in a transparent box (41cm X 17cm X 29cm) that was half-filled with 10L distilled water containing a nutrient stock solution (Smart & Barko, 1985), and 25 *E. densa* apical shoot tips, collected from plants maintained at the Waainek Research Facility. A yeast hydrolysate/sugar mixture (4 g Bacto^{TM} TC yeastolate/7 g sugar/10 ml H_2O) (Buckingham and Okrah 1993) for adult feeding was provided on a floating station. Adults were allowed to mate and oviposit, leaves with eggs were harvested and placed in a petri-dish containing distilled water with nutrient solution (Smart & Barko, 1985). Neonate larvae or ready to hatch eggs were transferred to test plants by excising the leaf material around it, and pinning the excised leaf with the larva/egg, onto the experiment plant. Eggs were checked for larval emergence after initiation of the replicate.

After a growth period of six weeks under the three experimental ambient CO_2 concentrations, *E. densa* shoots from high density treatments were harvested and brought into quarantine for rearing of *H. egeriae*. Water from the high density planting treatments was also collected and used for the experimental setup in quarantine to keep conditions similar to those in the OTC. Two to three shoots, 5cm to 10cm in length, were placed in round, 1.5 litre containers half-filled with water from each of the respective treatments. One *H. egeriae* neonate instar within a leaf, or mature egg on a leaf, was pinned to an *E. densa* leaf with a minuten pin

to ensure containers were enclosed with netting, held in place with a rubber band. Twenty replicates per CO_2 x temperature treatment were set up.

Hydrellia egeriae individuals were left to complete development. After 20 days, containers were checked daily for adult emergence. Adults were caught with an aspirator, anesthetized by placing them in a freezer for a few minutes and subsequently sexed and weighed (mg) with a Sartorius microbalance. The leaf area mined (¼, ½, ¾ or 1) for each leaf per shoot was recorded by observational estimation and then totalled to give an overall leaf area mined (Smith *et al.* 2019).

After weighing, the newly emerged F_1 adults were placed in rearing chambers according to the respective OTC treatment, to allow the second generation to develop under the experimental conditions. Rearing chambers were set up by placing OTC treated plants and water in 15L transparent boxes (41cm x 17cm x 29cm). Again, a yeast hydrolysate/sugar mixture (4 g Bacto^{TM} TC yeastolate/7 g sugar/10 ml H_2O) provided on a floating feeding station was placed in each box for adult feeding (Buckingham *et al.* 1993). Water from each CO_2 and temperature treatment was collected and added to the respective rearing chambers every week. Similarly, F_2 adults were placed in new rearing chambers upon emergence. Twenty eggs (F_3) were harvested from F_2 chambers and placed in petri-dishes with distilled water with nutrient solution to use in F_3 feeding trials. The same procedure used for F_1 feeding trials was followed to set up the *H. egeriae* F_3 feeding trials. After weighing and sexing, F_3 adults were reared until the F_5 generation to conduct the last feeding trials. Thus, feeding trials were conducted every second generation, using individuals from F_1, F_3 and F_5. The DIC from water in feeding experiments (quarantine) was measured for comparison to OTC treatments.

To determine if *E. densa* leaf C/N composition could have changed after 20 to 30 days in quarantine, shoots were collected from each respective OTC treatment, placed in 1.5L

containers filled with OTC treated water and grown for 30 days. Shoots were subsequently collected and prepared for analysis as above.

5.2.5 Part 2: Statistical analysis

5.2.5.a *The effect of elevated CO_2 on Hydrellia egeriae feeding and mass*

Analysis of covariance (ANCOVA) was used to determine if *H. egeriae* feeding (predictor variable) was influenced by ambient CO_2 and generation (predictor variables), with C/N ratios as the covariate. C/N ratios of *E. densa* leaves were only obtained during F_1 and F_5, and thus F_3 was excluded from the statistical analyses. A Pairwise Post-hoc Tukey test was used to tease out significant differences between treatments.

5.3 **Results**

5.3.1 The effect of damp course on water temperature

The temperature in this experiment was increased manually through the addition of black damp course in experimental pools. Thus, temperature differences within CO_2 treatments was only determined *ad hoc*. In general, the black damp course did not significantly increase temperatures (Table 5-1). The only treatments that experienced a significant increase in temperature were the 400ppm low planting density (+1.56°C) and 400ppm control (+1.58°C). Thus, for the remainder of the statistical analyses, temperature was not used as a predictor variable.

Table 5-1: Mean water temperature of each treatment combination, and results of the *t*-test to significant differences between heated and non-heated treatments. Significant treatments are highlighted in bold.

CO_2 level (ppm)	Planting density	Mean temperature (°C)		*t*-test output		
		Not heated	Heated	*t*-value	*df*	*P*-value
400	**Low**	**21.48 ± 0.35**	**23.04 ± 0.36**	**-3.13**	**79.91**	**< 0.01**
	High	21.85 ± 0.33	22.70 ± 0.35	-1.76	79.67	0.08
	Control	**21.63 ± 0.34**	**23.21 ± 0.36**	**-3.21**	**79.88**	**< 0.01**
600	Low	22.40 ± 0.34	22.63 ± 0.35	-0.47	79.82	0.64
	High	22.51 ± 0.34	22.71 ± 0.35	-0.41	79.88	0.68
	Control	22.42 ± 0.34	22.57 ± 0.35	-0.31	79.95	0.76
800	Low	22.30 ± 0.34	22.91 ± 0.35	-1.24	79.80	0.22
	High	22.20 ± 0.34	22.44 ± 0.35	-0.49	79.93	0.63
	Control	22.17 ± 0.34	22.52 ± 0.35	-0.72	79.97	0.47

Dissolved inorganic carbon was higher in control buckets, and increased with ambient CO_2 levels (Table 5-2). However, in general DIC in the experimental buckets were low, despite bubbling CO_2 into the water.

Table 5-2: The pH and quantity (mmol) of dissolved inorganic carbon species (CO_2, HCO^{3-}, CO_3^{2-}) measured in the different experimental treatments at the end of the experiment. NH: not heated treatments; H: heated treatments; DIC: dissolved inorganic carbon.

