

THE POTENTIAL OF  
*HYDRELLIA EGERIAE* RODRIGUES  
(DIPTERA: EPHYDRIDAE)  
AS A BIOCONTROL AGENT FOR  
*EGERIA Densa* PLANCH.  
(HYDROCHARITACEAE)  
IN SOUTH AFRICA

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ROSALI SMITH

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## Abstract

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The integrity of South Africa's valuable freshwater ecosystems has been threatened by aquatic invasive plants since the 1900s. Floating aquatic weeds, such as *Eichhornia crassipes* (C. Mart) Solms (Pondederiaceae), *Pistia stratiotes* L. (Araceae), *Salvinia molesta* D.S. Mitchell (Salviniaceae), *Azolla filiculoides* Lam. (Azollaceae), and the emergent weed, *Myriophyllum aquaticum* Verdc. (Haloragaceae) benefited from open, nutrient-rich water bodies. Due to the limitations of mechanical and chemical control in aquatic environments, classical biological control has been a huge asset in managing these weeds; consequently bringing them under complete or substantial control. However, submerged aquatic weeds are widely distributed through the aquarium trade in South Africa; facilitating their invasion into new habitats. The removal of surface mats following the successful management of floating weeds has enhanced the growth and competitive ability of submerged aquatic weeds, such as *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae), *Myriophyllum spicatum* L. (Haloragaceae) and *Egeria densa* Planch. (Hydrocharitaceae) in South Africa. Of these species, *E. densa* has become the most widely distributed, invading numerous systems across South Africa. Compared to other exotic submerged aquatic plants, *E. densa* is the only species capable of inhabiting freshwater systems in every province and therefore, it is vital to manage existing populations and prevent its further distribution and invasion. *Hydrellia* spp. (Diptera: Ephydriidae) biological control agents have been used extensively in the management of submerged aquatic weeds elsewhere, particularly those in the Hydrocharitaceae (Balciunas and Burrows 1996; Wheeler and Center 2001. *Hydrellia egeriae* Rodrigues (Diptera: Ephydriidae) has been identified as a promising candidate for *E. densa* and was imported into quarantine at Rhodes University, Grahamstown in 2014. The aims of this study were to conduct a pre-release assessment of the potential of *H. egeriae* as a biological control agent for *E. densa* in South Africa.

The first objective of this study was to establish the life history of the agent under controlled conditions on *E. densa* found in South Africa, as well as its population growth parameters to predict its invasion success in the field. Secondly, laboratory host-specificity testing was conducted to validate the host range of the agent, in view of published native range host-specificity testing, and to establish potential risks to non-target species, should it be released. Finally, a biological control agent should also effectively reduce the fitness of its host plant, and therefore, impact studies were conducted. Laboratory impact studies have been limited in the past, in that they only investigate agent damage for short ecological periods, thus underestimating the damage capacity of the agent under investigation. Therefore, the damage capacity of *H. egeriae* was investigated over three consecutive generations in multi-generational impact trials.

In a controlled environment of  $22 \pm 2^\circ\text{C}$ , *H. egeriae* exhibited the ability to rapidly increase in population size within a short period of time, which will enhance agent establishment and build-up in the field. Host-specificity trials indicated that *H. egeriae* has a host range restricted to the Hydrocharitaceae, with exploratory feeding and development on *Lagarosiphon major* Ridley, *L. muscoides* Harvey and *Vallisneria spiralis* L. However, only *L. major* supported agent development during paired larval choice tests, and continuation trials showed that the test species was not physiologically capable of supporting viable agent populations. Risk analysis illustrated that the feeding and reproductive risks that *H. egeriae* pose to non-target species are very low and therefore, *H. egeriae* should be safe for release in South Africa. Additionally, significant damage to vital plant structures (shoot growth and side shoot length) was only recorded under high (five larvae) agent abundances. Encouragingly, the number of leaves mined at the end of the experiment was similar for both intermediate (three) and high (five) larval abundances, suggesting that cumulative leaf-mining under

intermediate larval abundances has the potential to reduce the fitness of *E. densa*, given sufficient time.

Results from pre-release assessments provide a robust understanding of the specialization of the potential biological control agent to its host plant. Nevertheless, the absolute success of a biological control programme depends on the many factors after pre-release assessments that determine agent establishment, persistence and target weed suppression, e.g. mass-rearing, release protocols and a/biotic factors within the recipient community. Considering these factors, the best mass-rearing and release protocols are proposed here and future research priorities are identified. Finally, the long term success for managing *E. densa* in South Africa will require a holistic approach to address the underlying factors, such as eutrophication and human-mediated distribution that drive submerged aquatic plant invasions.

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## Chapter 1: General Introduction

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### 1.1 INVASIVE ALIEN PLANTS

Human well-being, social development and economic growth are intimately intertwined with goods and services provided by ecosystems. However, urbanization and agriculture places tremendous pressure on ecosystems (Keane & Crawley, 2002; Van Driesche *et al.*, 2010). Globalization and climate change mediate species invasions that threaten biodiversity; reducing ecosystem service benefits (Lodge 1993; Mack *et al.*, 2000; Richardson *et al.* 2000; Begon *et al.*, 2006). In South Africa, ecosystem services are estimated at R152 billion per annum (De Lange & van Wilgen, 2010; Chamier *et al.*, 2012) of which R6.5 billion are lost in the presence of invasive alien plants. In total, 63% of this estimate is attributed to water loss, 22% to loss in grazing capacity and 15% to biodiversity (De Lange & van Wilgen, 2010; Chamier *et al.*, 2012). The impacts of invasive alien plants throughout the world are extensive and cost governments millions to control every year. Pimentel *et al.* (2000) estimated that approximately 5 000 alien plants had invaded the United States (USA) and were responsible for the loss and damage of US\$24.4 billion worth of ecosystem services per annum. Additionally, costs for managing invasive alien plants were estimated at US\$9.7 billion per year. In the United Kingdom (UK), management costs for only three invasive species, *Hydrocotyle ranunculoides* L.f. (Apiaceae), *Myriophyllum aquaticum* Verdc. (Haloragaceae) and *Crassula helmsi* Kirk (Crassulaceae), cost £3 million per year (DEFRA, 2008).

In their introduced range, invasive alien plants exhibit higher growth and distribution rates that aid competitive ability over native species (Williams, 1954; Keane & Crawley, 2002). In the USA, *Lythrum salicaria* L. (Lythraceae), purple loosestrife, expands its distribution by 115 000ha per year and has reduced the biomass of 44 native species

(Pimentel *et al.*, 2005). Invasive alien plants also alter ecosystem processes, such as fire regimes, soil erosion, water loss and hydrology regimes. For example, in the western parts of the USA, *Bromus tectorum* L. (Poaceae) increased the frequency of fires in the invaded area from every 60 to 110 years to every three to five years, which resulted in a drastic reduction in the ability of native vegetation to establish (Whisenant, 1990).

Higher water utilization and transpiration rates exhibited by invasive alien plants reduce river flow (Chamier *et al.*, 2012). For example, in South Africa, invasive alien plants utilize approximately 3.3 billion m<sup>3</sup> more water annually compared to native species (Van Wilgen & de Lange, 2011). De Groot (1993) showed that the floating aquatic weed, *Eichhornia crassipes* (C. Mart) Solms (Pondederiaceae) has a transpiration rate that is three times higher than native plants. In freshwater systems, the negative effects caused by invasive aquatic weeds are detrimental. They impede navigation, recreational activities, choke water channels, obstruct energy generation, facilitate water-borne diseases and altogether degrade the aquatic ecosystem (Scheffer *et al.*, 2003; Pejchar & Mooney, 2009; Ray & Hill, 2012). Dense monoculture stands prevent sunlight from penetrating the water surface, reducing dissolved oxygen supply in the water that kills other aquatic organisms (Scheffer *et al.*, 2003; Van Driesche *et al.*, 2010; Chamier *et al.*, 2012; Ray & Hill, 2012). A study on the effect of *Salvinia molesta* D.S. Mitch. (Salviniaceae) infestations in the Sepik River, Papua New Guinea, revealed that oxygen levels were reduced by 37%, killing the submerged vegetation below (Van Driesche & Bellows, 1996).

## **1.2 MANAGEMENT OPTIONS**

The best approach in reducing the negative effects associated with invasive plants is to prevent their arrival into a new habitat through the enforcement of policies that prohibit their importation (Luken & Thieret, 1997; Gettys *et al.*, 2014; García-de-Lomas & Vilà, 2015), followed by rapid eradication where small populations had already established (Pleuss

*et al.*, 2012; SANBI, 2013). Early detection and rapid response cost far less than managing extensive established populations. For example, early detection and eradication of *Fallopia japonica* Houtt. Ronse Decr. (Polygonaceae) in Wales reduced management efforts by £23 million (DEFRA, 2008). However, Pleuss *et al.* (2012) found that eradication success decreased significantly when control measures were executed four years after the arrival of a species into a new environment, and in many cases invasive alien plants have to be managed continuously, due to range expansion and establishment. Generally, four management methods are used. These include mechanical, chemical, biological control (hereafter, biocontrol) and integrated pest management (IPM) (van Wilgen & de Lange, 2011). Each control method is discussed briefly.

#### Mechanical/manual control

Mechanical or manual control is the removal of invasive alien plants through human labour, human driven machinery or eco-physiological manipulation (Lancar & Krake, 2002). Numerous techniques have been developed in relation to the different invasive alien plant life traits and the various habitats they invade (terrestrial or aquatic). In terrestrial habitats, mechanical control is best suited if the weed infestation is sparsely distributed or occurs in isolated patches, due to the risk of disturbing and/or removal of native vegetation (van Driesche *et al.*, 2010). In most cases, mechanical control is accompanied by herbicide treatment to curb weed growth from trunks, seeds or any vegetative parts (Lancar & Krake, 2002; Csurhes *et al.*, 2008).

The ubiquitous nature of aquatic habitats increases the difficulty of employing mechanical control in these types of habitats. Control may be obtained in small water bodies, but only provides short-term relief for larger infestations (Lancar & Krake, 2002), requires frequent treatments due to the fast regeneration rates of aquatic weeds, and is time-consuming, making it an expensive practise. For example, the submerged aquatic weed,

*Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae) grows 2.54cm daily and regrows to the same density prior to mechanical control within two months (Gettys *et al.*, 2014). Mechanical control for *E. crassipes* is also not a viable option due to its ability to double in biomass every 11 to 18 days in nutrient enriched habitats (Hill & Coetzee, 2009).

Mechanical control also has non-selective effects, because of the removal of native species and juvenile fish during operations. If not employed with caution, mechanical control could facilitate weed dispersal through fragmentation or “hitchhiking” *via* equipment (Caffrey & Mohanan, 2006; Gettys *et al.*, 2014). Manipulating water levels to expose weeds to unfavourable conditions (drawdowns) has obtained some measure of control for aquatic weeds (Hussner *et al.*, 2017). However, this method is not effective for weeds that produce seeds, turions or tubers, because they only stimulate the development of these reproductive structures. Drawdowns are mostly effective in controlled systems, like irrigation channels and reservoirs (Lancar & Krake, 2002; Gettys *et al.*, 2014).

#### Chemical control

Herbicide application is a fast-acting method that is less time-consuming and labour intensive compared to mechanical control (Lancar & Krake, 2002; Hill & Coetzee, 2008). There are two types of herbicides; contact herbicides that kill plant structures they are applied to, and systemic herbicides that translocate through the plant, disrupting physiological processes and even affecting underground structures. Chemical control is most effective for small infestations (van Driesche *et al.*, 2010). For larger infestations, control can be achieved, but costs more and longer exposure times are required (Luken & Thieret, 1997; Gettys *et al.*, 2014). Subsequently, frequent treatments are required for weeds that regrow. This may result in the deterioration of underground or open waters, and the entry of toxins into the food web (Ray & Hill, 2013). Additionally, non-selective herbicides may also kill desirable native species, and in some cases, weeds become resistant to chemical treatment. For example, *H.*

*verticillata* has recently become resistant against fluridone and endothall in the USA (Berger & MacDonald, 2011). Because fluridone inhibits the enzyme phytoene desaturase (PDS), *H. verticillata* underwent somatic mutations in the gene PDS, making it two to six times more resistant to fluridone (Michel *et al.*, 2004).

### Biocontrol

One of the explanations for the invasiveness of exotic plants is the enemy-release hypothesis (ERH) (Williams, 1954). In their introduced habitat, top-down pressure from coevolved specialist enemies is reduced, which enhances weed population growth and competitive ability (Keane & Crawley, 2002; Colautti, 2004; Liu & Stiling, 2006; Halbritter *et al.*, 2012). Studies have shown that in the native region of the weed, plant damage is higher (Halbritter *et al.*, 2012), with proportionally more specialist herbivores that feed on reproductive structures (Liu & Stiling, 2006). The ERH forms the foundation for classical biocontrol, and therefore, specialist herbivores from the native range of the weed are introduced to restore top down pressure (Julien & White, 1997; Eilenburg & Hokkanen, 2006; Schoonhoven *et al.*, 2006). Specialists are essential because they express host use conservatism, which reduces non-target risk in the introduced range (Jermy, 1984; Bernays & Graham, 1988; Bordeur, 2012; Tingle *et al.*, 2016). In general, insects and pathogenic microorganisms (fungi and bacteria) are used as weed biocontrol agents (Begon *et al.*, 2006).

### Integrated pest management

The use of management options relies heavily on the availability of financial resources, equipment, trained personnel, environmental conditions and the source of the infestation. Therefore, control programmes are case-specific and should be employed accordingly (Hill & Coetzee, 2008). Adjustments are often made in the form of integrating control methods, a method referred to as integrated pest management (IPM). IPM is

ecologically focused, with the aim of using all available control options, while minimizing the use of herbicides (Begon *et al.*, 2006). It is especially employed where landscape-level control is required (Van Driesche *et al.*, 2010). A benefit: cost analysis of *E. crassipes* control programmes showed that IPM produced the best return on investment (US\$ 39/ha), compared to purely chemical (US\$209/ha) and biocontrol (US\$44/ha) (van Wyk & van Wilgen, 2002).

### 1.3 WEED BIOCONTROL IN SOUTH AFRICA

South Africa has a long history of species invasions. The influx of exotic plant species into South Africa started in the 17<sup>th</sup> century when the Dutch East India Company (VOC) established a refreshment station in Cape Town. After the depletion of indigenous forests for fuel and building material, seeds of exotic trees were imported from Norway and Sweden to supplement timber resources (Zimmermann *et al.*, 2004; Moran *et al.*, 2013; Townsend, 2015). During the 19<sup>th</sup> century, a second wave of species introductions and distribution was attributed to the growing interests in exotic species and the subsequent establishment of exotic gardens. For example, the well-known garden of Von Ludwig hosted 1 600 exotic species (Townsend, 2015). Today, approximately 660 exotic plants have been declared invasive (SANBI, 2013; South Africa Department of Environmental Affairs, 2014).

The practice of biocontrol in South Africa was initiated in 1913 with the release of the cactus scale, *Dactylopius ceylonicus* Green (Hemiptera: Dactylopidae) against *Opuntia monacantha* Haw (Cactaceae) (Moran *et al.*, 2013; Winston *et al.*, 2014). During this time, *O. monacantha* had invaded approximately 1 million hectare in only two provinces (South African Department of Environmental Affairs, 2007). The drastic reduction of *O. monacantha* was an excellent example of the potential of biocontrol. However, it was not until 1933, that the first research-based biocontrol agent, *Cactoblastis cactorum* (Berg) (Lepidoptera: Pyralidae), was released against *Opuntia ficus-indica* (L.) Mill (Cactaceae) in 1933 (Klein, 2011; Moran *et al.*, 2011). Despite the success of the agent in reducing *O. ficus-*

*indica* populations, biocontrol in South Africa only gained momentum some 40 years later in the 1970s (Moran *et al.*, 2011). Now ranked as the third most active country in the practice of biocontrol, South Africa has a record of 106 biocontrol agent releases against 48 weeds, which include cacti, shrubs, herbs, climbers, trees and water weeds. In total, 75 of these agents established, and have brought 18 weeds under substantial control, meaning supplementary control efforts are reduced. Ten weeds are under complete control, and no other control methods are needed (Moran *et al.*, 2013; SANBI, 2013).

South Africa's freshwater systems remain one of its most vulnerable ecosystems (Coetzee *et al.*, 2011a; Moorhouse & Macdonald, 2015). Although South Africa has almost no natural lakes and thus co-evolved free floating plants, the construction of reservoirs and impoundments has created ideal conditions for aquatic weed invasions. Additionally, urbanization, farming waste, industrialization and improper sewage treatments has enriched the majority of freshwater systems with nutrients, thus aiding their invasibility (Oberholster & Ashton 2008; Moran *et al.*, 2013; Ray & Hill, 2013). South Africa's freshwater systems have mostly been dominated by floating aquatic weeds since the mid-1900s and include *E. crassipes*, *S. molesta*, *Pistia stratiotes* L. (Araceae), *M. aquaticum* and *Azolla filiculoides* Lam. (Azollaceae) (Coetzee *et al.*, 2011a). The first aquatic weed biological programme was initiated in 1974 against *E. crassipes* with the release of the weevils, *Neochetina eichhorniae* Warner and *Neochetina bruchi* Hustache (both Coleoptera: Curculionidae). Since then, eight biocontrol agents have been released, the latest being *Megamelus scutellaris* Berg (Hemiptera: Delphacidae) in 2013 (Coetzee, pers. comm.). *Salvinia molesta*, *P. stratiotes*, *M. aquaticum* and *A. filiculoides* were also targeted for biocontrol and a review of these programmes in 2011 showed that these weeds have been successfully suppressed (Coetzee *et al.*, 2011b).

With the successful control of floating weeds, it was anticipated that available resources would enhance native species growth, but instead, a new suite of aquatic weeds has benefited from open, nutrient-rich water systems (Charudattan, 2001; Scheffer *et al.*, 2003; Coetzee *et al.* 2011a; Moran *et al.*, 2013; Bownes, 2014). For example, *E. crassipes* on the Vaal River was replaced by the submerged aquatic weed, *Myriophyllum spicatum* L. (Haloragaceae), following successful control (Coetzee *et al.*, 2011b). Since the early 2000s, an increasing number of waterbodies have been invaded by submerged aquatic species, which include *Egeria densa* Planch. (Hydrocharitaceae), *H. verticillata* and *M. spicatum*. These three species are also considered the three most notorious submerged aquatic weeds in the USA (Cuda *et al.*, 2008). Submerged aquatic plants are used extensively as aquarium plants and are easily accessed through the trade (Coetzee *et al.*, 2011a; Martin & Coetzee, 2011). Improper disposal after use and flooding of ornamental ponds increase the propagule pressure of exotic aquatic plants in freshwater systems, thus enhancing their invasiveness (Lockwood *et al.*, 2005; Martin, 2013). For example, 34% of the aquarium plants imported into the Netherlands were exotic species of which an estimated 1.7 million *E. densa* plants were imported (Netherlands Department of Environmental Science, 2014). Additionally, Martin & Coetzee (2011) found that 43% of pet stores in South Africa traded with the prohibited *Cabomba caroliniana* A. Gray (Cabombaceae), 38% with *Elodea canadensis* Rich. in Michx. (Hydrocharitaceae) and 48% with *E. densa*. Currently, *E. densa* is the most widely distributed submerged aquatic weed in South Africa. In addition, MAXENT distribution modelling also showed that of all the submerged exotic species recorded in South Africa, *E. densa* has the widest predicted range; capable of inhabiting freshwater systems in every province (Martin, 2013).