CO_2 treatment	Planting treatment	Temperature	pH	CO_2 (mmol)	HCO_3^- (mmol)	CO_3^{2-} (mmol)	DIC (mmol)
400ppm	High	NH	7.68	0.0043	0.0955	0.0003	**0.1000**
		H	7.51	0.0081	0.1316	0.0003	**0.1400**
	Low	NH	8.56	0.0002	0.0292	0.0006	**0.0300**
		H	5.57	0.1245	0.0257	0.0000	**0.1501**
	Control	NH	8.20	0.0060	0.4597	0.0043	**0.4700**
		H	5.79	0.1308	0.0391	0.0000	**0.1699**
600ppm	High	NH	7.89	0.0047	0.1843	0.0009	**0.1900**
		H	5.15	0.1400	0.0099	0.0000	**0.1500**
	Low	NH	5.41	0.1223	0.0177	0.0000	**0.1400**
		H	7.16	0.0272	0.1926	0.0002	**0.2200**
	Control	NH	6.30	0.1619	0.1581	0.0000	**0.3200**
		H	7.02	0.1145	0.5457	0.0003	**0.6605**
800ppm	High	NH	7.24	0.0246	0.2054	0.0002	**0.2302**
		H	7.10	0.0275	0.1525	0.0001	**0.1801**
	Low	NH	5.30	0.1358	0.0142	0.0000	**0.1500**
		H	7.52	0.0086	0.1213	0.0002	**0.1301**
	Control	NH	7.19	0.0450	0.3550	0.0004	**0.4004**
		H	7.60	0.0148	0.2845	0.0007	**0.3000**

5.3.2 The effect of elevated CO_2 and planting density on *Egeria densa* nutritional value

The main effect between CO_2 and planting density was used to explain *E. densa* leaf C/N ratios, because the interaction was not significant when tested. The model was significant ($F_{3:58} = 7.69$; $P = 0.001$; $R^2 = 0.25$), with only 800ppm as a significant predictor variable ($t = 4.60$; $P < 0.001$). Furthermore, model selection statistics indicated that a model containing only the predictor variable CO_2 had the lowest ΔAICc value (0.00) and largest weight (0.69) from all possible models (Table 5-3). This means that a model with predictor variable CO_2 is 2.2

times (0.69/0.31) more likely to explain the variation in *E. densa* leaf C/N ratio than a model containing CO_2 and Planting density. From these results, it is apparent that the planting density treatment did not affect *E. densa* leaf C/N ratios.

Table 5-3: Model selection output for *Egeria densa* C/N ratio linear model. Model selection was performed using Akaike's information criterion corrected for small sample sizes (AICc). K = number of parameters specified in the model. Plausible models (ΔAICc < 6) are highlighted.

Model structure	**K**	**LogLik**	**AICc**	**ΔAICc**	**Weight**
CO_2	4	-185.9	380.5	0.00	0.69
CO_2 + Planting density	5	-185.5	382.1	1.64	0.31

Carbon content of leaves, and thus C/N ratios increased significantly with elevated CO_2 levels (Figure 5-4). The lowest leaf C/N ratios were obtained from plants grown at 400ppm, with values ranging from 15 and 16. Plant nutritional value was not significantly different between planting densities within a CO_2 level; 400ppm (t_{58} = -0.83; P = 0.96), 600ppm (t_{58} = -0.83; P = 0.96) and 800ppm (t_{58} = -0.83; P = 0.96). However, plants grown at 800ppm had a significantly higher C/N ratio compared to plants grown at 400ppm and 600ppm. Planting density did not affect leaf C/N ratios.

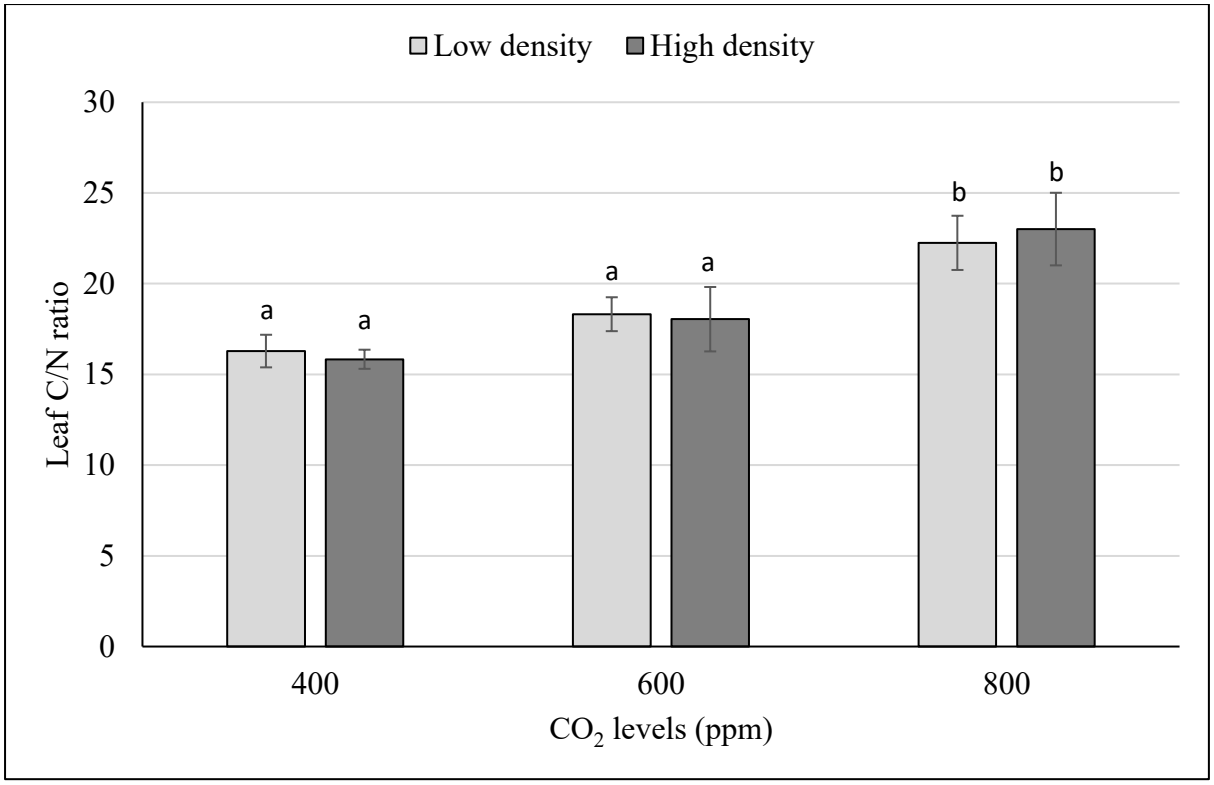


Figure 5-4: *Egeria densa* leaf nutritional value when grown at different planting densities (low density n=12; high density n=12) under elevated CO_2. Letters indicate statistical differences at $P < 0.05$. Error bars indicate standard errors.

The mean percentage nitrogen and carbon measured in *E. densa* leaves are given in Table 5-4. Nitrogen content of *E. densa* leaves decreased with increasing CO_2 levels, with the exception of plant grown at high density under 600ppm. The nitrogen content of these leaves were similar to plants grown at 400ppm.

Table 5-4: The mean percentage nitrogen and carbon (±SE) measured in *Egeria densa* leaves grown at different planting densities under elevated CO_2.

CO_2 level	**Planting density**	**% Nitrogen**	**% Carbon**
400	Low	2.79 ± 0.16	38.12 ± 0.17
	High	2.86 ± 0.09	37.70 ± 0.37
600	Low	2.46 ± 0.11	37.72 ± 0.20
	High	2.78 ± 0.34	37.49 ± 0.45
800	Low	2.12 ± 0.19	37.50 ± 0.33
	High	1.98 ± 0.19	37.39 ± 0.23

5.3.3 The effect of elevated CO_2 on *Hydrellia egeriae* feeding

Only main effects between variables CO_2, generation and C/N ratios (covariate) were used in an ANCOVA to measure *H. egeriae* response, as interactions produced high correlation values. *Hydrellia egeriae* larval feeding for both sexes changed significantly over generations (Table 5-3; Figure 5-5). Effect sizes refers to the total variation in leaf-mining that was accounted for by a significant variable when leaf C/N ratio (covariate) was taken into consideration. Because leaf C/N ratio is a covariate, effect sizes for this variable cannot be calculated. In this case, only generation was significant ($P < 0.001$), and accounted for 12% in female herbivory, and 32% in male herbivory. Interestingly, the generation treatment accounted for nearly 3 times more herbivory by males than by females.

Table 5-5: ANCOVA output illustrating the effect of CO_2 and number of generations on the total number of leaves consumed by *Hydrellia egeriae* during their immature stage leaf C/N ratio (covariate) was taken into account. Significant terms are highlighted.

Effect	**Sum sq**	***df***	***F***	***P***	**Effect size sq**
Females					
CO_2	123.6	2	2.75	0.07	0.05
Generation	**286.1**	**1**	**12.75**	**<0.001**	**0.12**
Leaf C/N ratio	**300.9**	**1**	**13.40**	**<0.001**	**-**
Residuals	2042.7	91			
Males					
CO_2	43.15	2	1.69	0.19	0.03
Generation	**128.55**	**1**	**10.04**	**< 0.01**	**0.32**
Leaf C/N ratio	**286.34**	**1**	**22.37**	**< 0.001**	-
Residuals	1267.36	99			

5.3.4 The effect of elevated CO_2 on *Hydrellia egeriae* adult mass

Both female and male adult mass was significantly affected by only generation (Table 5-5; Figure 5-6). Similar to *H. egeriae* herbivory, the generation treatment had a larger effect on male mass than females, with effect sizes of 32% compared to 14%. Interestingly, females that were given plants from the 600ppm treatment, visually consumed less leaves than females from 400ppm and 800ppm, and also had a higher mass. However, this can be correlated to high nitrogen and low carbon leaf content from plants reared at 600ppm (Table *5-4*).

Table 5-6: ANCOVA output illustrating the effect of CO_2 and number of generations on the mass (mg) of *Hydrellia egeriae* after emergence when leaf C/N ratios (covariate) was taken into account. Significant terms or interactions are highlighted.

Effect	Sum sq	*df*	*F*	*P*	Effect size sq
Females					
CO_2	0.01	2	0.38	0.68	0.01
Generation	**0.17**	**1**	**15.21**	**< 0.001**	**0.14**
Leaf C/N ratio	0.02	1	1.96	0.16	-
Residuals	1.00	91			
Males					
CO_2	0.02	2	2.46	0.09	0.03
Generation	**0.17**	**1**	**49.82**	**< 0.001**	**0.32**
Leaf C/N ratio	0.00	1	0.12	0.73	-
Residuals	0.34	99			