#### 1.4 *EGERIA DENSA* (BRAZILIAN WATERWEED)

##### Background and distribution

The first record of *E. densa* in South Africa was in 1963 in KwaZulu-Natal, Durban (Coetzee *et al.*, 2011a) and since then, it has been recorded in ponds, rivers and dams in the Eastern Cape, Western Cape, KwaZulu-Natal and Mpumalanga (Fig. 1.1).

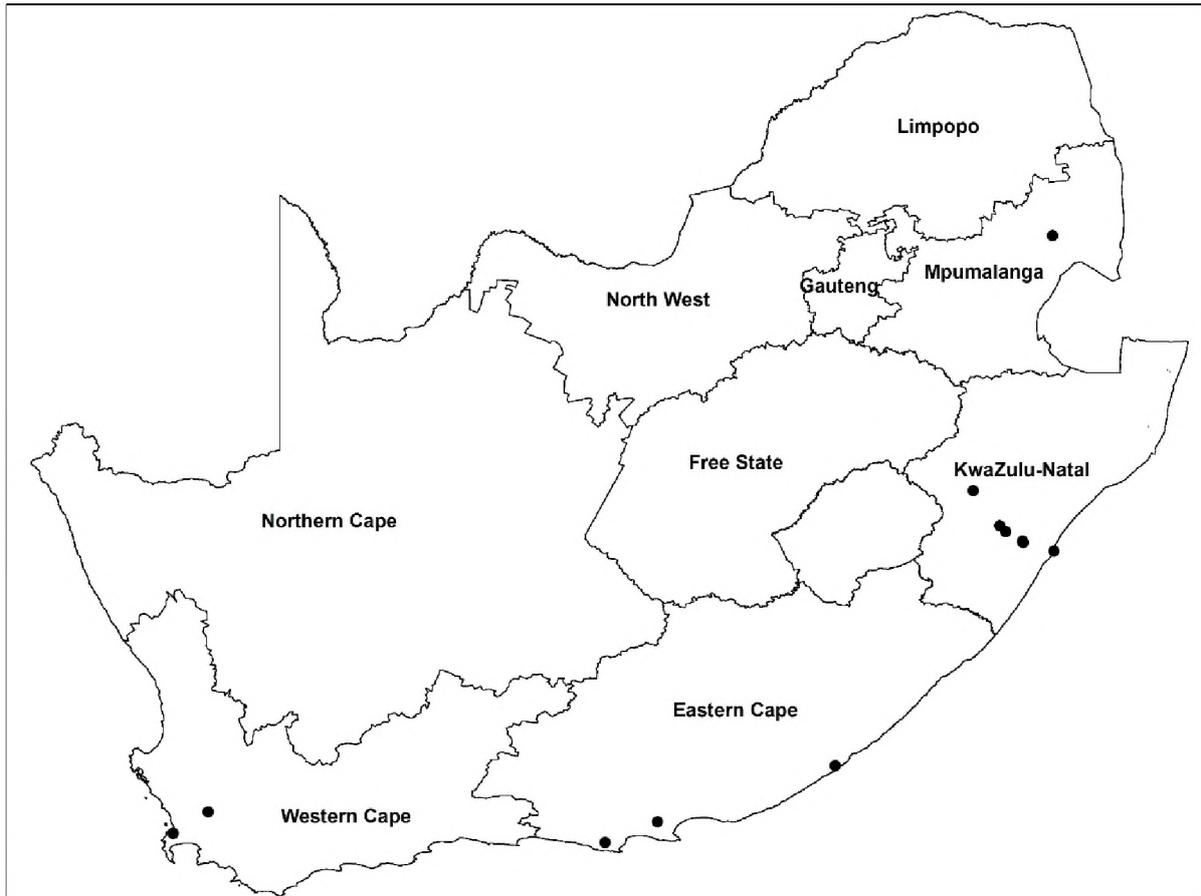


Figure 1.1: Distribution records of *Egeria densa* in South Africa.

*Egeria densa* is a submerged, freshwater perennial, native to South America (Cook & Urmi-König, 1984). Its native range is restricted to the subtropical regions of Brazil, and temperate and subtropical areas of Argentina and Uruguay. It was initially recorded outside its native habitat in 1893 (Cook & Urmi-König, 1984), but currently *E. densa* is so widely distributed around the world that it is found on all continents except Antarctica. This may be attributed to the use of *E. densa* as an oxygenators to rear fish for mosquito control

programmes, its dispersal through the aquarium trade and frequent use in biological school experiments (Cook & Urmi-König, 1984; Yarrow *et al.*, 2009; Martin & Coetzee, 2011; Gettys *et al.*, 2014). In South Africa, it has been declared a Category 1b weed according to the National Environmental Management of Biodiversity Act (NEMBA) (Act 10 of 2004, amended 2014).

### Biological description

*Egeria densa* is a member of the Hydrocharitaceae (frog-bit) family, and one of three species within the genus *Egeria* (Yarrow *et al.*, 2009). It is commonly referred to as Brazilian waterweed, dense waterweed or Brazilian elodea and is easily confused with similar looking species within the Hydrocharitaceae, for example *H. verticillata*, *Elodea* spp. and *Lagarosiphon* spp. (Netherlands Department of Environmental Science, 2014). These species are distinguishable based on the number of leaves per whorl. Generally, *Egeria* spp. have four leaves per whorl (Fig. 1.2a), whereas *Elodea* spp. have three, *H. verticillata* five and *Lagarosiphon* spp. have pseudo-whorls that vary between three to four leaves per whorl (Netherlands Department of Environmental Science, 2014).



Figure 1.2: (a) Line diagram of *Egeria densa* (i) male flower, (ii) female flower, (iii) single pointed oblong leaf and (iv) a characteristic whorl with four leaves. (b) Photograph of a single *E. densa* shoot with (c) male flowers. (Line drawing provided by University of Florida/IFAS Center for Aquatic and Invasive Plants. Used with permission. Photograph source: Rosali Smith).

The stems of *E. densa* are rooted, approximately 3mm thick, simple or irregularly branched and erect. They grow until they reach the water surface, therefore their length differs according to the depth of the water body (Yarrow *et al.*, 2009; Netherlands Department of Environmental Science, 2014). Leaves are smooth, minutely serrated, bright green (Fig. 1.2b), 10mm to 30mm in length and 3mm to 6mm in width (Fig. 1.2a) (Cook & Urmi-König, 1984; Cabrera Walsh *et al.*, 2013; Netherlands Department of Environmental Science, 2014). Double nodes on stems develop into adventitious roots (Cabrera Walsh *et al.*, 2013), whereas fertile nodes develop into leaves, flowers or branches (Yarrow *et al.*, 2009). Internode lengths vary between 2.5mm to 24mm, depending on the availability of light and

nutrients (Cook & Urmi-König, 1984; Yarrow *et al.*, 2009). *Egeria densa* has a “leafy” appearance if its internodes are short (Csurhes *et al.*, 2008).

*Egeria densa* is dioecious, but only female plants occur in South Africa (Coetzee *et al.*, 2011a). The female flowers are small and white, ranging between 15mm to 25 mm in size (Fig. 1.2c) (Yarrow *et al.*, 2009). Flowers are pushed above the water surface on a thin hypanthium and close when submerged by waves to keep the stigmas dry (Cook & Urmi-König, 1984; Yarrow *et al.*, 2009). Similar to other species in the Hydrocharitaceae family, *E. densa* reproduces asexually through stem fragmentation (Stanley & Shaw, 1986; Balciunas *et al.*, 2002; Yarrow *et al.*, 2009; Mangan & Baars, 2013). Fragments easily disperse into new regions, contributing to its invasiveness (Cook & Urmi-König, 1984; Cabrera Walsh *et al.*, 2013).

#### Environmental and growth characteristics

*Egeria densa* grows in a wide range of fresh water systems (Parsons & Cuthbertson, 2001), but prefers shallow, slow to moderate moving water (Cook & Urmi-König, 1984; Netherlands Department of Environmental Science, 2014). Its growth rate is optimal between 16°C and 28°C, whereas temperatures above 32°C reduce its growth and net photosynthesis rate (Yarrow *et al.*, 2009). Another important growth requirement is attenuated light, as too much light causes senescence (Washington State Department of Ecology, 2015). Under low light conditions, *E. densa* invests more energy in the elongation of its shoots, creating a dense canopy, instead of developing more shoots (Barko & Smart, 1981; Yarrow *et al.*, 2009). Nutrients may be utilized from both the water column and the sediment (Barko & Smart, 1981; Yarrow *et al.*, 2009) and limiting nutrients for *E. densa* growth are phosphorous, nitrogen and inorganic carbon (Feijoó *et al.*, 2002; de Freitas & Magela Thomaz, 2011; Netherlands Department of Environmental Science, 2014)

Phenological studies of *E. densa* in warm temperate Japan (Haramoto & Ikusima, 1988) showed that *E. densa* expresses different morphologies in response to seasonal changes; a robust grass type in winter and an herbaceous type with weak stems in summer. In autumn, the upper half of the shoots “die-off” while the lower part of *E. densa* stays dormant on the bottom of the water body during winter (Netherlands Department of Environmental Science, 2014). However, in tropical and subtropical regions, *E. densa* does not exhibit bimodal biomass patterns but grows actively year-round with its highest biomass in summer (Feijóo *et al.*, 1996; Mazzeo *et al.*, 2003). *Egeria densa* does not have specialized storage organs (tubers and turions), but stores carbohydrates in its leaves, stems and roots to survive colder temperatures and even frozen water surfaces (Haramoto & Ikusima, 1988; Pennington & Systma, 2009; Netherlands Department of Environmental Science, 2014). In spring, temperatures above 10°C initiate shoot development and elongation (Haramoto & Ikusima, 1988). Observations from the field in South Africa show that *E. densa* is present year-round, even in cool climate zones, such as the Western Cape (pers.obs.).

#### Association with other plant species

Knowledge of the species that a target weed is regularly associated with in its native and introduced ranges allows practitioners to predict ecosystem responses to its control. For successful control, the biocontrol agent should not only reduce the target weed population, but also facilitate native vegetation growth (Morin *et al.*, 2009). *Egeria densa* is commonly associated with *M. aquaticum*, *Alternanthera philoxeroides* (Mart.) Griseb. (Amaranthaceae) and *Sagittaria montevidensis* Cham. & Schltdl. (Alismataceae) in its native range. It has also been associated with the same species outside of its native range, a phenomenon referred to as an “imported plant community” (Cook and Urmi-König, 1984). Studies indicate that in its introduced range, *E. densa* co-occurs with aquatic species like *Ceratophyllum demersum* L. (Ceratophyllaceae), *Elodea nuttalli* Planch. St. John (Hydrocharitaceae), *Stuckenia pectinata*

(L.) Börner (Potamogetonaceae), *Potamogeton natans* L. (Potamogetonaceae) and *M. spicatum* (Hussner & Losch, 2005; Santos *et al.*, 2011; Netherlands Department of Environmental Science, 2014).

In South Africa, field surveys revealed that aquatic vegetation in water bodies infested with *E. densa* include native *Berula erecta* (Huds.) Coville (Umbelliferae), *Commelina* sp. (Commelinaceae), *Hydrocleys nymphoides* Willd. (Limnocharitaceae), *Ludwigia repens* J.R. Forst. (Onagraceae), *Nasturtium officinale* R. Br. (Brassicaceae), *Persicaria senegalensis* (Meisn.) Soják (Polygonaceae), *Potamogeton crispus*, L. (Potamogetonaceae), *S. pectinata*, *Potamogeton pussilus* L. (Potamogetonaceae), *C. demersum* and *Typha* sp. (Typhaceae). It also grew with invasive species like *A. filiculoides*, *S. molesta* and *M. aquaticum* (Martin & Smith, unpubl. data).

#### Positive effects

As an “ecosystem engineer”, *E. densa* may stabilize suspended particles and reduce water turbidity (Yarrow *et al.*, 2009). It also has the ability to absorb ammonium and phosphorous from the water column, which may reduce nutrient loading (Feijoó *et al.*, 2002; Netherlands Department of Environmental Science, 2014). *Egeria densa* may curb the reproduction and bloom of cyanobacteria due to its allelopathic effects on the growth of blue-green algae, specifically *Anabaena flos-aquae* (Lyngb.) Breb. (Nostocaceae) and *Mycrocystis aeruginosa* (Kützinger) Lemm (Mycrocystaceae) (Jansen van Vuuren *et al.*, 2006). It may also serve as shelter for fish and provide microhabitats for phytoplankton and zooplankton, but at high densities, fish may disappear (Lancar & Krake, 2002; Yarrow *et al.*, 2009). Even so, South Africa has many native submerged macrophytes to fulfil this role.

### Negative effects

The rapid growth rate of *E. densa* allows it to form dense monospecific stands, which impairs the use of the water (Fig. 1.3 & 1.4). High densities alter the flow and morphology of the water body (Dillon *et al.*, 1988; Netherlands Department of Environmental Science, 2014) and disrupt recreational activities like boating, fishing and swimming (*Egeria densa* Control Program, 2006; DiTomaso *et al.*, 2013). Even in its native range, dense stands have affected electric power generation (Bini *et al.*, 1999). Owing to its characteristics as an ecosystem engineer, it also modifies the availability of light, nutrients and dissolved oxygen that subsequently change ecosystem processes (Dillon *et al.*, 1988). Despite being an oxygenator, respiration at night depletes oxygen levels and under high density stands, may result in anoxic conditions during the day (Mazzeo *et al.*, 2003; Gettys *et al.*, 2014). In 2001, anoxic conditions caused by dense stands of *E. densa* killed freshwater mussels in Lake Omapere, Northland, New Zealand (Champion & Burns, 2001). A number of closely related invasive macrophytes are frequently cited causing similar damage, altering the composition and distribution of invertebrates (Keenan, 2010; Kelly & Hawes, 2005) and affecting the structure of fish populations (Bickel & Closs, 2009; Caffrey *et al.*, 2009).

It also increases surface water temperature during summer that creates unsuitable conditions for other organisms (Gettys *et al.*, 2014). In autumn, large numbers of dying shoot tips increase the amount of suspended material in the water column. Decomposing plant material not only reduces the oxygen supply in the water, but also creates an unpleasant smell (Lancar & Krake, 2002; Yarrow *et al.*, 2009; Washington State Department of Ecology, 2015).



Figure 1.3: Infestation of *Egeria densa* early spring of 2016, in the Liesbeeck River, Western Cape. (Source: R. Smith).



Figure 1.4: Dense *Egeria densa* monoculture stands in the Kouga River, Eastern Cape, July 2015. (Source: J.A. Coetzee).

Through its high competitiveness, *E. densa* crowds out indigenous plants. A dominance experiment between *E. densa* and *Lagarosiphon major* Ridley (Hydrocharitaceae) at different temperatures indicated that *E. densa* is highly competitive in warm temperatures

of 30°C (Riis *et al.*, 2012). *Lagarosiphon major* is indigenous to South Africa (Cook, 2004) and the dominance of *E. densa* over *L. major* in subtropical climates poses a threat to the persistence of native species.

### Management options

Controlling *E. densa* is expensive, especially for dense infestations. For example, costs associated with managing *E. densa* in various small water bodies in the USA from 1994 to 2000 were estimated at US\$530 300. However, for larger control efforts, costs were estimated at US\$3 million (Netherlands Department of Environmental Science, 2014).

### *Mechanical control*

A few management strategies have been used to control *E. densa* abroad, for example drawdowns in reservoirs and lakes (Csurhes *et al.*, 2008; Coetzee *et al.*, 2011a). In Black Lake, Louisiana, drawdowns proved to be highly successful (Goldsby and Sanders, 1977), however, drawdowns are not a viable option in South Africa due to its limited water resources. Other mechanical control methods include mowing, cutting, digging, chaining and pulling (Fig. 1.5) (Lancar & Krake, 2002; Csurhes *et al.*, 2008; Hussner *et al.*, 2017). However, these methods cause stem fragmentation that facilitate the dispersal of the weed (Cabrera Walsh *et al.*, 2013), and are short-term relief strategies that must be executed with caution.



Figure 1.5: Mechanical removal of *E. densa* in the Berg river, Western Cape (Source: J.A. Coetzee).

### *Chemical control*

Several herbicides have been recommended for controlling submerged and floating aquatic weeds; penoxsulan, fluridone, disquat, paraquat, 2,4-D (translocated herbicide), glyphosate, amitrole, and acrolein (achrylaldehyde) (Bowmer & Sainty, 1977; Lancar & Krake, 2002; Williams & Hackey, 2005; DiTomaso *et al.*, 2013). For example, disquat has been researched for *H. verticillata* control in the USA since the 1960s (Mackenzie & Hall, 1967), and has been used against most aquatic plants (Lancar & Krake; 2002). Finding a herbicide that is environmentally suitable and persistent has proven difficult in the past (Vernon & Hamilton, 2011). Chemical control in aquatic systems can be unsafe as it may expose organisms, both terrestrial and aquatic, to toxic chemicals if label rates are exceeded (Mullison, 1970; Williams & Hackey, 2005). A massive load of decomposing plant material

and non-target organisms leaves the water body in an anoxic state. The effect of chemical control may be less in smaller, isolated systems, but on a larger scale it may be detrimental. In a water-scarce country, herbicides may remove the aquatic weed temporarily, but on a long-term scale, irreparably harm or alter ecosystem services. The only herbicide registered in South Africa for use against submerged aquatic weeds, is the dibromide salt of diquat (Scuba/Midstream/Sonar), but these products are not registered for use against *E. densa* (Sharp, pers. comm).

### *Biocontrol*

There is a short list of organisms that has been found to damage *E. densa*, including a fungus, *Fusarium graminearum* (Shwein.) Petch (Hypocreales: Nectriaceae), the generalist triploid grass carp, *Ctenopharyngodon idella* Valenciennes (Cypriniformes: Cyprinidae), and a specialist ephydrid fly, *Hydrellia egeriae* Rodrigues (Diptera: Ephydridae) (Barreto *et al.*, 2000; Csurhes *et al.*, 2008, Cabrera Walsh *et al.*, 2013). Borges Neto & Pitelli (2004) used *F. graminearum* to create a bioherbicide that is damaging to *E. densa*, but it is not available for retail yet (Cabrera Walsh *et al.*, 2013). Grass carp feeds on submerged plants, but due to its ability to remove native species and whole macrophyte communities, it is not seen as a safe control option (Cuda *et al.*, 2008). To date, no host-specific biocontrol agents have been released against *E. densa* anywhere in the world (Hussner *et al.*, 2017).

### **Management of *E. densa* in South Africa**

During a national review of invasive and incipient submerged aquatic weeds in South Africa in 2011 (Coetzee *et al.*, 2011a), *E. densa* was targeted for biocontrol due to its increasing range expansion. Considering the wide application and biological success (establishment and range expansion) (McClay & Balciunas, 2005) of *Hydrellia* species in the biocontrol of submerged aquatic weeds (Grodowitz *et al.*, 2003), *H. egeriae* was imported into quarantine at Rhodes University, Grahamstown in September 2014, from the Exotic and

Invasive Weeds Research (EIW) facility of the Agricultural Research Service in California, USA (Strange, pers. comm.).