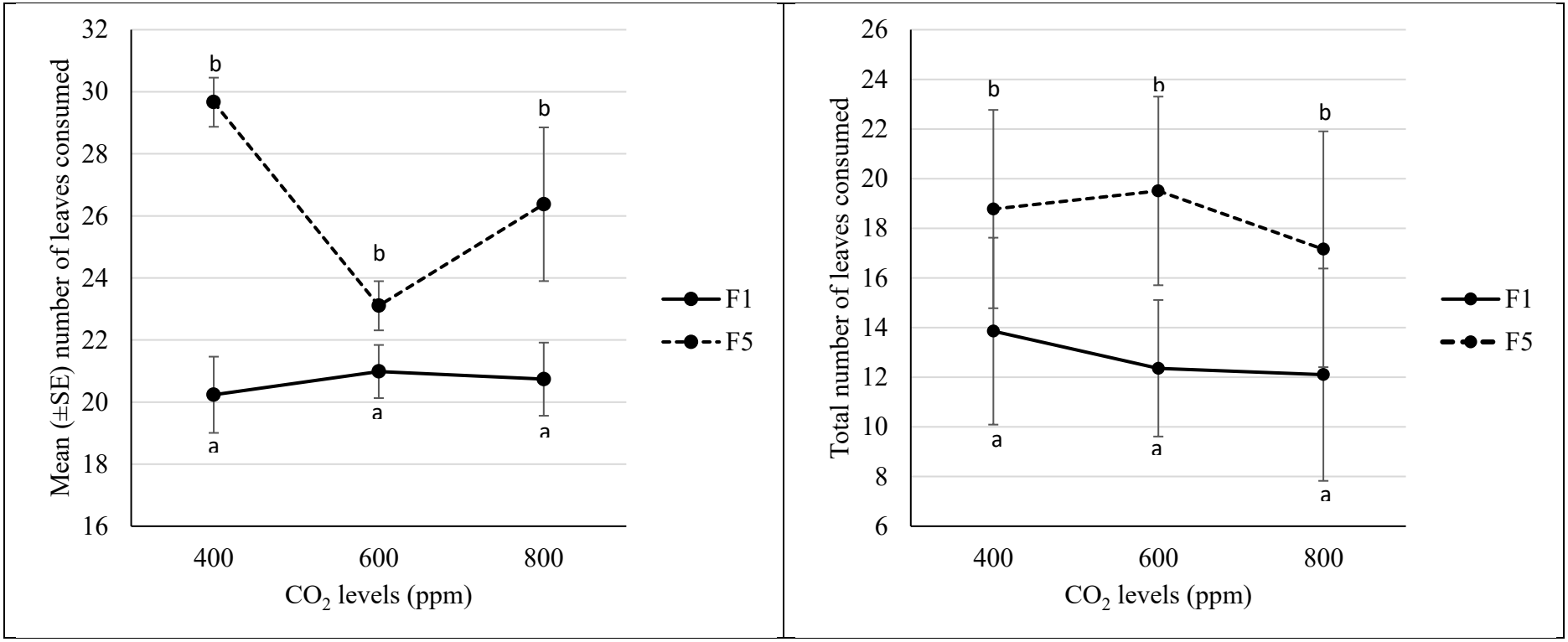


Figure 5-5: Mean (±SE) number of leaves mined by female (left) and male (right) *Hydrellia egeriae* during their larval stage for each CO_2 level and generation.

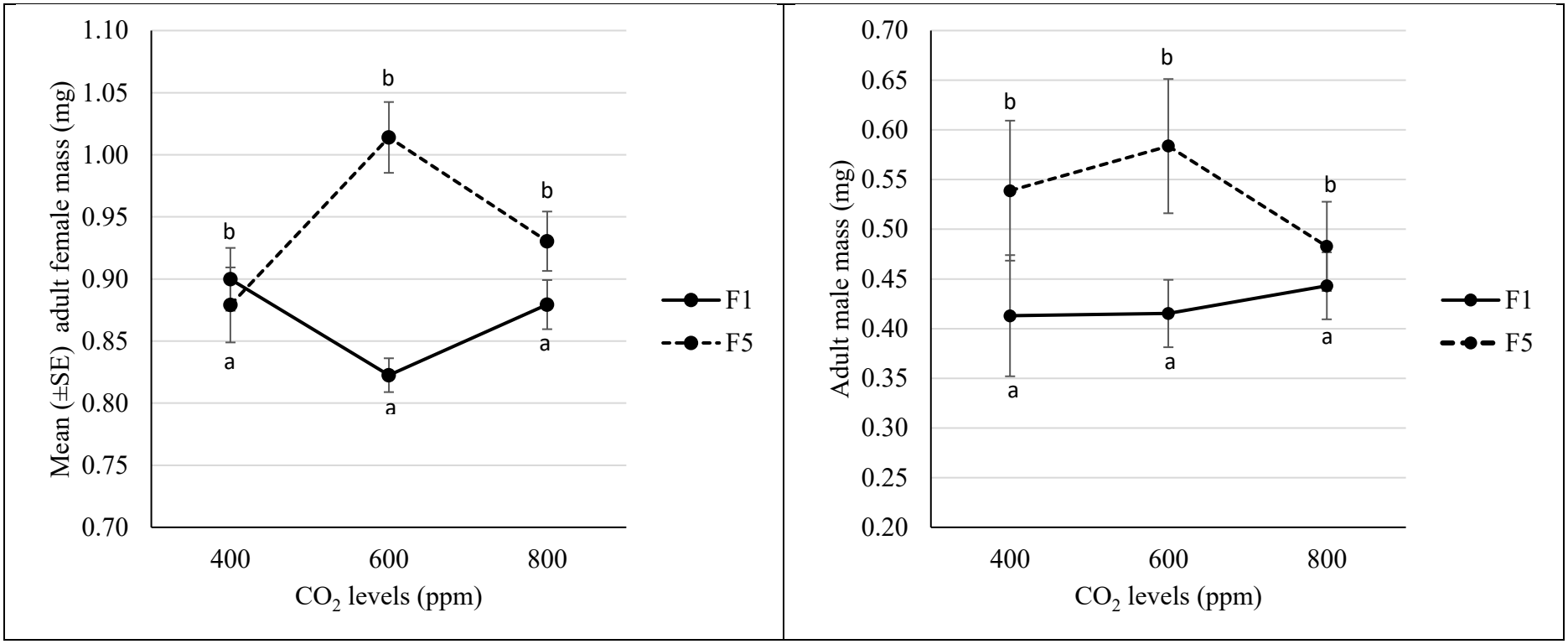


Figure 5-6: Mean (±SE) adult female (left) and male (right) mass (mg) of *Hydrellia egeriae* after emergence for each CO_2 level and generation.

5.4 Discussion

5.4.1 The effect of elevated CO_2 and planting density on *Egeria densa* nutritional value

This study tested the effect of elevated CO_2 and different planting densities on the nutritional quality, measured as leaf C/N ratio, of *E. densa*. These factors are particularly important within the context of *E. densa* invasion, climate change and biological control efforts. In a meta-analysis on the effect of elevated CO_2 on plant-insect interactions, Robinson *et al.* (2012) highlight the importance of looking beyond one-way studies, as interactions with environmental and biotic variables give more robust answers regarding the predicted impact of climate change. Although Silveira & Thiebaut (2020) found that in monoculture and mixed stands of *E. densa*, *Elodea canadensis* Michx. (Hydrocharitaceae) and *Lagarosiphon major*, an increase in planting density stimulated interspecific, rather than intraspecific competition, this study found that *E. densa* C/N ratios were not affected by population density within CO_2 levels, but predominantly by ambient CO_2 concentration that was bubbled into the water. Under elevated CO_2 levels, leaf carbon content was significantly increased from 400ppm and 600ppm to 800ppm. This study confirms results from Hussner *et al.* (2019), which found that elevated CO_2 (400ppm, 600ppm and 800ppm) did not increase or decrease the relative growth rate (g g^{-1} DM d^{-1}) of *E. densa*, but increased leaf C/N ratios at 800ppm at high DIC (3.0mM). In comparison to Hussner *et al.* (2019), the measured DIC levels in experimental buckets were lower, and thus any shifts in C/N ratios could be understated.

For biological control efforts, this implies that *E. densa* leaves will be less nutritious, not necessarily because of different densities of weed stands. When mixed into the water, CO_2 binds with H_2O to form carbonic acid or free CO_2^* (H_2CO_3), which reduces the water pH, depending on alkalinity (Talling 2010). The proportion of CO_2^* and HCO_3^- in the DIC pool shifts depending on pH, with more CO_2^* available at a pH < 7.0 (Talling 2010; Pedersen *et al.* 2013). CO_2^* is less diffuse in water, but costs aquatic plants metabolically less than HCO_3^-

(Pederson *et al.* 2013), and thus at 800ppm, carbon enrichment stimulated *E. densa* carbon sequestration. Previous studies have also found that starch accumulates in leaves at increased CO_2 levels, and thus nitrogen content decreases due to downregulated photosynthesis (Hussner *et al.* 2016; Dulger *et al.* 2017; Hussner *et al.* 2019). However, many freshwater systems are already functioning at DIC levels well above ambient CO_2 (Maberly 1996; Hasler *et al.* 2016). For example, Demars & Tremolieres (2009) found that carbon within ground-fed rivers systems ranged between 3 to 43 times atmospheric levels. Thus, naturally *E. densa* stands are already subjected to a heterogeneous DIC mosaic in the field, depending on the external and internal characteristics of the freshwater system. This implies, that *E. densa* leaf nutritional value might already differ between invaded sites.

5.4.2 The effect of elevated CO_2 on *Hydrellia egeriae* herbivory

Factors that affect plant nutritional quality are of interest for biological control efforts. This study showed that *Hydrellia egeriae* feeding between CO_2 levels within a generation was not significantly different. However, within CO_2 levels, feeding increased between F_1 and F_5. In the context of this experiment, the change in leaf consumption between generations is a result of diminishing nutrients in experimental buckets. Nutrient-rich sediment was used for initial planting, but nutrients were not added to treatments during the six month course of the experiment. Therefore, it is possible that sediment nutrients became depleted, which limited plant nutrient availability. Despite this experimental limitation, most of South Africa's freshwater systems are eutrophic (Coetzee & Hill 2012), and a major driving force for aquatic macrophyte invasions. Therefore, in the field, nutrients will not be a limiting factor.

Larvae of holometabolous insects have a high capacity to store nutrients (Sun *et al.* 2015). These insects emerge as adults with fat storages (C-rich lipids) obtained from larval feeding (Arrese & Soulages 2010). For example, fat bodies are used in the physiological age-grading system of *H. pakistanae* females (Lenz *et al.* 2007). The fat body of newly emerged

female flies are diffuse, green in colour and can make up almost half of the abdomen. The green colour is linked to leaf pigments consumed during the larval feeding stage. Fat bodies help insects overcome starvation within the first few hours of emergence, assist with female reproduction (Arres & Soulages 2010) and are reduced with accumulative oviposition (Lenz *et al.* 2007). For example, Aguila *et al.* (2013) found that ovary development and thus fecundity was prohibited when programmed cell death of larval fat bodies were inhibited. Adult mass did not significantly increase over CO_2, however *H. egeriae* expressed compensatory feeding between generations, which resulted in mass gain. In a review on the effect of elevated CO_2 levels on plant-insect interactions, categorised according to feeding guild, Bezemer & Jones (1998) found that leaf-miners, within a terrestrial system, only slightly increased food consumption, and that insect mass and population density was reduced. In contrast to this, the results from this aquatic system illustrate that nutrient limitation had a larger effect on leaf-mining than CO_2 levels. Increases in mass, as seen during F_5, can be linked to higher levels of carbon in leaves. For example, previous studies have found that fruit flies, grasshoppers and caterpillars store more fat when fed on a nutrient-poor or carbohydrate-rich diet (Warbrick-Smith *et al.* 2006; Le Gall & Behmer 2014; Smith *et al.* 2014). Within the context of this study, *H. egeriae* adults with more fat storage may be able to survive starvation for longer, as has been seen with *D. melanogaster* (Liu *et al.* 2012). However, other aspects of *H. egeriae* fitness need to be tested, such as development and survival of progeny.

Feeding trials in this study were conducted under quarantine conditions, and not directly under elevated CO_2 conditions (open top chambers). Thus, future studies could investigate *H. egeriae* feeding and development under such conditions. A limitation to this study was the generally low DIC level in experimental buckets, and thus plants could have experienced DIC limitation. In addition, this study failed to significantly mody temperature. Past studies have shown that *E. densa* responds positively to increases in temperature (Haramoto & Ikusima

1988; Silveira & Thiébaut 2017). Therefore, it will be of great importance to investigate the interaction between *E. densa* and *H. egeriae* under both elevated ambient CO_2 and temperatures. Warmer conditions could change *H. egeriae* survival if they are close to critical or lethal limits (Chapter 2).