*Hydrellia egeriae* is native to Argentina and has only been described recently (Rodrigues *et al.*, 2015). Similar to other *Hydrellia* species, females lay their eggs on protruding leaves of their host plant (Deonier, 1971). *Hydrellia egeriae* oviposit eggs on protruding *E. densa* leaves in clusters of 15 to 45 eggs. Larvae are phytophagous and feed on the photosynthetic tissue under the epidermis of a leaf; they undergo three instars and pupariate at the base of the last *E. densa* leaf mined. Adults are polyphagous; feeding on nectar, fungi, cyanobacteria, plant material and smaller insects (Deonier, 1971; Cabrera Walsh *et al.*, 2013). Male adults are between 1.86 and 2.07 mm in length and females between 1.7 to 2.09mm. Adults are dark brown, with a golden-brown coloured faces (Rodrigues *et al.*, 2015) (Fig. 1.6).



Figure 1.6: Profile of *Hydrellia egeriae* Rodrigues (holotype) (Photograph source: Rodrigues-Junior *et al.*, 2015).

## 1.5 PROGRAMMES INITIATED AGAINST INVASIVE SUBMERGED AQUATIC WEEDS

Submerged aquatic weeds that have become invasive throughout the world include *H. verticillata*, *L. major*, *M. spicatum* and *C. caroliniana*. In view of the knowledge gap in the biocontrol of submerged aquatic weeds in South Africa, a review of these biocontrol programmes is given.

The two largest programmes initiated against submerged aquatic weeds are those against *M. spicatum* in northern USA and Canada, and *H. verticillata* in southern USA (Grodowitz *et al.*, 2003; Cuda *et al.*, 2008; Alwin & Cheruvilil, 2009). *Myriophyllum spicatum* is one of North America's most problematic weeds and has been controlled by the native weevil, *Euhrychiopsis lecontei* Dietz (Coleoptera: Curculionidae) (Winston *et al.*, 2014). Adults of *E. lecontei* reduce the biomass of *M. spicatum* through leaf- and stem feeding, while larvae feed on internal stem tissue (Alwin & Cheruvilil, 2009). In a study on the chemical mediated host-plant selection of the weevil, *E. lecontei* expressed higher preference for the invasive *M. spicatum* than indigenous *Myriophyllum* species. This is the first record of a native herbivore used for the biocontrol of a submerged aquatic weed. However, augmentative releases of the agent are still required due to fish predation. Also, some challenges exist for the overwintering adults because in some released areas, there are no sites for overwintering or, if available, no damage is inflicted on the target weed in that period (Hairston & Johnson, 2001; Winston *et al.*, 2014).

The biocontrol of *H. verticillata* in the USA was the first biocontrol programme initiated against a submerged aquatic weed in the 1980s. *Hydrilla verticillata* is referred to as the world's worst submerged aquatic weed (Langeland, 1996), and in 2003, had invaded 40% of all water bodies in Florida, USA (Purcell & Goolsby, 2003). Mechanical and chemical costs to control *H. verticillata* in Florida, USA were estimated at between US\$10 and

US\$14.5 million for the years 1994 to 1996 (Pimentel, 2005; Langeland, 1996). Four agents were released in the USA; two weevils, *Bagous affinis* Hustache and *Bagous hydrillae* O'Brien (both Coleoptera: Curculionidae), and two leaf-mining flies, *Hydrellia balciunasi* Bock and *Hydrellia pakistanae* Deonier (both Diptera: Ephydriidae) (Balciunas *et al.*, 2002; Forno & Julien, 2000; Hussner *et al.*, 2017).

*Bagous affinis* is a tuber-feeding weevil that was released in 1987 and 1988, but given its narrow temperature tolerance and lack of dewatered sites for pupation, the weevil failed to establish (Buckingham & Bennett, 1994; Godfrey & Anderson, 1994; Forno & Julien, 2000). Additionally, *B. hydrillae* was released from 1991 to 1996 in various locations in Florida, Texas, Georgia and Alabama (Center *et al.*, 2013). Adults can persist on both submerged and exposed *H. verticillata* and feed on its leaves and stems. Females lay their eggs within the stems and once hatched, larvae feed on the internal tissue of the stems (Balciunas *et al.*, 2002). Larval and adult feeding cause *H. verticillata* stems to break off and drift to the shoreline, creating a "mowed-like" effect (Balciunas *et al.*, 2002). Pupation and further feeding by larvae and adults occur on fragments on the shoreline (Balciunas *et al.*, 2002). For many years, no permanent populations of *B. hydrillae* were confirmed until 2009 when adults were collected in southern Louisiana (Center *et al.*, 2013). The weevils had migrated almost 580km from the nearest release site. Despite their presence, no evidence exists for a suppressive effect on the invasive macrophyte (Center *et al.*, 2013).

*Hydrellia pakistanae* was first released in Florida in 1987 and then in five other states in the USA (Louisiana, Alabama, Georgia, Texas and California) (Center *et al.*, 1997). Subsequently, *H. balciunasi* was released at several sites in Florida and Texas in 1989 (Grodowitz, 1999; Freedman *et al.*, 2001). Only the larval stage of the flies inflicts damage to *H. verticillata* by mining its leaves, which reduces its photosynthetic capacity and induces secondary infection (Grodowitz *et al.*, 2004). Post-release studies (establishment, distribution,

weed damage) of *H. pakistanae* and *H. balciunasi* in the USA indicated that *H. pakistanae* is the most biologically successful agent of the two species (Grodowitz *et al.*, 1999; Grodowitz *et al.*, 2003), due to its wider range expansion (Grodowitz *et al.*, 2009).

With the discovery of *H. verticillata* in Pongolapoort dam in South Africa, *H. pakistanae* was also imported as a biocontrol agent via the “Short Route” (Harley & Forno, 1992), in the hope that South Africa would benefit from experience gained in the USA. *Hydrilla verticillata* can either be monoecious (Malaysian and Indonesian origin) or dioecious (Indian origin) (Madeira *et al.*, 2007), and due to the difference in biotype between South Africa and North America, where *H. pakistanae* was released, another *Hydrellia* fly, *Hydrellia purcelli* Deeming, was imported from a similar region as monoecious *H. verticillata* (Bownes & Deeming, 2016). Performance tests between the two *Hydrellia* biocontrol agents confirmed that *H. purcelli* had a higher performance (survival, longevity, fecundity and development time) on monoecious *H. verticillata* in South Africa than its congener *H. pakistanae* (Bownes, 2015). Quarantine trials also showed that the shore-fly was host-specific and permission for release was granted. However, the agent was shelved for later use, since *H. verticillata* is only known from one site in South Africa (Bownes, 2015)

A close relative of *H. verticillata*, *Lagarosiphon major* is native to South Africa but has become invasive in New Zealand (McGregor and Gourlay, 2002), Australia (Bowmer *et al.*, 1995), the UK and several European countries (Symoens & Triest, 1983; Reynolds, 2002; van Valkenburg & Pot, 2007). Field surveys for natural enemies associated with *L. major* in its natural habitat were conducted in 2007 and 2008 (Baars *et al.* 2010), and yielded two promising control agents, an ephydrid fly, *Hydrellia lagarosiphon* Deeming (Baars *et al.*, 2010) and a stem-mining midge, *Polypedilum* n. sp. (Diptera: Chironomidae) (Earle *et al.*, 2015). The life cycle and type of damage that *H. lagarosiphon* inflicts to its host plant is similar to other *Hydrellia* biocontrol agents (Martin *et al.*, 2013; Mangan & Baars, 2013).

Host-specificity tests have also shown that the agent has a narrow host range (Mangan, 2012). *Polypedilum* n. sp. is the first chironomid that has been successfully cultured in quarantine as a potential biocontrol agent. Only the immature stage is aquatic with neonate instars that feed on the stems and leaves of *L. major* and later instars that burrow into shoots for feeding and pupation (Earle *et al.*, 2013). Applications for the release of both agents are in the process of being submitted in New Zealand (Mangan, pers. comm.).

The biocontrol programme against *C. caroliniana* is in the early stages of development, in comparison to the previous examples. It has become problematic in Australia, USA, Japan, China, The Netherlands, India, Canada and Greece, and is reported to occur in South Africa, but no permanent populations have been confirmed (Martin & Coetzee, 2011; Schooler *et al.*, 2012). To date, no agents have been released. The only host-specific agent identified thus far is a stem-feeding weevil, *Hydrotimetes natans* Kolbe (Coleoptera: Curculionidae) (Schooler *et al.*, 2012). Immatures of the weevils feed internally in the stem that causes stem breakage whereas adults feed on the stems and young leaves of the shoot tips (Cabrera Walsh *et al.*, 2011). *Hydrotimetes natans* completes its life cycle on *C. caroliniana*, and only emerges from the water to mate (Cabrera Walsh *et al.*, 2011).

The biocontrol of submerged aquatic weeds in South Africa is a new programme within the practice of biocontrol and was initiated with the identification of a massive infestation (600ha) of *H. verticillata* in the Pongolapoort Dam, KwaZulu-Natal in 2006 (Henderson, 2006). Since the initiation of the biocontrol programme against *H. verticillata*, the importation of *H. egeriae* into quarantine established the second biocontrol programme initiated against a submerged aquatic weed in South Africa. Fortunately, practitioners can build on experiences from biocontrol programmes elsewhere, especially from those in the USA (Grodowitz *et al.*, 2003; Cuda *et al.*, 2008).

Shore-flies of the genus *Hydrellia* have been investigated extensively for the biocontrol of submerged aquatic weeds. Thus far, four species have been investigated for classical biocontrol, of which all have proven to be host-specific. Two have been released (*H. pakistanae* and *H. balciunasi* in the USA), a third (*H. purcelli*) has been granted permission for release (in South Africa) and a release application is in the process of being submitted for a fourth *Hydrellia* biocontrol agent (*H. lagarosiphon* in New Zealand) (Okrah & Buckingham, 1993; Mangan, 2012, Bownes, 2015; Mangan, pers. comm). Considering the host-specificity of *Hydrellia* flies and their wide application in biocontrol programmes against submerged Hydrocharitaceae, the discovery of a *Hydrellia* species in the native range of *E. densa* is promising (Cabrera Walsh *et al.*, 2013). *Egeria densa* is the most widely distributed submerged aquatic weed in South Africa, and in contrast to *H. verticillata*, *E. candensis*, *C. caroliniana* and *M. spicatum*, is the only submerged aquatic weed capable of invading water bodies in every province (Martin, 2013). Therefore, it is imperative to manage established populations and prevent its further dispersal.

## 1.6 THESIS OUTLINES AND AIMS

The aim of the thesis was to investigate the suitability of the leaf-mining fly *H. egeriae* as a biocontrol agent for *E. densa* in South Africa. Thus far, the only available information regarding the agent is from native range studies conducted by Cabrera Walsh *et al.* (2013) and a description of the species (Rodrigues *et al.*, 2015). This study will serve as an *a priori* study that aims to establish the life history traits, host-specificity and damage capacity of *H. egeriae* for release in South Africa. This will guide decision-making for its release or rejection as a biocontrol agent.

For any biocontrol programme, an initial step is to establish the life history traits of the agent and identify life stages that need to be host-specific for non-target safety (Sheppard, 2003). Therefore, the first objective was to give an in-depth account of the biology of the fly,

construct a life table and determine its population growth parameters. These results are presented in Chapter 2, and supplemented with temperature dependent development tests from native range studies (Cabrera Walsh *et al.*, 2013) to assist predictions regarding the invasion success of *H. egeriae*.

Once a better understanding of the biology of the fly is obtained, host range testing is necessary to validate the specificity of *H. egeriae* for *E. densa* and any potential ecological risks related to its release (van Lenteren *et al.*, 2006). This is accomplished by establishing the range of host plants used by the agent through native range studies and laboratory-based host-specificity testing (McEvoy, 1996; Van Driesche & Bellows, 1996; McFayden, 1998; Louda *et al.*, 2003; Moran *et al.*, 2013). In Chapter 3, results from quarantine-based host-specificity trials are presented and used to calculate feeding and reproductive risks on non-target species (Wan & Harris, 1997). Results are also interpreted in view of host-specificity trials conducted in the native range of the agent (Cabrera Walsh *et al.*, 2013).

Furthermore, the impact of *H. egeriae* on *E. densa* fitness was investigated. Impact studies often underestimate the damage capacity of an agent when tests are only conducted for one generation or short periods of time (McClay & Balciunas, 2005). Therefore, the effect of *H. egeriae* leaf-mining on *E. densa* was assessed for three consecutive generations at intermediate and high agent densities. Results are presented and discussed in Chapter 4.

Finally, the suitability of *H. egeriae* as a biocontrol agent for *E. densa* in South Africa on the basis of its life-history traits, host-specificity and damage capacity is discussed. Limitations for the successful biocontrol of *E. densa* are presented and recommendations are made for the further development of the programme.

## Chapter 2: Life history of *Hydrellia egeriae* Rodrigues

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### 2.1 INTRODUCTION

The shore-fly genus, *Hydrellia* Robineau-Desvoidy, is the most species rich within the family Ephydriidae (Diptera) (Hesler, 1995). It is the only member within the Hydrellini tribe, subfamily Notiphilinae (Deonier, 1971). The genus was established by Robineau-Desvoidy in 1830, and consists of more than 200 species that occur throughout the world (Deonier, 1971; Mathis, 2010). *Hydrellia* spp. larvae are phytophagous and mine leaves and stems of plants that generally occur within or near aquatic environments (Mathis *et al.*, 2016). These include plants from the families Alismataceae, Brassicaceae, Cruciferae, Gramineae, Hydrocharitaceae, Lemnaceae, Poaceae and Potamogetoneaceae (Deonier, 1971; Rodrigues Junior *et al.*, 2014; Mathis *et al.*, 2016).

Adults are polyphagous; feeding on nectar, fungi, cyanobacteria, plant material and smaller insects (Deonier, 1971; Mangan *et al.*, 2015). *Hydrellia* spp. adults are considered important pollinators for aquatic plants. They frequently congregate in flowers for feeding and mating, transferring pollen between aquatic plants in the process (Katzenberger & Zacharias, 2015). In Germany and the Netherlands, declining population of *Stratiotes aloides* L. (Hydrocharitaceae) due to habitat destruction and eutrophication, prompted an investigation into the arthropod species that pollinate the aquatic plant. The most abundant and actively pollinating species was the shore-fly, *Hydrellia tarsata* Haliday (Katzenberger & Zacharias, 2015).

Several species within the genus are also serious pests that have caused massive crop losses (Hesler, 1995; Sain, 2000). These include *Hydrellia philippina* Ferino, *Hydrellia sasakii* Yuasa & Isitani, *Hydrellia tomiokai* Miyagi, *Hydrellia wirthi* Korythkowski and the rice pest *Hydrellia griseola* Fallén, (Hesler, 1995; Sain, 2000; Mathis *et al.*, 2016). *Hydrellia*

*griseola* has received a lot of attention due to its detrimental effects on various cereal crops, and in particular, rice (*Oryza sativa* L. (Poaceae)). In Europe, *H. griseola* damage accounted for a 50% loss in wheat, barley and oats production (Hesler, 1995), and in 1953, it caused 10 to 20% loss in rice crops in California (USA) valued at US\$ 16 000 000 (Mathis *et al.*, 2016).

The endophytophagous larvae produce significant changes in aquatic plant communities either in combination with other microinvertebrates (Deonier, 1971) or on their own (Grodowitz *et al.*, 2003). Additionally, leaf-mining species often express a degree of specialization in host-plant choice (Deonier, 1971; Davies, 1988). Therefore, the close association of *Hydrellia* species with aquatic plants has made them favourable candidates for biocontrol programmes, particularly of submerged aquatic weeds from the Hydrocharitaceae family (Buckingham & Okrah; 1993; Mangan & Baars, 2013; Cabrera Walsh *et al.*, 2013; Martin *et al.*, 2014).

*Hydrellia* species tolerate a wide range of temperatures (-2°C and 44°C), but optimal temperatures range between 25°C and 32°C (Deonier, 1971; Cabrera Walsh *et al.*, 2013, Martin *et al.*, 2013). Results of biological studies of *Hydrellia* spp. biocontrol agents indicate that female fecundity is variable, depending on the species and temperature. In an optimal environment of 27°C, females of *H. balciunasi*, *H. pakistanae* and *H. purcelli*, control agents of *H. verticillata*, oviposited 35.5, 68.4 and 102.5 eggs, respectively (Buckingham & Okrah, 1993; Bownes & Deeming, 2016). Additionally, the female fecundity of *H. lagarosiphon*, a potential control agent of *L. major*, was tested at cooler temperatures and decreased from 25.5 eggs at 20°C to 9.6 at 13.5°C and 3.3 at 10°C (Mangan & Baars, 2013).

Under ideal temperatures, larvae hatch after 2 to 8 days, larval development ranges between 9 to 14 days and pupariation is completed after 6 to 15 days (Grigarick, 1959; Deonier, 1971; Buckingham & Okrah, 1993; Cabrera Walsh *et al.*, 2013; Mangan & Baars,

2013; Martin *et al.*, 2013; Bownes & Deeming, 2016). Cooler temperatures prolong the development time of *Hydrellia* flies, for example total development time for *H. lagarosiphon* decreased from 57 days at 20°C to 73 days at 16.5°C, 120.7 days at 13.5°C and 171.1 days at 10°C (Mangan & Baars, 2013). Despite prolonged development times, surveys in the native range of *H. lagarosiphon* revealed that both adult and immature stages of the fly were abundant in the field, even at temperatures below 0°C (Martin *et al.*, 2013). Encouragingly, the presence of adult and all larval stage of the fly during winter surveys in South Africa indicates that *H. lagarosiphon* is capable of surviving sub-zero overnight temperatures (Earle, 2013).

Successful biocontrol relies on the ability of an agent to establish, proliferate and reach high abundances in the introduced range (McClay & Balciunas, 2005). Temperature is the most influential climatic variable on insect performance (Watt *et al.*, 2016). Knowledge of a potential agent's biology, development time and reproduction is fundamental for any biocontrol programme (Julien & White, 1999). It assists predictions of its biological potential and contributes to effective mass-rearing and agent release efforts (McClay & Balciunas, 2005; May & Coetzee, 2013; Mangan *et al.*, 2015; Watt *et al.*, 2016). This is obtained through insect performance studies at constant temperatures (Campbell *et al.*, 1974). Life tables are useful, because they summarize the life-time pattern of survivorship for a species and allow comparisons between different temperatures and/or species (Begon *et al.*, 2006; Pilkington and Hoddle, 2006).

In its native range, temperature-dependent biological studies showed that *H. egeriae*'s development was optimal at 15°C with 100% survival rate for all immature stages and a total development time of 73.7 days (Cabrera Walsh *et al.*, 2013). The shortest total development time was at 30°C, with 21.2 days and a survival rate above 84% for all immature stages (Cabrera Walsh *et al.*, 2013). The temperature development threshold for *H. egeriae* was

12.8°C, 8.0°C and 9.7°C for eggs, larvae and puparia, respectively (Cabrera Walsh *et al.*, 2013).