This study was an initial step in understanding aquatic plant-herbivore interactions under climate change. If *E. densa* is already subjected to a heterogeneous mosaic of water DIC in the field, determining leaf nutritional qualities of field collected *E. densa* will add to the knowledge basis of this study. As such, the continuation of this study will also benefit other submerged invasive aquatic biological control programmes, such as *H. verticillata*, *L. major* and *M. spicatum*, which have become problematic in freshwater systems worldwide (Hussner *et al.* 2017).

6. General Discussion

Egeria densa is a secondary invaderto systems once invaded by the "Big Bad Five" aquatic weeds in South Africa. It quickly forms monoculture stands in open niches that alter nutrient stoichiometry water flow and cause sedimentation (Yarrow *et al.* 2009; Drexler *et al.* 2020). Because of the negative effects of *E. densa* (Hill & Coetzee 2017), a biological control programme was initiated as part of a rapid response to its establishment and dispersal in South Africa. The South American ephydrid fly, *H. egeriae* was released for the first time in South Africa, and worldwide in October 2018. This thesis presented the findings of the sequential stage of a biological control programme, after a potential biological control agent received permission for release (Moran *et al.* 2013; Smith *et al.* 2019).

Laboratory experiments that explored the thermal physiology of the agent have shown that *H. egeriae* is climatically suitable (i.e. abiotic filter) to its novel range and should be able to establish and proliferate at all sites in South Africa. Post-release surveys confirmed that the agent has established at two sites, but field populations diminished without augmentative releases. Due to low field populations, especially in the Nahoon River, herbivory levels were almost negligible, and thus had little effect on *E. densa*. In the Midmar Dam, herbivory levels were almost as high as in its native range, but has not yet translated into target weed population-level effects, since high agent numbers were short-lived. Nonetheless, the agent indicated potential to inflict high levels of damage to its host plant in its introduced range, but such effects will require augmentative releases. This study has also shown that biotic factors, such as parasitism are affecting *H. egeriae* field population build up, but not establishment.The acquisition of parasitoids was predicted, butparasitism rates and seasonality is essential information to refine augmentative release strategies with the aim of attaining sufficiently large enough field populations and top-down control.

This study only captured the initial stages after releasing the agent. Post-release evaluations require years of investment (Martin *et al.* 2018; Hinz *et al.* 2019). Continuing post-release surveys will give a clearer indication of establishment patterns across the country and adjusted augmentative releases should reduce biological control parasitism and assist in field population build-up. Long-term post-release evaluations will also be necessary to monitor the effect of the biological control agent on its target weed, the response of the surrounding community and to calculate return on investment values.

Finally, this study aimed to predict the effect of elevated CO_2 on the biological control of *E. densa*. Results indicated that *E. densa* will become less nutritious at 800ppm, should nutrients be limited. Furthermore, larvae consumed less plant material when given less nutritious plant material, but despite this, *H. egeriae* adult mass did not change. This study was limited by nutrient availability, which will not be limited in South Africa's eutrophic waters. Plant-herbivore interactions between *E. densa* and *H. egeriae* is harder to predict due to the nature of carbon in freshwater systems. For example, many freshwater systems are already functioning at DIC levels well above ambient CO_2 (Maberly 1996; Hasler *et al.* 2016). Ground-fed river system can range between 3 to 43 times atmospheric levels. It would not be surprising that *E. densa* stands are already subjected to a DIC heterogeneous mosaic in the field, depending on the external and internal characteristics of the freshwater system. Thus, elevated CO_2 will have variable effects on *E. densa* and its biological control agent, depending on the characteristics of the water body. It will be important to establish the current and future water DIC levels and *E. densa* leaf qualityfor comparison within and between sites.

The goal of this biological control programme is to reduce the abundance (cover, density) of *E. densa* at invaded sites, and measure community response to its management. However, within the short post-release survey period, constraining factors to *E. densa* management were established, and should be incorporated into an adjusted biological control

strategy. Future work should aim to increase release events for agent population build-up, especially after winter when parasitism is high. To optimize the success of managing *E. densa* and push toward long-term ecosystem recovery, a more holistic approach is suggested.

6.1 Biological control and ecosystem restoration

Biological control is only one of the management options for invasive species (Hussner *et al.* 2017). Similarly, biological control success is context dependent (chapter 1), and it has often been anticipated that it will indirectly allow native vegetation growth i.e. passive ecosystem recovery (Zavaleta *et al.* 2001; Denslow & D'Antonio 2005; Carson *et al.* 2008). For example, post-release evaluations 7 and 14 years after the release of four biological control agents against the invasive tree, *Melaleuca quinquenervia* (Cav.) Blake (Myrtaceae) in southern Florida, USA showed native species returned to the study site. Although melaleuca was not completely eradicated, native diversity correlated positively with reduced invader densities (Rayamajhi *et al.* 2011). Nonetheless, a review of biological control post-release evaluations showed that more than half of the plants that grew after successful biological control suppression were exotic species (Clewley *et al.* 2012), and in some cases, no recolonization of any species occurred (Reid *et al.* 2009). This is not necessarily inefficiency on the part of biological control, but a reminder that ecosystem recovery can be complex and nonlinear (Scheffer 2009; Chapter 3), are under constant threat in a rapidly changing world (Chapin III *et al.* 2000) and not always given sufficient time to stabilize and recover, especially when expected to do so passively (Pelletier *et al.* 2020). In addition, systems that are subjected to chronic disturbances experience secondary invasion or invasion meltdown. Thus, aiming for passive restoration in the biological control of *E. densa* or any programmeis not always a viable option and an integrated approach is necessary (Stephens *et al.* 2009; Zavaleta *et al.* 2001).