Population growth parameters complement biology studies, because they give insight into the proliferation potential of an insect under investigation. Parameters include net reproduction rate ( $R_o$ ), mean generation time ( $T_c$ ), intrinsic rate of increase ( $r_m$ ), finite rate of increase ( $\lambda$ ) and population doubling time ( $T_d$ ). The net reproduction rate is in essence the number of daughters a female produces during the course of her life. If  $R_o > 1$ , the population increases, if  $R_o < 1$ , the population is declining and  $R_o = 1$  depicts a stagnant population (Deevey, 1947). The mean generation time is an expression of the time elapsing between the birth of a female and her offspring (Deevey, 1947). Intrinsic rate of increase indicates the maximum exponential population growth potential in the absence of density-dependent factors, while population doubling time is the number of days a population requires to double in size when at given  $r_m$  (Carey, 1989).

Host plant and agent biotype matching is crucial for the success of the biocontrol programme (Byrne *et al.*, 2011; Patterson *et al.*, 2011; Bownes, 2015). For example, performance experiments showed that *H. pakistanae* was not suitable for the biocontrol of *H. verticillata* in South Africa, as the agent and its host plant have separate biotypes (Bownes, 2015). Consequently, another *Hydrellia* species (*H. purcelli*), was imported from Singapore, and exhibited better performance (female fecundity, adult longevity and shorter development time) than its congener *H. pakistanae* (Bownes, 2015). Therefore, although Cabrera Walsh *et al.* (2013) investigated the biology of *H. egeriae* in its native range, it is necessary to establish the life history traits of the potential biocontrol agent on *E. densa* found in South Africa under ideal conditions. This will provide baseline information for the development of the biocontrol programme against *E. densa*.

The aim of this study was to investigate the basic biology of *H. egeriae* at a controlled temperature of 22°C. Life history traits of particular interest were female fecundity, development time and adult longevity. These characteristics are often seen to best describe the biotic potential of an insect (Southwood & Henderson, 2000). Furthermore, the aim was to construct a life table for *H. egeriae* and to calculate its population increase parameters as measurements of its potential establishment in the field.

## 2.2 MATERIAL & METHODS

### Plant and insect cultures

Plant material was collected throughout the year from Kouga River, Patensie, Eastern Cape (S 33°44'54.622"; E 24°38'7.605") and cultured in a flow-through system in a polytunnel at the Waainek Biological Control Research Facility in Grahamstown. Thirty shoots were individually planted in 13.5L round tubs (41cm x 24cm) with field-collected sediment and fertilizer. The slow release fertilizer, Multicote™ (Haifa) was used in quantities of 0.7g per 1kg sediment. A 1cm layer of silica sand was placed over the sediment to prevent water clouding and algal growth. Planted tubs were placed in 600L tanks connected to a flow-system. Plants were also given a fluid nutrient stock solution every third month that consisted of calcium chloride (91.7mg/L), magnesium sulphate (69.0mg/L), sodium bicarbonate (58.4mg/L) and potassium bicarbonate (15.4mg/L) (Smart & Barko, 1985). Plant material from this *E. densa* culture was used for all of the experiments in this study.

In order to start a culture of the potential control agent for biological, host specificity and impact studies, larvae of *H. egeriae* were imported under permit (P0063110) in September 2014, from the Exotic and Invasive Weeds Research (EIW) facility of the Agricultural Research Service in California, USA in 2014 and brought into quarantine at Rhodes University, Grahamstown. Larvae were placed in transparent boxes (41cm X 17cm X 29cm) equipped with a mesh window and kept in a controlled environment of  $22 \pm 2$  °C under fluorescent lighting and a 12:12 day: night cycle. Each box was half-filled with spring water and contained stems of *E. densa* and a floating petri-dish with approximately six dead *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) and a yeast hydrolysate/sugar mixture (4 g Bacto™ TC yeastolate, 7 g sugar, 10 ml H<sub>2</sub>O) to provide nutrition for the adult flies (Mangan *et al.*, 2015). Water and new plant material were added as needed. Individuals in each box were left to complete their life cycle and newly eclosed adults were transferred to

new boxes to start the cycle over. Every week, two new boxes were set up to maintain high insect numbers.

All tests conducted with *H. egeriae* were conducted in the quarantine facility at Rhodes University and used individuals from the fly colony reared as described above.

#### *Hydrellia egeriae* fecundity

In order to determine female fecundity, development time and survival of *H. egeriae*, a pair of newly eclosed adults was sexed and placed in a transparent 1.5 L container. Five *E. densa* apical shoot tips, 6cm in length, were placed in each container, which were filled with 3 L of spring water. Flies were also provided with 50% diluted honey and a yeast hydrolysate/sugar mixture on a 2cm X 2cm white polystyrene float. Ten replicates were prepared. Each pair of adults was allowed to mate and the females to oviposit. These adults were transferred to a new container every second day and the number of eggs were counted and then monitored every second day until adult eclosion. This continued for the duration of the female's lifespan.

The number of eggs oviposited (fecundity), number of larvae that hatched (fertility), original adult mortality, larvae that pupariated and adult emergence were recorded every second day. Eclosed adults were removed from the containers with a pooter, frozen and sexed. This was done until no more adults emerged.

#### Life table and population increase parameters

Female fecundity, egg to adult development time, survival rate for each life stage and offspring sex ratio of *H. egeriae* was used to construct a life table (Deevey, 1947). The proportion of the original cohort surviving ( $l_x$ ) or dying ( $d_x$ ) during each life stage was used to calculate the percentage apparent mortality ( $m_c = \frac{d_x}{l_x} \times 100$ ). Furthermore, life table data were used to calculate the net reproductive rate ( $R_o = \sum l_x m_x$ ). The number of surviving

females is represented by  $l_x$ , where  $x$  is female age in days, and  $m_x$ , the number of daughters born (Deevey, 1947). From the life table data, the intrinsic rate of increase ( $1 = \sum x l_x m_x \exp(-r_m)$ ), mean generation time ( $T_c = \frac{\sum x l_x m_x}{R_0}$ ) (Deevey, 1947) and population doubling time ( $T_d = \frac{\ln(2)}{r_m}$ ) were calculated (Carey, 1989). The bootstrap pseudo-replication technique was used to obtain standard errors for each mean value of the population parameters (Pilkington & Hoddle, 2006).

#### Male/female leaf consumption

An experiment was conducted to determine the number of leaves that male and female flies consume during their larval stage. This required standardised *E. densa* plants, so 30 apical shoots, 20cm in length, were planted individually in 3cm x 5cm vials, filled with sediment at the Waainek Research Facility polytunnels. The sediment contained a slow release fertilizer, Multicote<sup>TM</sup> (Haifa), according to the abovementioned ratio and was covered with washed silica sand. The vials were placed in a tub, connected to the flow through system. Shoots were left to grow for 3 weeks to allow root growth. After this growth period, the plants were taken to the quarantine facility. Each vial was placed individually in 600ml transparent containers (24cm x 7.5cm) that were filled with spring water.

Eggs were collected from the *H. egeriae* culture by excising the leaf with the eggs and placing it in a petri-dish with spring water. Eggs were checked daily for larvae hatching. One neonate larva was transferred to a replicate by excising the leaf material around it, and pinning the excised leaf with the larva, onto the test plant. Each container was enclosed with netting held in place with a rubber band to prevent adult escape after eclosion. Containers were placed in a controlled environment of  $22 \pm 2^\circ\text{C}$  under fluorescent lighting and a 12:12 day: night cycle. Larvae were left to feed and develop to adulthood. Newly eclosed adults

were individually removed from each container with a pooter and sexed. The number of leaves mined by each larva was counted.

#### Statistical analysis

Differences in leaf consumption between male and female larvae of *H. egeriae* were used for statistical analysis. The distribution of the data set was tested for normality using the Shapiro Wilk test STATISTICA 13.0 (STATSOFT Inc., 2015). Statistical differences between leaf consumption by males and females were determined using a Student's *t*-test.

## 2.3 RESULTS

### Biology

*Eggs:* Females oviposited eggs singly or in groups on *E. densa* leaves that breached the water surface. The eggs are elongate and approximately 0.4mm in length and 0.1mm in width. When observed with the naked eye, eggs always appeared white. However, when observed under a microscope, the egg colour is variable and reflective of the developmental processes under the transparent chorion. Initially, eggs appeared white (Fig. 2.1), but turned golden brown (Fig. 2.2) when neonate larvae were ready to eclose. It took  $4.78 \pm 0.34$  days (Table 2.1) for larvae to hatch and prior to hatching, the fully developed neonate started to move within the chorion. Larvae used their mouth hooks to cut an opening in the chorion at the micropylar end to facilitate hatching.



Figure 2.1: A cluster of *Hydrellia egeriae* eggs on an *Egeria densa* leaf tip.



Figure 2.2: Fully developed larvae within eggs that are ready to hatch. The transparent chorion allowed for observation of the larva inside.

*Larvae:* Upon emergence, larvae were approximately the same size as the chorion. Larvae immediately entered *E. densa* leaves and mined in the crown of the shoot, where the leaves were softer. To enter the mesophyll layer of the leaf, larvae penetrated the leaf epidermis vertically with their mouth hooks and expanded the opening horizontally to create an “entry scar” (Fig. 2.3).



Figure 2.3: A neonate larvae that entered an *Egeria densa* leaf for feeding. Abbreviations: ES, entry scar; FA, feeding apparatus.

*Hydrellia egeriae* larvae mined alongside the midrib of a leaf, and generally mined through the whole leaf before moving onto a new leaf. They resided within leaves throughout the entire larval stage. The only time they were found outside a leaf was when they sought out a new leaf to mine. Instars only mined leaves and gradually increased in size throughout the process. Based on the larval exuviae left within the first leaf, first instars developed into second instars before moving onto their second leaf (Fig. 2.4). The first instar stadium lasted approximately 2 days. Second instars were approximately 1.5mm in length and 0.3mm in width; the stadium was completed in approximately 7 days.

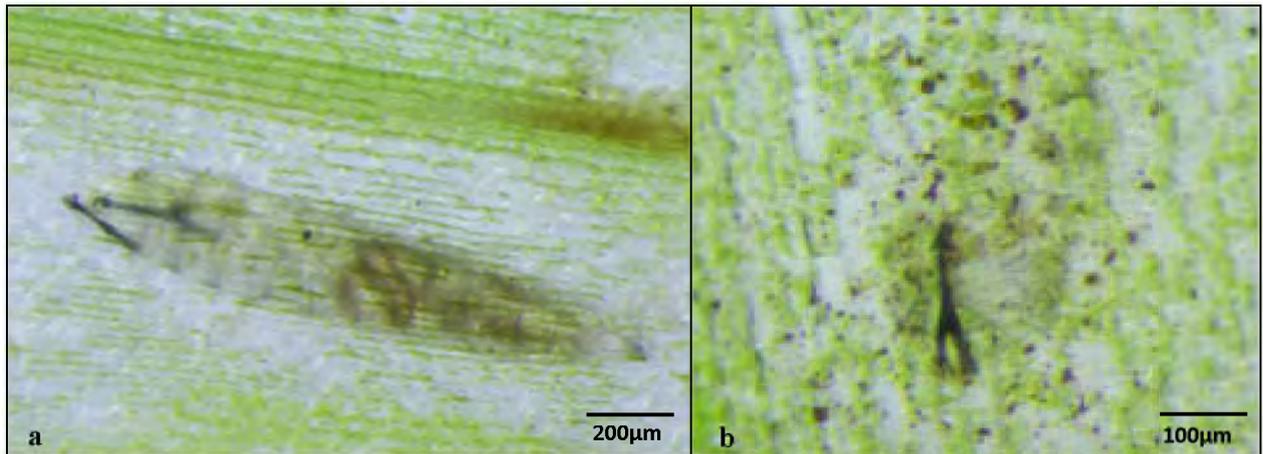


Figure 2.4: (a) Second instar shedding its first instar exuvium and mouth hook. (b) First instar exuvium and mouth hook remains in larval mine.

The third instar is the last of the actively feeding immatures of *H. egeriae*. They were 2.4mm in length and 0.8mm in width and fed for approximately six to seven days before pupariation (Fig. 2.5).



Figure 2.5: (a) Third instar mining an *Egeria densa* leaf. The larval mines are light green and contain larval frass. (b) Anterior view of a third instar and its mouth hook.

Morphologically, instars did not differ from each other with the exception of their anal spines and feeding apparatus (Fig. 2.6). The hook-like peritremes of the posterior

spiracles of first instars were slightly curved and completely sclerotized, giving them a dark appearance. Those of the second and third instars were only sclerotized at the tips, and the anal spines of the third instars are more “needle-like”. The lengths of the feeding apparatus also differed between instars.

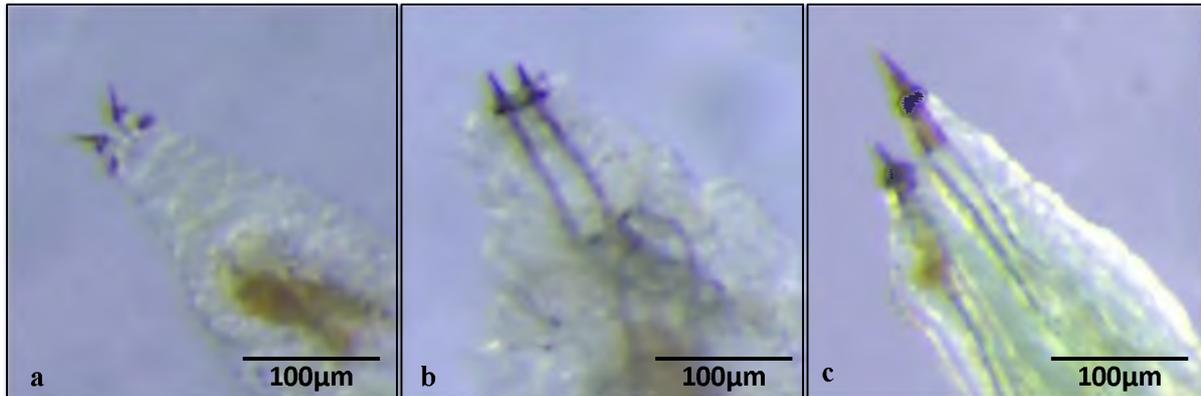


Figure 2.6: (a) The curved, completely sclerotized hook-like peritremes of the posterior spiracles of a first instar. (b) Only the tips of the second and (c) third instars are sclerotized.

The duration of the larval stage, from first to third instar, was  $16.40 \pm 0.42$  days (Table 2.1), during which they consumed on average  $24.56 \pm 8.07$  leaves. Female larvae consumed significantly more leaf tissue than males with  $32.85 \pm 2.21$  leaves compared to  $18.96 \pm 2.07$  leaves ( $t_8 = 4.44$ ;  $P = 0.002$ ).

*Pre-pupa:* Prior to pupariation, third instars underwent a short, non-feeding stage. This stage is morphologically different from the preceding third instar and the succeeding puparia and is referred to as the pre-pupa. After  $16.40 \pm 0.42$  days, the mature third instar anchored itself to the base of the last leaf it mined with its anal spines. The larva became inactive and its cuticle started to contract and sclerotize to form the puparium (Fig. 2.7).



Figure 2.7: Pre-pupa of *Hydrellia egeriae*, the short fourth instar that precedes pupariation.

*Puparium:* The puparium was golden/brown and fusiform (Fig. 2.8). The remnant mouth hook of the third instar is clearly illustrated in Figure 2.8 as well as the operculum; which is the structure that the pharate adult will open to emerge.



Figure 2.8: Lateral view of a puparium of *Hydrellia egeriae* anchored at the base of an *Egeria densa* leaf with its anal spines. Circle indicates the position of the operculum.

*Adults:* After  $10.22 \pm 0.35$  days (Table 2.1), adults emerged from the puparia. Pharate adults opened the puparium with their ptilinum and floated to the water surface in an air bubble. Adults moved around with a combination of walking, hopping and short flights. Male adults (Fig. 2.9a) are between 1.86 – 2.07mm in length and female adults (Fig. 2.9c) 2 – 2.3mm. Their face is oval-shaped and silver/greyish. The thorax and abdomen are silver/brown coloured on the anterior side and golden/silver on the ventral side. Adults have dark setae on their head, thorax abdomen and legs.

*Hydrellia egeriae* adults are distinguished from each other by their genitalia. Males have a concavity in the middle of the abdomen, protected by the cercus that opens during mating to expose the phallus (Fig. 2.9b). The abdomen of females is smooth and uniform

with distinct sternal segments (Fig. 2.9d). Adults mated within 24 hours of eclosion and females started to oviposit within 48hours.

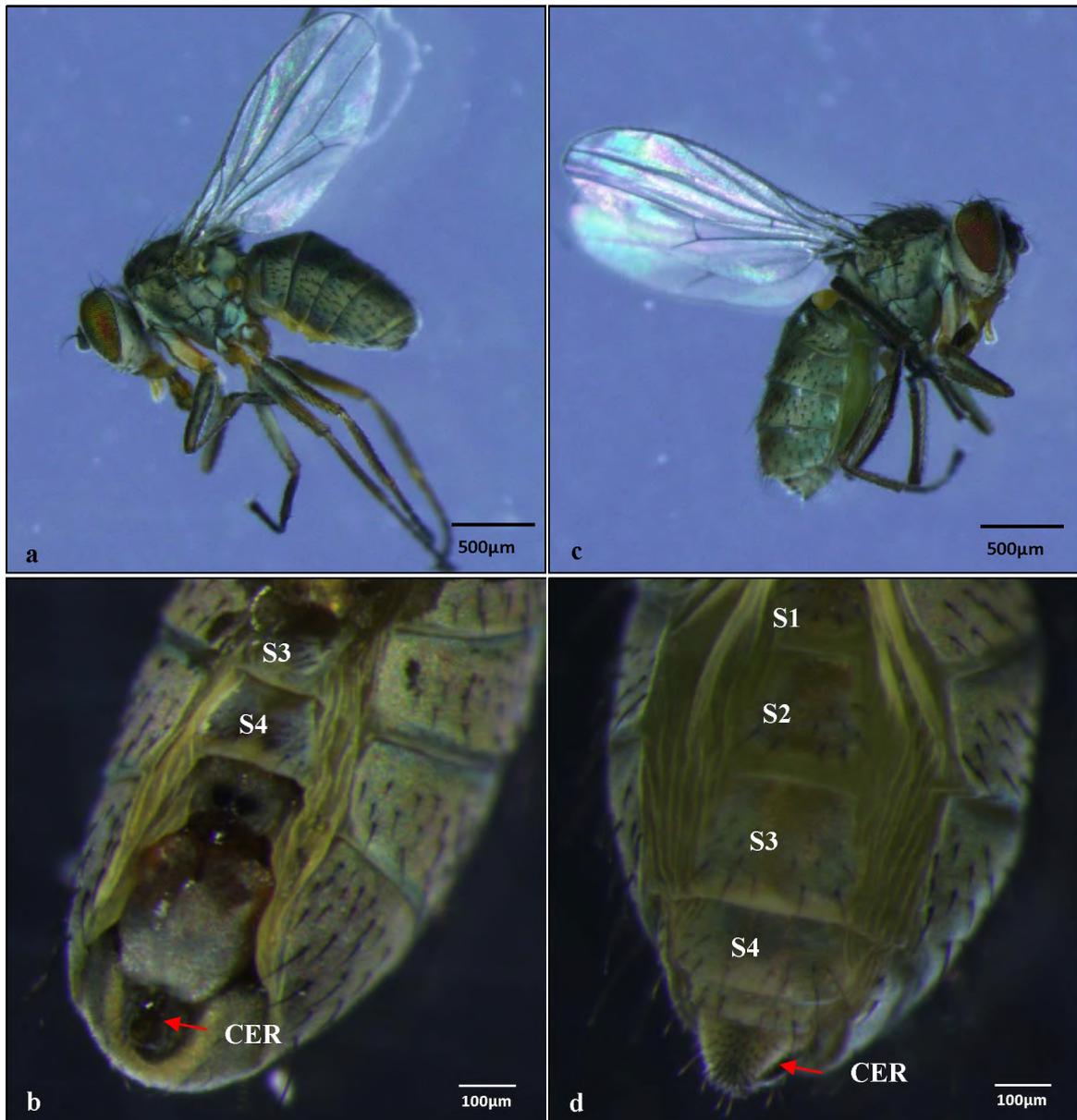


Figure 2.9: Lateral view of a (a) male and (c) female adult of *Hydrellia egeriae*. Ventral view of the (b) male and (d) female genitalia. Abbreviations: CER, cercus; S1-S4, Sterna 1 to 4.