6.2 Ecological engineering

An integrated management plan needs to incorporate active recovery to prevent invasion treadmill or shifts between invasive stable states. Such an approach could include

native species reseeding after suppression of the target weed, as have been seen in some biological control programmes (DeLoach *et al.* 2000; Dudley *et al.* 2011; Rayamajhi *et al*. 2011). For example, active revegetation has been an important component to managing *Tamarix* species in riparian ecosystems in the United States, as it assists functional redundancy, and hence resilience (DeLoach *et al.* 2000; Dudley *et al.* 2011). This is in line with the four factors that were identified for maintaining or enhancing ecosystem resilience: connectivity, habitat heterogeneity, functional redundancy and diversity (Pelletier *et al.* 2020). Functional redundancy refers to the ability of species to perform similar roles. In a greenhouse experiment, Petruzzella *et al.* (2020) showed that macrophytes native to the Netherlands, are able to competitively reduce the establishment of the invasive species *L. major*, and that resistance was enhanced by native community richness and functional similarity between native and invader species. Ultimately, these four factors interact with the spatial and temporal properties of the system (Scheffer 2009; Pelletier *et al.* 2020). For example, Alofs & Jackson (2014) found that herbivory/predation intensity was stronger in lentic systems, compared to lotic water bodies due to spatial confinement. In contrast, lotic systems may be more subjected to disturbances, for example changes in water velocity, which reduces herbivory/predation intensity.

Reseeding of native species, i.e. active recovery, can overcome some of the shortages within systems that have undergone long periods of biological invasion. Native species do not necessarily have a lower propagule pressure than exotic species (Heidbüchel *et al.* 2016), but when connectivity between systems are lost, native species recolonization may be slow and outcompeted by exotic species (Zavaleta *et al.* 2001; Levine *et al.* 2004). In addition, reseeding can assist in preventing open niches for exotic species colonization. This gives practitioners the opportunity to choose species that are ecological engineers themselves and that are functionally equivalent to the invasive species. Although it is unfavourable to have functional

homogenous communities (Pelletier *et al.* 2020), the initial recolonization of native species could stabilize the system (Emery-Butcher *et al.* 2020), before working towards species diversity and functional heterogeneity.

Complete eradication and restoration are seldom the result of management practices (Hussner *et al.* 2017; Pelletier *et al.* 2020), and in some cases diverse communities contain invasive species (Levine *et al.* 2004). As mentioned by Sailer (1972), significant progress towards ecosystem recovery is made when invasive aquatic plants, with their natural enemies, become a part of the aquatic community. In contrast to engineering resilience, where ecosystems are expected to "bounce back" to a former state (Holling 1973), ecological resilience offers the opportunity for systems to learn and adapt (Angeler *et al.* 2014). In this regard, biological control together with reseeding could facilitate communities, where invasive species are contained and even contributes to ecosystem resilience and functioning (Sailer 1972).

6.3 **Resilience paradigm**

In the age of humankind or the Anthropocene, humans have a significant effect on the environment, which is exacerbated by an ever-increasing population. In return, humans are dependent on the services provided by the environment (Millennium Ecosystem Assessment 2005; Brauman 2007; Pejchar & Mooney 2009; Geist 2011). Although it would be important to consider ecological resilience, success will be limited when stakeholders operate differently around environmental issues. Therefore, it will be important to no only strengthen ecological resilience, but more holistically, socio ecological resilience (Biggs *et al.* 2012; Folke *et al.* 2016; Hussner *et al.* 2017; Pelletier *et al.* 2020). The social aspect refers to humans and their activities such as politics, culture, economy and technology, whereas ecology refers to the earth, and the abiotic and biotic components that it is comprised of (Folke *et al.* 2016). Thus, economic and social prosperity and development are intertwined with the earth. For example,

this study aims to manage *E. densa* because of the adverse effects it has on freshwater systems, yet invaded sites are modified by humans and highly polluted through sewage leakages and agricultural and urban runoff. This highlights that society and ecosystems can function very different from each other, and socio ecological resilience aims to unite these functions in a panarchy (Holling 1973; Biggs *et al.* 2012). With increasing demand for freshwater in a changing climate, perspectives on invasive alien plants and freshwater conservation may be different among various stakeholders (Chapin III *et al.* 2000; Convention on Biological Diversity 2006; Hussner *et al.* 2017). Thus, long-term aquatic weed management should be viewed through the lens of socio ecological resilience (Figure 6-1; Folke *et al.* 2002; Folke *et al.* 2016). This thesis presents findings of an *E. densa* biological control agent that exhibits potential to suppress its target weed. This management option will contribute limited resilience to ecosystem recovery (dashed arrow Figure 6-1). When this biological control program is conducted within an aquatic weed management framework, i.e. action taken from the inside of the circle outwards, resilience is strengthened (two dashed arrows). However, resilience is strongest when aquatic weed management is approached by working from the outside of the circle to the inside. This is emphasized by Albert *et al.* in 2020 when addressing the freshwater biodiversity crisis:

> "Conservation actions are most effective when they are implemented with full recognition of the genuine fragility of ecosystems. We currently have technologies to manage and amerliorate many aspects of the freshwater biodiversity crisis – what we lack is political will."

There are seven principles for building social ecological resilience, which was suggested by Biggs and colleagues in 2012. Before I briefly mention them, it is important to highlight that the application of these principles are context-dependant and will change over time, since socio ecological systems are adaptive systems (Biggs *et al.* 2012). Therefore, I will only apply some principles to this study as examples of how aquatic weed management should be integrated into socio ecological resilience.