Population development parameters

*Hydrellia egeriae* females oviposited  $68.29 \pm 11.35$  during their lifespan of  $13.27 \pm 1.93$  days (Table 2.1). Development from egg to adult for *H. egeriae* required approximately 1 month (31 days). The sex ratio was biased towards males, with 1.27:1 males to females.

Table 2.1: Population development parameters of *Hydrellia egeriae* ( $\pm$ SE) at  $22 \pm 2^\circ\text{C}$ .

<b>Female longevity (days)</b>	$13.27 \pm 1.93$
<b>Total progeny</b>	$68.29 \pm 11.35$
<b>Mean development time (days)</b>	
<b>Egg stage</b>	$4.78 \pm 0.07$
<b>Larval stage (1<sup>st</sup> – 3<sup>rd</sup>)</b>	$16.40 \pm 0.42$
<b>Pupal stage</b>	$10.22 \pm 0.35$
<b>Total development time</b>	$31.40 \pm 0.35$
<b>Sex ratio (% female)</b>	1.27:1 ( $44.37 \pm 0.22\%$ )

Demographic life table/growth parameters

The calculated demographic parameters at a temperature of  $22 \pm 2^\circ\text{C}$  are listed in Table 2.2. The net productive rate ( $R_o$ ) for *H. egeriae* was greater than one, since females produced  $28.35 \pm 0.05$  daughters during their lifespans. The time that elapsed between the birth of a female and birth of her offspring was 36 days. In the absence of density dependent factors, the maximum exponential population growth ( $r_m$ ) for *H. egeriae* was 0.09. Furthermore, *H. egeriae* expressed the potential to double in population size every week (7.58 days).

Table 2.2: Population growth parameters ( $R_o$ ,  $r_m$ ,  $T_c$ ,  $T_d$ ) for *Hydrellia egeriae* at  $22 \pm 2^\circ\text{C}$ 

<b>Growth parameters</b>	<b><i>n</i></b>	<b>Duration (days <math>\pm</math> SE)</b>
$R_o$ : net reproductive rate	7	$28.35 \pm 0.05$
$r_m$ : intrinsic rate of natural increase	7	$0.0914 \pm 0.000$
$T_c$ : mean generation time	7	$36.59 \pm 0.01$

$T_d$  : population doubling time

7

 $7.58 \pm 0.00$ 

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*n*: Number of *H. egeriae* female used

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Survival rate of *H. egeriae* survival rate from egg to adult was 42.76%. Out of a cohort of 421, only 180 survived to adulthood (Table 2.3). The developmental stage with the highest percentage apparent mortality ( $M_c$ ), was the larval stage with 51.16% (Table 2.3). The apparent mortality in the egg and pupal stage were much lower with 8.08% and 4.76%, respectively.

Table 2.3: Life table of *Hydrellia egeriae* at a constant temperature of  $22 \pm 2$  °C

<b>Life stage</b>	<b>Number of individuals entering stage (<math>l_x</math>)</b>	<b>Number of individuals dying in stage (<math>d_x</math>)</b>	<b>Percentage (%) apparent mortality (<math>M_c</math>)</b>
<b>Egg</b>	421	34	8.08
<b>Larvae</b>	387	198	51.16
<b>Pupae</b>	189	9	4.76
<b>TOTAL</b>	421	241	57.24

## 2.4 DISCUSSION

*Hydrellia egeriae* exhibited the potential to proliferate rapidly. One female produced 28 daughters ( $R_0$ ) and the population doubling time ( $T_d$ ) was just over a week. Female fecundity of *H. egeriae* at  $22 \pm 2^\circ\text{C}$  is similar to that of *H. pakistanae* at  $27 \pm 1^\circ\text{C}$ , with 68.29 and 68.40 eggs, respectively (Buckingham & Okrah, 1993). Females of *H. egeriae* have a longer lifespan than *H. pakistanae* with 13.27 and 10.20 days, respectively (Buckingham & Okrah, 1993). *Hydrellia pakistanae* has been the most successful of the *Hydrellia* biocontrol agent to date, due to its high establishment rates and ability to expand its range (Center *et al.*, 1997). However, its impact on *H. verticillata* is debatable (see Forno & Julien, 2000).

Female *Hydrellia* species consume approximately 50% more leaves than males during their immatures stages. This is solely because of sexual dimorphism (Buckingham & Okrah, 1993; Bownes, 2015; Bownes & Deeming, 2016). Females have larger bodies to accommodate the female reproductive system. A study on the reproductive system of *H. pakistanae* showed that females have two ovaries, each with up to 10 ovarioles that host several developing follicles (Lenz *et al.*, 2007). The same study showed that females emerged with a fully mature reproductive system that allows them to oviposit their first offspring within a few hours of emergence (Lenz *et al.*, 2007).

The high mortality rate (51%) of *H. egeriae* larvae was unexpected, since *H. egeriae* larvae exhibited a survival rate of 100%, 84% and 84.6% at  $20^\circ\text{C}$ ,  $25^\circ\text{C}$  and  $30^\circ\text{C}$  in studies conducted in their native range (Cabrera Walsh *et al.*, 2013). Compared to *H. lagarosiphon*, larval mortalities above 50% were only recorded at temperatures of  $13.5^\circ\text{C}$  and below (Mangan & Baars, 2013). Because insect performance is temperature dependent, higher mortality rates are expected at extreme temperatures (see Cuda *et al.*, 2008). Therefore, the high mortality rate during the larval stage of *H. egeriae* in this study may be a result of intra-specific competition for resources.

The total development time for *H. egeriae* is comparable to that reported by Cabrera Walsh *et al.* (2013). In this study, *H. egeriae* completed its development in 31.4 days at an ambient temperature of  $22 \pm 2^\circ\text{C}$ . Similarly, Cabrera Walsh *et al.* (2013) established that the total development time for *H. egeriae* at  $25^\circ\text{C}$  was 30.22 days. However, the fly's development and population growth parameters may be positively affected by warmer temperatures, considering that development time of *H. egeriae* was reduced from 45 days at  $20^\circ\text{C}$ , to 30.22 days at  $25^\circ\text{C}$  and 21.2 days at  $30^\circ\text{C}$  (Cabrera Walsh *et al.* 2013). Population growth parameters for *H. lagarosiphon* at four different temperatures also followed this pattern. The net reproductive rate of this *Hydrellia* fly at  $20^\circ\text{C}$  was 31% higher than at  $16.5^\circ\text{C}$ , and the population doubling time was 37% shorter (Mangan & Baars, 2013).

The physiological and metabolic processes of insects are dependent on their immediate temperature (Davies, 1988). Biocontrol is enhanced when agents are selected from areas that are climatically similar to that of the invaded area (Williamson, 1996). *Hydrellia egeriae* occurs naturally in water bodies of the Paraná Delta, Buenos Aires (Cabrera Walsh *et al.*, 2013; Rodrigues *et al.*, 2015). This region has a subtropical climate with winter minimum temperatures below  $0^\circ\text{C}$  and summer minimums lower than  $18^\circ\text{C}$  in the lower Delta (Cabrera Walsh, pers. comm.). *Egeria densa* infestations are found across various climate regions in South Africa, with heavily infested sites concentrated in the coastal regions that include the Western Cape, Eastern Cape and Kwa-Zulu Natal provinces. The latter two provinces have subtropical climates and the Western Cape has a Mediterranean climate with warm, dry summers and mild, rainy winters. The average temperatures for sites in the Western Cape range between  $7.9^\circ\text{C}$  and  $27^\circ\text{C}$  and between  $7.7^\circ\text{C}$  and  $23^\circ\text{C}$  for the Eastern Cape and Kwa-Zulu Natal (Climate-Data.org., 2016). In the eastern central regions, conditions are drier and warmer with only summer rainfall. Minimum winter temperatures drop to sub-zero in these regions, with frequent frost events ( $>24$ ) (Coetzee *et al.*, 2007).

Cabrera Walsh *et al.* (2013) determined that *H. egeriae* development will continue unhindered in water temperatures above 13°C. Although winter temperatures are below the temperature threshold for *H. egeriae* development, native range surveys showed that larvae were abundant even at water temperatures near sub-zero (Cabrera Walsh *et al.*, 2013). *Hydrellia* species generally overwinter as larvae in their host plant. Cold temperatures prolong the development time from egg to adult or cause immatures to become dormant (Deonier, 1971; Harms & Grodowitz, 2011; Mangan & Baars, 2013; Cabrera Walsh *et al.*, 2013).

Native range studies showed that the only limiting factor for *H. egeriae* prevalence was host plant availability. Long periods of flooding and interspecific competition with floating aquatic weeds made *E. densa* inaccessible to gravid females for oviposition (Cabrera Walsh *et al.*, 2013). In addition to these factors, *E. densa* has a slower growth rate in the winter and generally resides prostrate on the bottom (Yarrow *et al.*, 2009). However, field surveys in South Africa showed that *E. densa* reaches the water surface or “tops out” year around (Martin, pers. comm.) so oviposition sites should not be limited.

Other factors that may limit the biological success and performance of *Hydrellia egeriae* in South Africa is parasitism. During native range surveys, approximately 10% of the field-collected *H. egeriae* puparia were parasitized. Parasitoids reared from samples included *Chaenusa aurantium* Kula and Martinez (Hymenoptera: Braconidae), *Hydrelliaeucoila egeria* Diaz and Gallardo (Hymenoptera: Figitidae), and a newly, undescribed pathogenic fungus that attacks puparia. Three parasitoids were reared from *H. lagarosiphon* during exploration surveys in South Africa. Two of these parasitoids are from the genus *Chaenusa* (*C. luteostigma* and *C. nigristigma*) and the third was identified as *Ademon lagarosiphonae* sp. n. (Opiinae) (Martin *et al.*, 2013).

Similarly, the introduced biocontrol agent *H. pakistanae* in Lewisville, Texas, USA, is parasitized by a generalist parasitoid wasp, *Trichopria columbiana* Ashmead (Hymenoptera: Diapriidae) (Grodowitz *et al.*, 2009; Coon *et al.*, 2014). Studies on the relationship between parasitoids and *H. pakistanae* population dynamics indicated that population growth of both the species corresponded. Nonetheless, *H. pakistanae* established and increased regardless of a parasitism rate of ~ 20% (Grodowitz *et al.*, 2009; Coon *et al.*, 2014). The potential effect of parasitism on *H. egeriae* populations in South Africa warrants further investigation and should be an important factor of consideration when determining field releases.

Results from this study show that *H. egeriae* has the potential to proliferate rapidly. Complementary temperature-dependent development studies conducted by Cabrera Walsh *et al.* (2013), suggest that optimal mass-rearing temperatures will be between 25°C and 30°C. Its minimum temperature thresholds and overwintering behaviour indicate that it has capacity to overwinter successfully, if released. Due to its ability to tolerate a wide range of temperatures, the prospect for its establishment in South Africa is promising, especially in sites along the coastal regions. Release attempts should aim to synchronize agent and host plant dynamics to enhance establishment success. Generally, warmer temperatures in spring and summer encourage extensive *E. densa* growth (Yarrow *et al.*, 2009). Therefore, *H. egeriae* should be released in spring when warmer temperatures are conducive to shorter agent development time, higher intrinsic rates of increase, and potential to inflict significant damage to the weed during vigorous growing periods (Day *et al.*, 2014).

Despite the favourable life characteristic traits of *H. egeriae*, non-target safety is the key determinant for its release. Host-specificity is pivotal to the science of biocontrol (van Wilgen *et al.*, 2013) and provides basic information upon which the safety of a biocontrol agent is assessed (van Driesche *et al.*, 2000). Non-target effects posed by the release of biocontrol agents remain a major concern for the practice (Wan & Harris, 1997; Simberloff &

Stiling, 1996; Pemberton, 2000; Louda *et al.*, 2003; Tingle *et al.*, 2016; Downey & Paterson, 2016) and has been a driving force for extensive refinement in host-specificity methodology (Simberloff & Stiling, 1996; McFayden, 1998; Louda *et al.*, 2003; Downey & Paterson, 2016). Host-specificity has also proven adequate to determine the safety of biocontrol agents, and is accepted by government regulatory authorities (McFayden, 1998). Therefore, the host-specificity of *H. egeriae* is tested and discussed in chapter 3.

## Chapter 3: Host-specificity of *Hydrellia egeriae*

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### 3.1 INTRODUCTION

Host specialization is in essence a behavioural process governed by chemoreception and followed by physiological adaptation (Ehrlich & Raven, 1964; Jermy, 1984; Futuyma, 1983; Bernays, 1998). Host-specificity tests validate the spectrum of host utilization at a behavioural and physiological level, i.e. host location, host acceptance, and host suitability (Louda *et al.*, 2003; van Driesche & Murray, 2004). This is executed by establishing the agent life stage that needs to be host-specific, validating the specificity of that life stage in laboratory assays (fundamental host range), and establishing if hosts used in the laboratory are included in the agent's realised host range (van Driesche *et al.*, 2000). Fundamental host range includes all the host species that are physiologically suitable to support agent development, whereas, realised or field host range is the subset of fundamental hosts used in the field (van Driesche *et al.*, 2000; Sheppard *et al.*, 2005).

Host-specificity testing was mostly focused on non-target effects to economically important crops and agronomic plants in the mid-1900s (McEvoy, 1996; McClay & Balciunas, 2005), but shifted to the safety of indigenous and congeneric plants from 1965 (Wan & Harris, 1997; van Driesche *et al.*, 2010). Initial test plant selection was based on taxonomic relatedness to the target species, a procedure referred to as the centrifugal phylogenetic method. Emphasis was also placed on those species that were economically important, rare, endangered or that possess similar morphologies, chemical properties and spatial distribution as the target weed (Wapshere, 1974). However, with a better understanding of insect-plant interactions and more plant phylogenetic publications, test plant selection shifted towards genetic relatedness to the target weed (Briese, 2003) because insect host utilization is more often phylogenetically conserved (Futuyma & Agrawal, 2009),

Host-specificity experiments are conducted using various no-choice and choice test conditions to determine the extent of the fundamental and realised host of the candidate agent. No-choice tests provide the most robust information regarding agent behaviour; exposing adults to potential hosts, assess feeding and oviposition, and larval feeding and development (Harris, 1991; Briese *et al.*, 2001). Confinement to a single test species evaluates if the agent is willing to oviposit or feed on the plant and the likelihood of offspring development (McFayden, 1998; Marohasy, 1998; Schaffner, 2001). Generally, ovipositing females are responsible for selecting the optimal host species and the quality of this decision is reflected in the performance of her progeny; a phenomenon referred to as the ‘preference-performance or mother-knows-best’ hypothesis (Futuyma, 1983; Bernays & Graham, 1988; Fox, 2000; Tilmon, 2008; Prager *et al.*, 2014). In addition, no-choice larval feeding tests establish the range of species that a deprived agent is willing to utilize, and is therefore, usually wider than the oviposition host range (Wan & Harris, 1997; Schaffner, 2001; van Driesche & Murray, 2004; Bordeur, 2012).

No-choice tests are prone to false-positives due to time-dependent effects (starvation) and, consequently choice tests are conducted to clarify ambiguous results and prevent the likelihood of rejected safe species (Marohasy, 1998; Schaffner, 2001; Briese, 2003). The addition of a test species to the test design accentuates the mode of choosing between hosts, and ranks species according to agent preference (Marohasy, 1998). Choice-tests require mobile life stages, predominantly feeding adults and ovipositing females (Schaffner, 2001; van Driesche & Murray, 2004).

In cases where agents utilize non-target species during choice tests, continuation tests are conducted to establish if these species are capable of sustaining a viable agent population (Buckingham & Okrah, 1993; Bownes, 2015). This is illustrated in the host-specificity testing of *H. pakistanae*, where larvae developed on the test species *P. crispus* during no-choice and

choice tests, but when reared solely on the test species, the agent population declined rapidly and died out after the seventh generation (Buckingham *et al.*, 1989).

Laboratory host-specificity tests are prone to overestimate (false-positive) or underestimate (false-negative) the host range of an agent, and tests have to be interpreted cautiously. Factors such as small cage sizes, bypassing steps in host location and agent experience or learning may produce agent behaviour that would not occur in natural conditions (Heard & van Klinken, 1998; McFayden, 1998; van Driesche *et al.*, 2000). However, open-field studies in the area of origin are used to overcome these artefacts and assist in the final interpretation of laboratory-based tests (McFayden, 1998; Schaffner, 2001). Additionally, host-selection and suitability parameters from laboratory tests are used to provide a numerical value of the relative feeding and reproductive risks of non-target species to the target weed in the field (Wan & Harris, 1997; Paynter *et al.*, 2015). Ultimately, all tests conducted should model the ecological context in which the agents will interact with the potential host (Louda *et al.*, 2003), while interpretation of results should be carefully considered to ensure they are representative of the natural host-range (McFayden, 1998; Marohasy, 1998).

#### Host specificity of *Hydrellia egeriae*

Adult *Hydrellia* flies are polyphagous and feed on nectar, fungi, cyanobacteria, plant material and smaller insects (Deonier, 1971; Mangan *et al.*, 2015). Only the larval stage is phytophagous and has, therefore, been the feeding life stage under investigation for all *Hydrellia* biocontrol agents (Buckingham & Okrah, 1993; Bownes, 2014; Bownes & Deeming, 2016). Past studies illustrated that *Hydrellia* spp. females express some degree of host preference during oviposition, but under high egg load, females oviposit indiscriminately, and in many cases even on inanimate objects (Deonier, 1971; Courtney *et al.*, 1989; Buckingham & Okrah, 1993; Mangan & Baars, 2013).