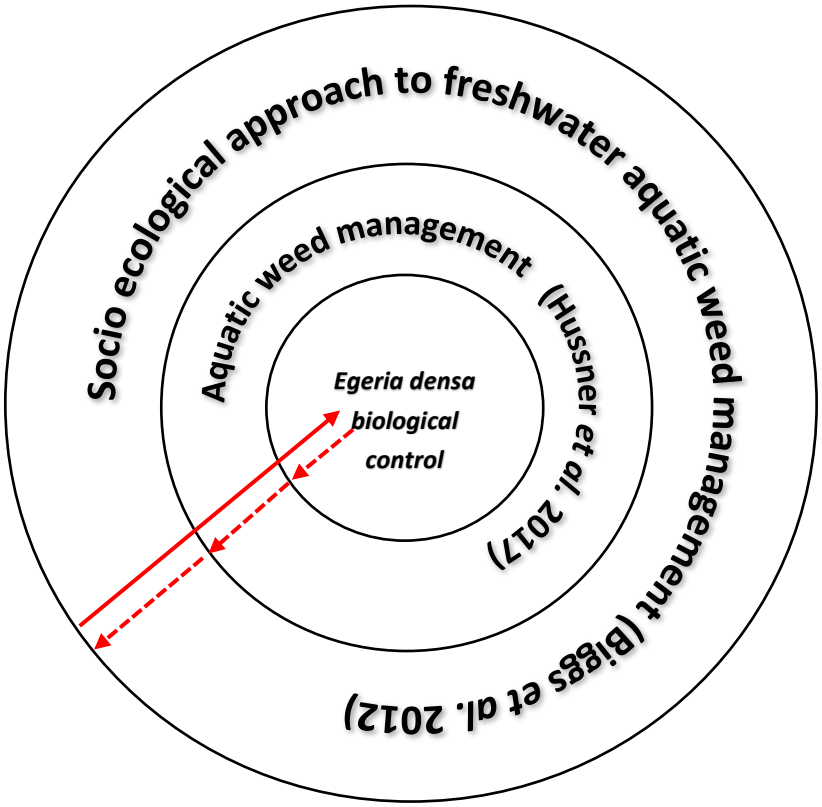


Figure 6-1: Theoretical diagram on freshwater conservation through social ecological resilience based thinking. Length of arrows indicate contribution to resilience. Extended from Biggs *et al.* (2012) and Hussner *et al.* (2017).

The first principle is to maintain diversity and redundancy. As mentioned earlier, in terms of active restoration, functional redundancy refers to the ability of system components to perform similar roles. Resilience is enhanced, because although components fulfil the same role, they may respond differently to change. In terms of this study, it will be important to not only practise *E. densa* weed management, but to involve local municipalities, residents, farmers etc. in attaining the same goal. For example, local municipalities are often guilty of not maintaining sewage stations, and thus effluent with high levels of nitrates and phosphate flow

into freshwater systems. High nutrient levels not only promote invasive species growth, but also becomes a chronic disturbance, which increases difficulty in attaining long-term aquatic weed management. Residents are often unaware of the status of aquatic plants and acts as carriers of exotic plants between systems.

The second principle is managing connectivity, which refers to the structure and strength in which resources, information or species are dispersed among system components. Connectivity can either be positive or negative. For example, the Nahoon River site used in chapter 3, is approximately 22.7km downstream from the Nahoon Dam. Along this connected system, non-point pollution, sewage leakage and invasive species propagules are easily distributed to the study site, which is not only a study site, but situated next to a residential area. Residents from this area have seen the degradation of the Nahoon River over the last couple of years, and some have even moved away because of no improvement. Over the course of the study period, attempts by residents and other local bodies have been made to clear water hyacinth and *E. densa* through herbicides.In some cases this has been done independently, and proved counteractive to biological control efforts and water quality improvements. This is a result of low connectedness. By increasing connectivity, different stakeholders can work together to address invasive alien plant infestations through integrated pest management and a more holistic approach.

Thirdly, slow variables and feedbacks must be managed. Slow variables can be positive or negative, may not be apparent and occur in small quantities. For example, small quantities of nutrients that run into the Midmar Dam or fishermen that disperse *E. densa* fragments to different parts of the dam. Positive slow variables would be school programmes that mass-rear and continuously release biological control agents. The fourth principle is to foster complex adaptive systems thinking, which acknowledges that systems are made up of complex

interactions between humans and ecosystems. This principle does not necessarily affect resilience, but is necessary for adaptive management during changes or devastating events.

Fifthly, learning should be encouraged. Socio ecological systems are developing, and thus learning through hypotheses testing and experimentation is important for adaptive management. Embedded into this principle is the need to continue post-release surveys on the biological control of *E. densa*. Similarly, this study aimed to also monitor the change in aquatic community composition at *E. densa* invaded sites, using the water surface as a middle ground. It would be beneficial to continue such monitoring for system-based aquatic weed management, which will assist adaptive management based on system stables states, e.g. floating dominated, submerged dominated or algae dominated. In addition, more investigations can be made into the response of *H. egeriae* and *E. densa* to climate change.

Finally, the sixth and seventh principles are to broaden participation and promote polycentric governance. This can be accomplished through community engagement, and involving multiple institutions that can individually address emerging problems at the right time and scale.

6.4 Conclusion

Hydrellia egeriae is the first biological control agent released against *E. densa* worldwide, and also the first biological control agent released against a submerged aquatic weed in South Africa. This study showed that the thermal physiology of the leaf-mining fly is wide and encompasses its host plant, contributing to its ability to establish and persist at all *E. densa* sites in South Africa. These thermal requirements and its simple life cycle contribute to its attractivess as a potential biological control agent in other countries. Post-release surveys indicate that *H. egeriae* requires augmentative releases to sustain and boost field populations and, especially to overcome parasitism. Leaf-mining damage have been recorded, but have not translated into *E. densa* population-level impacts due to low agent populations, time and

underlying factors such eutrophication. Therefore, other countries should investigate the potential for parasitism acquisition before importing *E. densa*, but more importantly, seek to address any underlying factors that contribute to *E. densa* invasiveness, i.e. eutrophication. It is harder to predict biological control agent response to climate change, but continued post-release surveys will provide information for future adaptive management.

The biological control against *E. densa* is in its infancy. Results from this study suggest that *H. egeriae* can be an effective biological control, but will require adjusted release strategies and continued field monitoring. This will require frequent release events, for example every three to four months, and especially after winter when insect development lag in comparison to its host plant. Considering the continued pollution and enrichment of South Africa freshwater systems, shifts between stable states and secondary invasions, a more holistic approach is necessary to push systems towards recovery. Integrating biological control efforts and active restoration within socio ecological resilience and thus freshwater conservation, will not only assist in the management of *E. densa*, but aquatic weed management in general. Considering the challenge to conserve freshwater systems worldwide, such an approach may provide useful information and an adaptable management for other countries.

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