Because *H. egeriae* is under consideration for release in South Africa, laboratory no-choice larval feeding tests in the native range of *H. egeriae* (Argentina) showed that larvae only mined and developed on three non-target species, all within the family Hydrocharitaceae. These included the congener *Egeria naias* Planch., *Eloдея callitrichoides* Rich. Casp. and *Najas guadalupensis* Spreng Magnus, of which *E. naias* had the highest survival percentage of 82%. During paired choice experiments with equal number of *H. egeriae* eggs on *E. densa* and *E. naias*, larvae showed a clear preference for their host plant, with significantly more larvae recorded on *E. densa* at the end of the experiment. Females expressed some degree of oviposition preference during choice and no-choice trials, where significantly more eggs were recorded on *E. densa*, however, gravid females readily oviposited on containers walls (Cabrera Walsh *et al.*, 2013). During open field choice trials with pools containing *E. densa*, *E. naias*, *E. callitrichoides*, *N. guadalupensis*, *Limnobium laevigatum* (Humb. & Bonpl. ex Willd.) Heine, *Vallisneria americana* Michx and *V. spiralis* L., all within the Hydrocharitaceae, colonization and larval mining were only observed in *E. densa* pools (Cabrera Walsh *et al.*, 2013). These native range results are very promising for the proposed release of *H. egeriae* in South Africa. Whereas congeneric species are generally viewed as most vulnerable for biocontrol agent release (Pemberton, 2000; Suckling & Sforza, 2014), there are no indigenous *Egeria* species in South Africa (Cook, 2004).

The aim of this study was to evaluate the host specificity of *H. egeriae* in a South African context, and to conduct a risk analysis of its release. The first objective was to conduct quarantine-based host-specificity testing of the agent. Based on test designs from previous *Hydrellia* spp. host-specificity trials (Buckingham & Okrah, 1993; Mangan, 2012; Bownes, 2014; Bownes & Deeming, 2016), results from native range studies and the plasticity in oviposition host range, oviposition preference trials were not deemed necessary to validate non-target safety. Thus, testing oviposition preference of *H. egeriae* will

potentially lead to an overestimation of the host range. Host preference expressed by larvae of *H. egeriae* and other *Hydrellia* spp. released as biocontrol agents warrant this life stage as the primary determinant of host-specificity. In addition, oviposition in itself does not usually cause significant damage, and therefore, test designs were focused on larval preference and performance. Secondly, results from host-specificity tests were used to determine the percentage feeding and reproductive risk to non-target plants in the field (Wan & Harris, 1997).

### 3.2 MATERIALS & METHODS

#### Test plant selection

The centrifugal phylogenetic method (Wapshere, 1974; Briese, 2003) was used for selecting the test plants for host specificity of *H. egeriae*. Phylogenetic trees of the order Alismatales (Petersen *et al.*, 2015) and the family Hydrocharitaceae (Chen *et al.*, 2012) were used to identify families and genera present in South Africa that are closely related to the target species (Table 3.1).

Six genera and 11 species were selected within the Hydrocharitaceae, mainly consisting of members from the genus *Lagarosiphon*. The most closely related families to the Hydrocharitaceae are the Aponogetonaceae and Alismataceae. Seven species were selected from these two families, with only one species indigenous to South Africa (*Aponogeton distachyos* L.f.). Five species of the Potamogetonaceae were included in the test plant list due to previous interactions of other *Hydrellia* spp. with *Potamogeton* species during host-specificity testing (Buckingham, 1994; Mangan, 2012; Cabrera Walsh *et al.*, 2013). *Myriophyllum spicatum* was selected as a representative species from the Haloragaceae based on similarities in morphology and habitat as the target plant. Additionally, one representative from the Araceae, *Lemna* sp., was selected based on its relatedness to the Hydrocharitaceae and its common distribution in water bodies (Table 3.1).

Table 3.1: Non-target species selected for host-specificity testing of *Hydrellia egeriae* on the basis of phylogenetic relatedness. Asterisks (\*) indicate exotic plant species.

Family	Test plant	Family	Test plant
<b>Hydrocharitaceae</b>	<i>Egeria densa</i> Planch	<b>Alismataceae</b>	<i>Echinodorus cordifolius</i> (L.) Griseb
	<i>Lagarosiphon major</i> Ridley		<i>Alisma plantago-aquatica</i> L.
	<i>Lagarosiphon muscoides</i> Harvey		<i>Sagittaria platyphylla</i> (Engelmann.) J.G.Smith*
	<i>Lagarosiphon cordofanus</i> Caspary	<b>Aponogetonaceae</b>	<i>Aponogeton distachyos</i> L. filius
	<i>Lagarosiphon ilicifolius</i> Obermeyer		<i>Lemna</i> sp.
	<i>Lagarosiphon verticillifolius</i> Obermeyer	<b>Haloragaceae</b>	<i>Myriophyllum spicatum</i> L.*
	<i>Hydrilla verticillata</i> Royle *		<i>Potamogeton schweinfurthii</i> A. Bennett
	<i>Najas horrida</i> A. Brown ex Magnus	<b>Potamogetonaceae</b>	<i>Potamogeton crispus</i> L.
	<i>Najas marina</i> L.		<i>Potamogeton pussilus</i> L.
	<i>Ottelia exserta</i> Ridley		<i>Stuckenia pectinata</i> L.
<i>Vallisneria spiralis</i> L.	<i>Potamogeton thunbergii</i> Chamisso & Schlechtendal		
<i>Blyxa aubertii</i> L.C.			

### Larvae for testing

First instars (neonates), instead of eggs, were used for the larval no-choice feeding trials. This was to ensure that viable individuals were used and prevent egg damage during transfer to test plants (Cabrera Walsh *et al.*, 2013; Mangan, 2012). To obtain first instars, ten pairs of newly eclosed adults were placed in a transparent box (41cm X 17cm X 29cm), half-filled with spring water (10L), 30 *E. densa* apical shoot tips, dead *D. melanogaster* as a protein source (Chapter 2) and a yeast hydrolysate/sugar mixture. Adults were allowed to mate and oviposit, and were removed after 3 days. Eggs were harvested, placed in a petri-dish and monitored daily for eclosion. Larvae were prepared for testing by excising excess leaf material around the larva with a sterilized razor blade.

### Plants for testing

Prior to experimental set up, test plants were planted in 3cm x 5cm vials containing sediment and a slow release fertilizer Multicote™ (Haifa) was used in quantities of 0.7g per 1kg sediment. Plants were placed in 600L tanks that are connected to a flow-system in a polytunnel at the Waainek Biological Control Research Facility, Grahamstown. A fluid nutrient stock solution as proposed by Smart & Barko (1985) was added to the tanks to ensure healthy plant growth. These plants were subsequently used during no-choice and paired choice trials in quarantine at Rhodes University. Where rooted test plants were not available, healthy leaves or plant fragments were used instead. Whole *V. spiralis* plants were used during host-specificity testing.

*Lagarosiphon ilicifolius* and *L. verticillifolius* could not be collected for host-specificity trials due to a drought in 2015/16 that resulted in low water levels in rivers and dams in which these plants usually occur. Similarly, *Ottelia exserta* and *Blyxa aubertii* could not be collected despite extensive efforts.

### No-choice larval feeding

In order to establish the fundamental host range of *H. egeriae*, no-choice larval feeding trials were conducted. Test plants were individually placed in 600ml containers (24cm x 7.5cm) filled with spring water. An excised *E. densa* leaf containing the first instar, was pinned to leaves on the test plants with Minuten pins. The containers were enclosed with netting held in place by an elastic band to prevent any eclosed *H. egeriae* adults from escaping.

One replicate consisted of sufficient test plant material for feeding and development and five *H. egeriae* larvae. After 30 days, the replicates were checked for larval mining and development. Larval mining was determined by dissecting test plants, placing plant fragments in a petri dish containing spring water and observation under a stereo microscope.. If larvae mined the test plant, the leaf area damaged ( $\frac{1}{4}$ ,  $\frac{1}{2}$ ,  $\frac{3}{4}$  or 1) was recorded and accounted for the whole test species. Subsequently the total number of leaves was counted, so that the percentage of the plant damaged could be calculated ( $\frac{\text{damaged leaves}}{\text{total number of leaves}} \times 100$ ). Survival was measured as the number of larvae that pupariated on the test plant.

### Paired choice larval feeding

In order to establish larval host plant preference, paired choice larval feeding trials were conducted with test plants (*L. major* and *L. muscoides*) that supported larval development during no-choice trials. However, paired choice larval feeding trials were not conducted with *V. spiralis* due the low percentage of larval development during no-choice trials. Sufficient test plant material and *E. densa* shoots were placed together in a 1.5L container. Shoot tips with young leaves were used as first instars generally feed on softer leaflets. Excised *E. densa* leaves with first instars were pinned to a 1cm x 1cm piece of sponge and placed in the middle of the container to drift in the water. The sponge allowed

instars to choose their feeding site. The number of mined leaves for each test plant was counted to establish larval damage, and the number of puparia per test plant was also recorded.

#### Multi-generation continuation trials

Multi-generations population persistence trials were conducted with test plants that supported larval development during paired-choice trials. This was to establish if non-target species were able to sustain viable *H. egeriae* populations in the field. Thirty excised stems of the test plant, 15cm in length, were placed in a transparent “culture” container (41cm X 17cm X 29cm) with spring water. One hundred *H. egeriae* eggs were placed in the container and left to feed and develop on the test plant. One replicate was set up for the target weed *E. densa* and for the non-target species, *L. major*. After 30 days, boxes were checked for adult eclosion every second day, during which eclosed adults were removed and placed into a new culture container with the test plant they emerged from, spring water and food (drosophila and yeast hydrolysate/sugar mixture) on a float. The same procedure used in the life history trials was further applied to determine the female fecundity and survival of *H. egeriae* for each test plant. The trials were conducted for three generations.

#### Risk assessment

Results from no-choice, choice and continuation trials were used to conduct an assessment of the risks posed by *H. egeriae* to non-target species in the introduced range (Wan & Harris, 1997). To calculate these risk percentages, the relative survival and damage were calculated using the mean survival (preference) and damage (performance) of a test plant species in proportion to that of the target weed during no-choice tests. The same method was used to calculate the relative survival (preference) for non-target species during choice larval feeding tests. The feeding risk was determined by multiplying the preference and

performance values of the no-choice and choice tests. Additionally, the reproductive risk of *H. egeriae* was determined by multiplying the preference values during no-choice tests with the number of viable reproducing adults during multi-generation tests.

### Statistical Analysis

All statistical analyses were conducted in the R environment (version 3.2.3; R Development Core Team, 2014; available at <http://cran.r-project.org> using R Studio (version 0.98.1103). The distribution of larval damage and mean survival for no-choice and choice feeding tests were tested for normality using the Shapiro Wilk test. Due to the non-normal distribution of all the independent variables, a non-parametric Kruskal Wallis test was used to determine statistical difference between test plants for larval feeding and mean survival during no-choice tests. The post hoc Kruskal-Dunn test was used to detect statistical differences ( $P < 0.05$ ) between test plants. Additionally, the Wilcoxon matched-pairs test was used to determine statistical differences ( $Z < 1.645$ ;  $P < 0.05$ ) for choice larval tests.

### 3.3 RESULTS

#### No-choice larval feeding

*Hydrellia egeriae* expressed significant preference for its host plant. Larval damage to *E. densa* was significantly higher (25.54%) than for all other test species ( $H = 55.05$ ;  $P < 0.001$ ) (Table 3.2) at 25.54%. During the no-choice tests, *H. egeriae* mined only closely related species within the Hydrocharitaceae. These included *L. major* ( $5.11 \pm 2.14\%$ ), *L. muscoides* ( $2.32 \pm 0.66\%$ ), *L. cordofanus* ( $0.04 \pm 0.02\%$ ), *H. verticillata* ( $0.83 \pm 0.17\%$ ) and *V. spiralis* ( $9.00 \pm 3.29$ ). Leaf-mining on *L. cordofanus* was marginal and significantly lower ( $H = 55.05$ ;  $P < 0.05$ ) than damage incurred to *L. major*, *L. muscoides* and *V. spiralis*. *Egeria densa* also supported significantly higher *H. egeriae* development to adulthood than all other test species ( $H = 68.41$ ;  $P < 0.001$ ), at  $82.22 \pm 4.04\%$ . Non-target species that supported larval development were *L. major*, *L. muscoides* and *V. spiralis* with  $11.43 \pm 5.95\%$ ,  $6.67 \pm 5.12\%$  and  $1.54 \pm 0.18\%$ , respectively. Only the two *Lagarosiphon* species, *L. major* and *L. muscoides* were subjected to choice larval feeding tests.

Furthermore, 13 of the 19 non-target species tested under no-choice conditions revealed no larval mining or development. Two of these species, *N. horrida* and *N. marina*, are within the Hydrocharitaceae and the remainder belong to less closely related families including the Potamogetonaceae, Alismataceae, Araceae, Aponogetonaceae and Haloragaceae.

Table 3.2: Mean ( $\pm$ SE) percentage (%) leaf-mining and survival of *Hydrellia egeriae* first instars on test plants during no-choice feeding trials

Test plant	<i>n</i>	% Feeding <sup>a</sup>	Relative damage	% Survival <sup>b</sup>	Relative survival <sup>c</sup>
<b>Hydrocharitaceae</b>					
<i>Egeria densa</i>	135	25.54 $\pm$ 1.62a	1.00	82.22 $\pm$ 4.04a	1.00
<i>Lagarosiphon major</i>	55	5.11 $\pm$ 2.14b	0.20	11.43 $\pm$ 5.95b	0.14
<i>Lagarosiphon muscoides</i>	60	2.32 $\pm$ 0.66b	0.09	6.67 $\pm$ 5.12b	0.08
<i>Lagarosiphon cordofamus</i>	50	0.04 $\pm$ 0.02c	0.001	0	-
<i>Lagarosiphon ilicifolius</i>	not tested	-		-	
<i>Lagarosiphon verticillifolius</i>	not tested	-		-	
<i>Hydrilla verticillata</i>	55	0.83 $\pm$ 0.17bc	0.03	0	-
<i>Najas horrida</i>	70	0		0	
<i>Najas marina</i>	50	0		0	
<i>Vallisneria spiralis</i>	65	9.00 $\pm$ 3.29b	0.35	1.54 $\pm$ 0.18b	0.02
<i>Ottelia exserta</i>	not tested	-		-	
<i>Blyxa aubertii</i>	not tested	-		-	
<b>Potamogetonaceae</b>					
<i>Potamogeton crispus</i>	65	0		0	
<i>Potamogeton pussilus</i>	55	0		0	
<i>Stuckenia pectinata</i>	50	0		0	
<i>Potamogeton schweinfurthii</i>	70	0		0	
<i>Potamogeton thunbergii</i>	45	0		0	

<b>Alismataceae</b>			
<i>Echinodorus</i>			
<i>cordifolius</i>	30	0	0
<i>Alisma plantago-</i>			
<i>aquatica</i>	40	0	0
<i>Sagittaria</i>			
<i>platyphylla</i>	40	0	0
<b>Aponogetonaceae</b>			
<i>Aponogeton</i>			
<i>distachyos</i>	15	0	0
<b>Araceae</b>			
<i>Lemna</i> sp.			
	30	0	0
<b>Haloragaceae</b>			
<i>Myriophyllum</i>			
<i>spicatum</i>	55	0	0

*n*: number of individuals tested

<sup>a</sup> Number of mined leaves/total number of leaves x 100

<sup>b</sup> Number of pupariate/5 x 100

<sup>c</sup> Relative survival determined using the mean survival on the test plants in proportion to that on the target weed. Means ( $\pm$ SE) within columns followed by the same letter are not significantly different ( $P < 0.05$ , post hoc pairwise comparisons).

### Paired choice larval feeding

First instars showed a significant preference for feeding and pupariation on *E. densa* during paired-choice trials. *Hydrellia egeriae* mined significantly more leaves of its host plant than *L. major* ( $Z = 3.857$ ;  $P < 0.001$ ) and *L. muscoides* ( $Z = 2.627$ ;  $P = 0.007$ ) (Table 3.3). When given the choice, larvae mined 8 and 48 times more the number of leaves on its host plant than of *L. major* and *L. muscoides*, respectively. Larval survival followed the same trend, with significantly higher numbers of puparia recorded on *E. densa* compared to *L. major* ( $Z = 5.402$ ;  $P < 0.001$ ) and *L. muscoides* ( $Z = 3.430$ ;  $P < 0.001$ ). The percentage of *H. egeriae* larvae that preferred *E. densa* to pupariate in was  $61.9 \pm 7.16\%$  and  $68.57 \pm 5.95\%$

for choice feeding tests. The only non-target species that supported some degree of larval survival was *L. major* with  $4.55 \pm 2.67\%$ .

Table 3.3: Number of mined leaves and percentage survival ( $\pm$ SE) of 1<sup>st</sup> instars during paired-choice trials.

Test plant	<i>n</i>	Number of mined leaves		Percentage (%) Survival <sup>a</sup>		Relative survival <sup>b</sup>
		<i>E. densa</i>	Non-target	<i>E. densa</i>	Non-target	
<i>Lagarosiphon major</i>	105	58.92 $\pm$ 10.27a	7.25 $\pm$ 3.13b	61.90 $\pm$ 7.16a	4.55 $\pm$ 2.67b	0.07
<i>Lagarosiphon muscoides</i>	35	82.80 $\pm$ 5.44a	1.80 $\pm$ 0.37b	68.57 $\pm$ 5.95a	0.00 $\pm$ 0.00b	0.00

*n*: number of individuals tested

<sup>a</sup> Number of puparia/5 x 100

<sup>b</sup> Relative survival determined using the mean survival on the test plants in proportion to that on the target weed. Means ( $\pm$ SE) within columns followed by the same letter are not significantly different ( $Z > \pm 1.645$ ;  $P < 0.05$ , Wilcoxon-Mann-Whitney test).

Due to complete larval development on *L. major* during paired choice trials, a multi-generations study was conducted to establish if the non-target species could sustain agent populations.

Multi-generation continuation trial

Results showed that *L. major* could not sustain *H. egeriae* for more than two generations. Out of a cohort of 100 eggs, only 18 eggs developed unto adulthood, which produced only one viable adult in the first generation (F<sub>1</sub>) (Table 3.4). Conversely, population growth for *E. densa* was positive. Seventy one individuals of the hundred eggs (71%) completed development during the first generation (F<sub>1</sub>) and population growth was 271% for the second generation (F<sub>2</sub>) with 192 adults.

Table 3.4: The number of eclosed *Hydrellia egeriae* adults reared on *Egeria densa* and *Lagarosiphon major* during continuation trials.

Test Plant	<i>n</i>	F <sub>1</sub>	F <sub>2</sub>
<i>Egeria densa</i>	1	71.00	192.00
<i>Lagarosiphon major</i>	1	18.00	1.00

*n*: one replicate consisted of 100 eggs

Risk assessment

The non-target risk posed by *H. egeriae* is very low. The feeding risk of non-target species *L. major* and *L. muscoides* in the field is 1.4% and 0%, compared to 100% for *E. densa* (Table 3.5). In addition, the reproductive risk of *H. egeriae* to *L. major* is 3.5% and, because no multi-generation trials were conducted for *L. muscoides*, percentage reproductive risk for *L. muscoides* could not be calculated.

Table 3.5: Risk assessment of non-target attack by *Hydrellia egeriae*, using its preference for and performance on test species during no-choice, choice and continuation trials.

Test species	Plant preference <sup>a</sup>	Feeding damage <sup>b</sup>	Feeding risk(%) <sup>c</sup>	Larval survival <sup>d</sup>	Viable reproducing adults <sup>e</sup>	Reproductive risk (%) <sup>f</sup>
<i>Egeria densa</i>	1.00	1.00	100	1.00	1.00	100
<i>Lagarosiphon major</i>	0.07	0.20	1.40	0.14	0.25	3.50
<i>Lagarosiphon muscoides</i>	0.00	0.09	0.00	0.08	<i>not tested</i>	*

<sup>a</sup> Relative survival of agent on test species during choice tests (Table 3.3).

<sup>b</sup> Feeding damage during no-choice tests (Table 3.2).

<sup>c</sup> Product of suitability indices for preference<sup>a</sup> and performance<sup>b</sup>.

<sup>d</sup> Relative survival of agent on test species during no-choice tests (Table 3.1).

<sup>e</sup> Viable reproducing adults (F<sub>1</sub>) from multi-generation tests (Table 3.4).

<sup>f</sup> Product of suitability indices for larval survival<sup>d</sup> and generational turnover<sup>e</sup>

### 3.4 DISCUSSION

Results from this quarantine-based study indicate that *H. egeriae* has a narrow host range. This supports native range specificity testing, where *H. egeriae* expressed a clear preference for, and higher performance on its host plant during no-choice, choice and open field trials (Cabrera Walsh *et al.*, 2013). Out of 18 non-target plant species tested, *H. egeriae* only mined five non-target species, all within the Hydrocharitaceae, and developed on three of these non-target species. During choice tests, marginal feeding was recorded on *L. major* and *L. muscoides*, but only *L. major* supported larval development. However, *L. major* proved physiologically unfit to support *H. egeriae* populations during continuation tests and risk assessment indicated that feeding risks for both *Lagarosiphon* species was 1.4% or less, and the reproductive risk for *L. major* was 3.5%.

No-choice tests are beneficial as they predict the range of species that are susceptible to *H. egeriae* damage in the field. Test species that supported larval development during no-choice tests are all within the Hydrocharitaceae family, which is similar to results from native range host specificity testing (Cabrera Walsh *et al.*, 2013). This includes two species from the genus *Lagarosiphon* and one species from the genus *Vallisneria*. Plant architecture is an important consideration when interpreting no-choice larval feeding trials. All of the species that *H. egeriae* mined are similar in architecture and has similar leaf-sizes, except for *V. spiralis*, which has much larger and fewer leaves per plant. Consequently, the percentage of plant damage for *V. spiralis* may be greater compared to *E. densa*, *H. verticillata* and the *Lagarosiphon* species. Therefore, it will be necessary to explore the full extent of *H. egeriae* leaf-mining on *V. spiralis* for a complete understanding of its susceptible to leaf-mining. Exploratory feeding was also recorded for *L. cordofanus* and the invasive weed, *H. verticillata*. Under field conditions, should *H. egeriae* be released, starved larvae isolated from their host plant, may feed on *L. major*, *L. muscoides*, *L. cordofanus*, *H. verticillata* and

*V. spiralis* and *L. major* may support limited reproduction. This may occur where *H. egeriae* disperse to new areas where the target weed is not available or where agent damage drastically reduced *E. densa* populations. However, feeding on *L. cordofanus* and *H. verticillata* was below 1% during no-choice tests and supported no agent development. In addition, *H. verticillata* is an invasive weed in South Africa.

Specialist herbivores often use closely related species due to similar morphological and chemical traits (Futuyma & Agrawal, 2009; Tingle *et al.*, 2016). A phylogenetic tree of the Hydrocharitaceae based on two plastid genes (*rbcL* and *matK*) and five mitochondrial genes (*atp1*, *ccmB*, *cob*, *mttB* and *nad5*) (Chen *et al.*, 2012), indicates that the genera *Lagarosiphon* and *Egeria* are within the same clade, while *Hydrilla* and *Vallisneria* are located within a sister clade. Feeding and development on the further related *V. spiralis* support the hypothesis that no-choice tests often produce false-positives due to small cage sizes and interference with natural host finding behaviour (Harris & McEvoy, 1995; Marohasy, 1998; McFayden, 1998). Larvae did not complete their development on *V. spiralis* during testing, leaving the plant and dying during the third instar stage. Open field choice tests indicated that *H. egeriae* only colonized *E. densa* pools, and no leaf-mining or adults were recorded in *V. spiralis* pools (Cabrera Walsh *et al.*, 2013). The genus *Lagarosiphon* is from the Afrotropics; species within the genus are morphologically similar to *E. densa* (Chen *et al.*, 2012; Netherlands Department of Environmental Science, 2014). Furthermore, the incidence of larval feeding and development on *L. major* was anticipated because biocontrol candidates of Hydrocharitaceae species such as *H. lagarosiphon* are closely associated with this plant/genus (Martin *et al.*, 2013; Mangan & Baars, 2013). The phylogenetic relatedness of the genus to *E. densa* predicted *H. egeriae* mining and development on *L. major* and *L. muscoides* during no-choice testing.

The utilization of closely related native species, i.e. agent spill-over, is a major concern for the release of biocontrol agents, especially for those species that are within the same genus as the target weed (Pemberton, 2000; Tingle *et al.*, 2016). The genus *Egeria* only consists of three species, *E. densa*; *E. naias* and *E. heterostemon* (Yarrow *et al.*, 2009). Studies within the native range of *H. egeriae* established that agent survival was second highest on the congeneric species. However, *E. naias* is from the Neotropical region and does not occur naturally in South Africa (Chen *et al.*, 2012).

No-choice tests often overestimate the host range of an agent (Marohasy, 1998; Sheppard *et al.*, 2005), therefore, choice tests are necessary to clarify any ambiguities (Schaffner, 2001; Briese, 2003). The low level of leaf damage and larval development on *L. major* compared to *E. densa*, illustrates that it is an inferior host plant for *H. egeriae*. Additionally, no larval survival was recorded on *L. muscoides* during choice tests, illustrating the specificity of *H. egeriae* for its host plant. However, results should also be interpreted with caution, as choice tests may produce false positives and/or false negatives (Marohasy, 1998; Sheppard *et al.*, 2005). For this study, test species were intertwined with the host plant during choice tests. It is possible that *E. densa* masked non-target species, or that larvae were not deprived sufficiently of food, resulting in target acceptance that would not occur in natural conditions (van Driesche *et al.*, 2000; Sheppard *et al.*, 2005). Continuation trials establish the likelihood of a non-target species sustaining a viable agent population in the field. In this study, continuation tests indicated that *L. major* is not physiologically able to produce viable populations of *H. egeriae* in the field.

Host-specificity testing conducted with *H. purcelli* for the biocontrol of *Hydrilla verticillata* in South Africa, produced similar pattern of non-target use (Bownes, 2014). During no-choice larval feeding tests, four species from the family Hydrocharitaceae incurred agent damage and supported larval survival, of which three species were from the genus

*Lagarosiphon*. Survival rates for *L. muscoides*, *L. ilicifolius* and *L. major* were 39, 37 and 13.5% and the only non-target species that produced adults during paired choice-tests, were *L. major* and *L. ilicifolius*. None of these species was able to sustain *H. purcelli* populations for more than three generations during continuation tests.

Risk assessment indicated that non-target feeding and reproductive risk in the field from *H. egeriae* is low. In addition, *L. major* has its own specific herbivore (*H. lagarosiphon*) that persists in high numbers year-round that may outcompete *H. egeriae* (Martin *et al.*, 2013). Hybridization of biocontrol agents with related species has been recorded in four cases (see Havill *et al.*, 2012), and is an undesirable non-target effect (van Wilgen *et al.*, 2013). In an extensive systematic and ecological study of the genus *Hydrellia*, Deonier (1971) never encountered interbreeding of *Hydrellia* species, in either laboratory, or natural conditions. This suggests that hybridization of *H. egeriae* and native *Hydrellia* species in the field is unlikely. Despite larval mining and development on *V. spiralis* during no-choice tests, native range studies showed that *V. spiralis* is not included in the realised host range of *H. egeriae* (Cabrera Walsh *et al.*, 2013). Additionally, biocontrol seldom completely eradicates a weed (McFayden, 1998), and therefore, *E. densa* should always be available in the field to sustain agent populations.

*Lagarosiphon major*, *L. muscoides* and *V. spiralis* are well-known aquarium plants and are therefore of economic value due to their commercialization within the trade (Martin & Coetzee, 2011). However, in natural systems, *L. major*, *L. muscoides* and *V. spiralis* often become weedy, and in the case of the first two species, are considered noxious (Cook, 2004). According to the National Assessment red list of South Africa, all of the species used by *H. egeriae* during no-choice tests, with the exception of *H. verticillata*, are of least conservation concern (Cholo & Foden, 2006).

It is essential to consider the ecological characteristics of the target weed and non-target species during pre-screening analysis (Sheppard *et al.*, 2005). Pre-release surveys in South Africa showed that test plant species that co-occur with *E. densa* in the field, are *S. pectinata* and *P. pussilus* (section 1.2.3). Host-specificity testing showed that *H. egeriae* poses no threat to *Potamogeton* species, because no larval mining or development was recorded on any of these test species. This was an unexpected result considering *Hydrellia* spp. interactions with *Potamogeton* species from previous host-specificity testing conducted with *Hydrellia* spp. biocontrol agents in the past (Buckingham & Okrah, 1993; Bownes, 2015, Mangan, 2012). It is also possible that additional species could have been missed during surveys due to small population size, competitive exclusion, invisibility and seasonal variability, and therefore, the full spectrum of plants, and thus possible non-target species, in close proximity to *E. densa* may be underestimated. Nonetheless, risk analysis of *H. egeriae* suggests that the agent poses minimal to no threat to native species. Additionally, minimal damage to *L. major* or any other susceptible non-target species may be deemed an appropriate “trade-off” for the potential advantages of controlling *E. densa* in South African freshwaters.

In a review on the success of predicting non-target effects through relative performance scores, Paynter *et al.* (2015) found that incorrect risk predictions prior to agent release, was a result of asynchrony between the target weed and the biocontrol agent. This was particularly the case for seed-feeding agents that utilize ephemeral plant structures (Paynter *et al.*, 2015). Under normal conditions, *E. densa* should be available year-round and therefore, resource availability will not be a limiting factor for *H. egeriae* persistence.

Thus far, *H. egeriae* expressed a narrow host utilization range, and the probability of non-target effects in the field is minimal. It will be important to conduct host-specificity tests with species that could not be collected for testing during this study; *B. aubertii*, *O. exserta*,

*L. ilicifolius* and *L. verticillifolius*, as they are in the same clade as *E. densa* (Chen *et al.*, 2012). In addition to host-specificity, the release of ineffective agents should be avoided to prevent unnecessary expenditure and maintain the credibility of biocontrol as a management option. Potential agents should prove to be effective, i.e. inflict significant damage to reduce the fitness, reproduction and competitiveness of the weed (Harris, 1991; McClay & Balciunas, 2005; Sheppard, 2003; Morin *et al.*, 2009). Therefore, practitioners perform pre-release efficacy tests, which usually occur in conjunction with host-specificity trials (Balciunas, 2004), to establish the predicted damage capacity of the agent.

## Chapter 4: Impact study of *Hydrellia egeriae*

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### 4.1 INTRODUCTION

Like most natural resource management projects, biocontrol is expensive and requires long-term investments from stakeholders. Generally, a complete biocontrol programme requires between 10 to 20 years and initially, screening and pre-release studies require a minimum of three years (Kluge, 2000; van Driesche *et al.*, 2010). Regardless of the high initiation costs and extensive time-scale of biocontrol, reviews of its benefits have shown that it has positive benefit: cost ratios that grow over time and outweigh other control methods (McFayden, 1998; McConnachie *et al.*, 2003; Morin *et al.*, 2009; Lockwood *et al.*, 2010; van Wilgen & de Lange, 2011; van Klinken *et al.*, 2016). This is illustrated in the biocontrol of the floating aquatic weed, *A. filiculoides* (red water fern) in South Africa with the frond-feeding weevil, *Stenopelmus rufinasus* Gyllenhal (Coleoptera: Curculionidae). Costs associated with its biocontrol were valued at US\$7 976 per ha/year in the first year (1996) of the programme and US\$276 per year from 1996 to 2000. Within the first year of its release, the weevil caused a massive reduction in the aquatic weed, and after three years, the weed was no longer viewed as a threat (McConnachie *et al.*, 2003). The benefit: cost ratio for its control was 2.5:1 in 2000, and was estimated to increase to 13:1 in 2005 and 15:1 in 2010 (McConnachie *et al.*, 2003). In contrast, mechanical and chemical control were estimated at US\$ 1005 and US\$136 per ha/year, but due to the rapid regeneration of the weed, delivered no long term relief (McConnachie *et al.*, 2003). Other reviews of the benefits of biocontrol illustrated that management costs were 14% less than mechanical and 5% less than chemical control methods (van Wilgen & de Lange, 2011).. Benefits were mostly gained from the reduction in loss of water, grazing capacity and biodiversity (McFayden, 1998; Morin *et al.*, 2009; Lockwood *et al.*, 2010; van Wilgen & de Lange, 2011; van Klinken *et al.*, 2016).

Because biocontrol seldom eradicates a weed, agents that damage critical plant life traits (McEvoy & Coombs, 1999, Sheppard, 2003; Raghu *et al.*, 2006), or produce a source-sink imbalance and/or induce secondary infection or disease (Julien, 1991) should be selected. Plants have evolved many ways to tolerate, compensate or defend themselves from herbivory (Rosenthal & Kotanen, 1994; Schwachtje & Baldwin, 2008) and therefore, agent damage should offset these mechanisms (Mangan & Baars, 2016).

McClay & Balciunas (2005) proposed that an agent's predicted impact is the product of its climatic range, abundance and per-capita impact. Laboratory or greenhouse efficacy experiments only provide information on the response of plants to herbivory on an individual level. Therefore, these results are extrapolated to community levels with information on the agent's relative abundance and performance in its native range (Harris, 1991; McClay & Balciunas, 2005; Sheppard, 2003; Morin *et al.*, 2009).

In a review on the effectiveness of biocontrol, Clewley *et al.* (2012) concluded that beetles from the Curculionidae (weevils) and Chrysomelidae (leaf beetles) were most effective at reducing target plants and should be prioritized in future programmes. Weevils have been used extensively in terrestrial, floating and even submerged aquatic weed biocontrol programmes (Chapter 1) (Coetzee *et al.*, 2011a; Winston *et al.*, 2014). Yet despite the release of two weevils (*B. affinis* and *B. hydrillae*) for the control of *H. verticillata*, the most effective agent thus far has been the leaf-mining fly *H. pakistanae* (Doyle *et al.*, 2002).

A few members of the Hydrocharitaceae family have become invasive throughout the world (*H. verticillata*, *L. major*, *E. densa* and *E. canadensis*). Biocontrol programmes for these weeds have shown that *Hydrellia* species are frequently associated with submerged macrophytes from the Hydrocharitaceae family (Baloch *et al.*, 1976; Martin *et al.*, 2013; Cabrera Walsh *et al.*, 2013; Mangan & Baars, 2016). Compared to the needle-like leaves of

*C. caroliniana* and *M. spicatum*, species from the Hydrocharitaceae have lanceolate-shaped leaves that offer safe feeding niches for leaf-mining species. Although leaf-mining damage is less conspicuous (Deonier, 1971; Mangan & Baars, 2016), their feeding behaviour is of significant value for the biocontrol of weeds from the Hydrocharitaceae. Additionally, they possess favourable characteristics that enhance biocontrol; high female fecundity, small body size, multiple overlapping generations (multivoltine), short life cycles and completion of their life cycle on the target weed (Crawley, 1989; Harris, 1991; Julien, 1991; Cuda *et al.*, 2008; Mangan & Baars, 2016).

The major impact of *Hydrellia* species on their host plants is the direct consumption of photosynthetic tissue that reduces the host plant's biomass and photosynthetic capacity (Baloch *et al.*, 1976; Doyle *et al.*, 2002; Martin *et al.*, 2013; Cabrera Walsh *et al.*, 2013; Mangan & Baars, 2016). They also induce indirect damage by fungi and disease-induced stress and passively spread pathogen propagules (Shabana *et al.*, 2003). Damaged tissues of aquatic weeds that lack genetic diversity are more susceptible to secondary infection and deteriorate rapidly when exposed to chronic damage (Buckingham, 1994; Mangan & Baars, 2016). In addition, *Hydrellia* leaf-mining releases resources within the ecosystem that enhance native species growth and competitiveness (Louda, 1994; Doyle *et al.*, 2007; Grutters *et al.*, 2016).

Traits that contribute to the invasiveness of submerged macrophytes include dispersal through fragmentation, rapid growth rate, short life cycle and phenotypic plasticity (Yarrow *et al.*, 2009; Thomas *et al.*, 2015). *Egeria densa* lacks storage structures, like tubers and turions and only reproduces vegetatively in South Africa (Harris, 1991; Haramoto & Ikusima, 1988; Netherlands Department of Environmental Science, 2014). Therefore, for successful biocontrol, a potential agent should inflict sufficient damage to reduce the growth rate of *E.*

*densa*, open up dense canopies to benefit native vegetation regrowth and reduce the viability of its propagules.

In its native range, *H. egeriae* was recorded on 89.58% of sampled branches. Larvae usually mine in the top 15 to 25cm of *E. densa* stands, where the leaves are younger and more nutritious (Cabrera Walsh *et al.*, 2013). Under normal field conditions, 80-100% of sampled plants were actively mined and even completely mined under high *H. egeriae* abundances (Cabrera Walsh *et al.*, 2013). Post-release evaluation showed that *H. pakistanae* only reached intermediate larval abundances on *H. verticillata* (2 000 to 4 000 immatures per kg fresh weight) in the field (Doyle *et al.*, 2002; Grodowitz *et al.*, 2003). Mesocosm tank experiments showed that intermediate *H. pakistanae* abundances had a similar effect on light saturated photosynthesis than high agent abundances with a reduction of 30 to 40% and 40%, respectively (Doyle *et al.*, 2002).

The objective of this study was to determine the effect of *H. egeriae* mining on *E. densa* fitness. Considering the information of impact and post-release evaluations of other *Hydrellia* biocontrol agents (Grodowitz *et al.*, 1993; Mangan & Baars, 2016) and the nature of the damage incurred, this study was conducted for three consecutive generations at intermediate and high larvae abundances, to infer potential damage to *E. densa* in the field.

## 4.2 MATERIAL AND METHODS

### Multiple generation larval feeding

The effect of sustained larval damage on plant growth and side shoot growth was determined in a multiple generation damage experiment. In total, 90 apical *E. densa* shoots, 15cm in length, were planted in 3cm x 5cm vials. Two shoots were planted in each vial to ensure that plant material was not a limiting factor for larval feeding and survival. This resulted in 45 replicates. Plants were then placed in 600L tanks connected to a flow-through system in a polytunnel at the Waainek Biological Control Research Facility in Grahamstown. The nutrient stock solution recommended by Smart & Barko (1985) was added to the tanks. After a growth period of 21 days, the 45 vials with *E. densa* shoots were brought into the quarantine facility at Rhodes University, Grahamstown. The plants were placed in 600ml transparent containers (24cm x 7.5cm). Neonates were transferred to the plants at different abundances; 0 (control), three (intermediate) and five (high), with 15 replicates per treatment. Larvae were transferred using the same technique as in Chapter 3. Larvae were left to feed and checked for pupariation after 17 days.

To determine the effects of one generation of *H. egeriae* larvae mining on *E. densa*, five replicates per treatment were uprooted after the first generation. Puparia were removed, by excising the leaf containing the puparium. The number of puparia and leaves with mining scars were counted and recorded. The length of the shoots as well as the number and lengths of side shoots and roots were recorded in centimetres (cm). The shoots and roots of each replicate were separated, washed and placed into a brown envelope. Envelopes were frozen for 48 hours to kill any unseen larvae and oven dried (ProLab). Plant material was subsequently weighed with a micro balance (Adventurer® Ohaus) to determine the dry biomass of the shoots and roots.

Following the pupariation of the first generation of larvae, the remaining plants were treated with a new batch of neonate larvae at the same neonate density regimes to imitate a second generation of larval feeding; 10 replicates per treatment. After 17 days, five replicates per treatment were uprooted and processed as before to determine the effect of two generations of leaf-mining on *E. densa* plants. A new batch of neonate larvae was applied to the remaining replicates at the same larval abundances, to imitate a third generation of feeding. Upon pupariation, replicates were treated and processed as described above.

### Statistical analysis

The mean value of plant parameters (shoot length, shoot growth, number of side shoot, side shoot length, root biomass) of the two shoots per vial were calculated and used for statistical analysis. Statistical analyses were conducted in the R environment (version 3.2.3; R Development Core Team, 2014; available at <http://cran.r-project.org>) using R Studio (version 0.98.1103). The distribution of all dependent variables was tested for normality with the Shapiro Wilk test. The effect of sustained larval mining at different abundances on plant parameters was determined with the use a generalized linear model (GLM). Due to the non-normal distribution of the dataset, a log-link distribution was used for all the plant parameters, and a Poisson distribution link function was applied on the count data (number of mined leaves and mean larvae survival percentages). *Post hoc* pair-wise comparisons (least square means) were conducted for variables that had significant *P*-values ( $P < 0.05$ ). Graphs were produced in Microsoft Excel 2013.

### 4.3 RESULTS

Multiple generation mining at different larval abundances had a significant effect on the number of leaves *H. egeriae* damaged ( $Wald X^2 = 232.73$ ;  $df: 23$ ;  $P < 0.001$ ) and the mean percentage of larvae that pupariated ( $Wald X^2 = 365.35$ ;  $df: 25$ ;  $P < 0.001$ ) (Table 4.1). Significantly more leaves were damaged at high larval abundances during development of the first generation ( $F_1$ ) ( $Z = -12.27$ ;  $P < 0.001$ ) and second generation ( $F_2$ ) ( $Z = -7.17$ ;  $P < 0.001$ ), than intermediate larval abundances (Table 4.1). Intermediate larval abundances progressively attained similar damage levels by the end of the experiment with 132 mined leaves, compared to 145 leaves for high larvae abundances.

During larval-mining of the  $F_1$ , abundant food resources allowed 80% of individuals in the high larval abundance to reach the adult stage. Subsequently, survival rates decreased significantly to 56% for the  $F_2$  individuals ( $Z = 4.57$ ;  $P < 0.001$ ) and 48% for the  $F_3$  individuals ( $Z = 6.25$ ;  $P < 0.001$ ), indicating that intraspecific competition and density-dependent population regulation occurred at high larval abundances. Survival rates for intermediate larval abundances were significantly lower ( $Z = -6.51$ ;  $P < 0.001$ ) than high larval abundances for the  $F_1$  with only 47% of larvae surviving to the adult stage. However, survival rates increased significantly for the  $F_2$  ( $Z = -7.62$ ;  $P < 0.001$ ) and the  $F_3$ , ( $Z = -3.97$ ;  $P = 0.002$ ) with 86% and 66% survival, respectively. These values were also significantly higher than the survival rates demonstrated for high larvae abundances for the  $F_2$  ( $Z = 5.71$ ;  $P < 0.001$ ) and the  $F_3$  ( $Z = 3.70$ ;  $P = 0.002$ ); suggesting that the plant material provided in this study could only sustain intermediate larvae abundances for three consecutive generations.

Table 4.1: Effect of increasing larval abundances on percentage ( $\pm$ SE) *Hydrellia egeriae* survival.

Larval abundance	Generation	<i>n</i>	Mean larval ( $\pm$ SE) survival <sup>a</sup>	Number ( $\pm$ SE) of mined leaves
Control		4	-	-
Intermediate	F1	5	46.67 $\pm$ 4.80a	30.80 $\pm$ 13.33a
High		5	80.00 $\pm$ 7.16b	96.00 $\pm$ 8.94b
Control		5	-	-
Intermediate	F2	5	86.67 $\pm$ 19.54c	109.20 $\pm$ 8.16c
High		5	56.00 $\pm$ 8.75d	162.40 $\pm$ 4.00d
Control		4	-	-
Intermediate	F3	4	66.67 $\pm$ 21.06e	132.25 $\pm$ 13.61d
High		5	48.00 $\pm$ 22.73d	145.60 $\pm$ 13.56d

<sup>a</sup>Survival was counted as the number of larvae that pupariated

Means ( $\pm$ SE) within columns followed by the same letter are not statistically different ( $P < 0.05$ , *post hoc* pair-wise comparisons).

The higher order interaction of different larval treatments and multiple generations on shoot length growth was significant (Fig. 4.1) ( $Wald X^2 = 238.46$ ,  $df = 33$ ,  $P < 0.001$ ), but no statistical difference was obtained between treatments and generations.

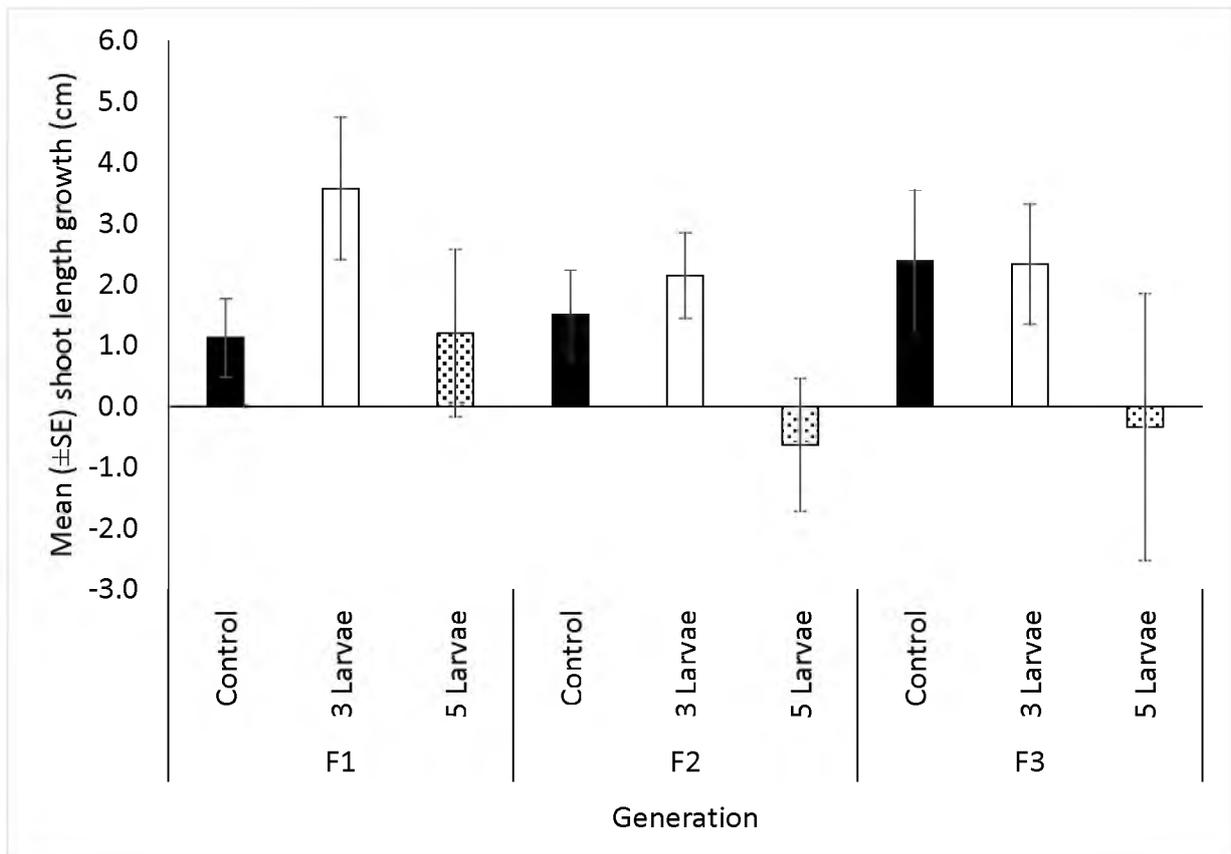


Figure 4.1: Mean ( $\pm$ SE) shoot length growth of *Egeria densa* subjected to no, intermediate (3 larvae/shoot) and high (5 larvae/shoot) larval density feeding for three consecutive generations. ( $Wald X^2 = 238.46$ ,  $df = 33$ ,  $P < 0.001$ ). Error bars represent S.E.

The higher order interaction of leaf-mining for three generations had no significant effect on the shoot biomass of *E. densa* ( $Wald X^2 = 0.04351$ ,  $df = 33$ ,  $P = 0.99$ ). However, pair wise comparisons within larval treatments, indicated that the shoot biomass of undamaged *E. densa* was significantly affected by generation time (Fig. 4.2) ( $Wald X^2 = 0.04351$ ,  $df = 33$ ;  $P = 0.017$ ). Control shoots weighed significantly more after larval mining of the F<sub>3</sub>, compared to larval mining of the F<sub>1</sub> ( $Z = -2.49$ ;  $P = 0.033$ ) and F<sub>2</sub> ( $Z = -3.32$ ;  $P = 0.002$ ). In the absence of herbivory, *Egeria densa* doubled its shoot biomass from 0.0684g to 0.1635g within one generation (17 days).

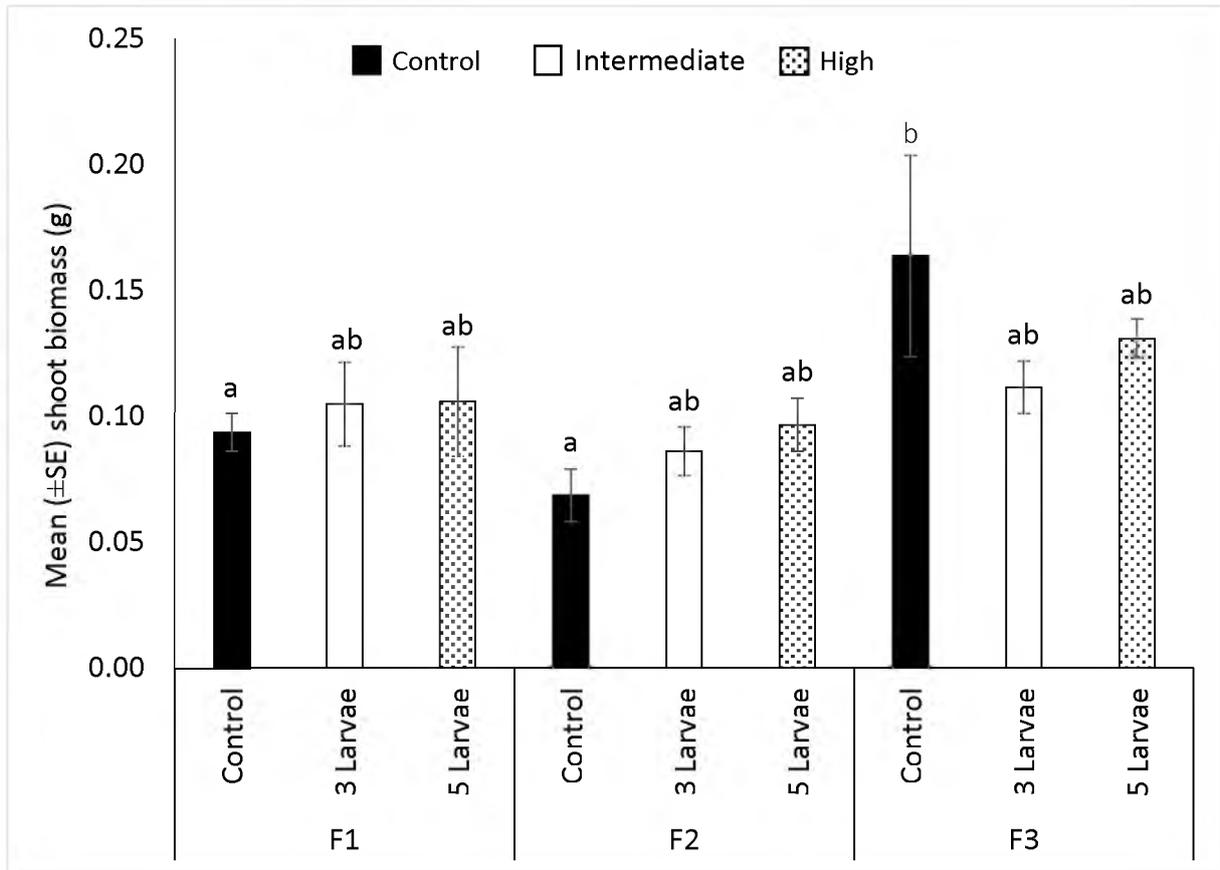


Figure 4.2: *Egeria densa* shoot biomass ( $\pm$ SE) for three consecutive generations (F1-F3) with no, intermediate and high larval density feeding ( $Wald X^2 = 0.04351$ ,  $df = 33$ ,  $P = 0.99$ ). Error bars represent S.E. Different letters above means, indicate statistical difference within larval treatments.

The number of side shoots ( $Wald X^2 = 12.35$ ,  $df = 33$ ,  $P = 0.878$ ) (Fig. 4.3) and root biomass ( $Wald X^2 = 0.00013$ ,  $df = 33$ ,  $P = 0.99$ ) (Fig. 4.5) were not significantly affected by consecutive *H. egeriae* leaf-mining at various abundances. Side shoot length was significantly affected by leaf-mining for three consecutive generations ( $Wald X^2 = 132.32$ ,  $df = 33$ ,  $P < 0.001$ ) (Fig. 4.4). Under high larval abundances, larvae mined young nutritious leaves located in the crown of growing sides shoots, resulting in side shoots that were significantly shorter than those of undamaged plants for the F<sub>2</sub> of leaf-mining ( $Z = 2.37$ ;  $P = 0.046$ ) (Fig. 4.4). However, *E. densa* compensated for herbivory during the F<sub>3</sub> of herbivory, as side shoots were not significantly shorter than those after F<sub>2</sub> of leaf-mining ( $Z = -0.66$ ;  $P = 0.78$ ) at high larval densities. Additionally, side shoot length at high larval densities were not

significantly shorter than plants treated with no ( $Z = 0.96$ ;  $P = 0.59$ ) and intermediate ( $Z = 0.66$ ;  $P = 0.78$ ) larval densities during the  $F_3$  of leaf-mining.

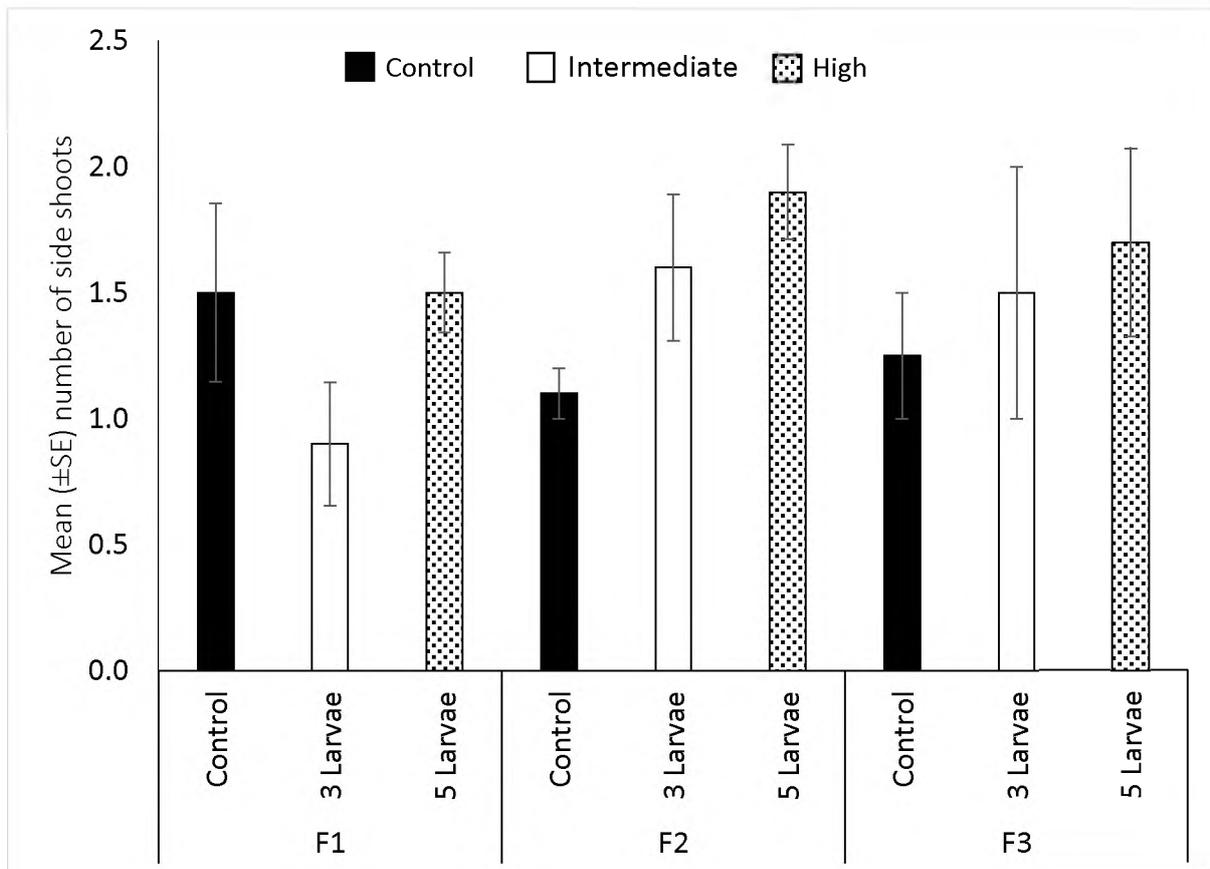


Figure 4.3: Mean ( $\pm$ SE) number of side shoots produced during three consecutive generations of no, intermediate and high larval density feeding. ( $Wald X^2 = 12.35$ ,  $df = 33$ ,  $P = 0.878$ ). Error bars represent S.E.

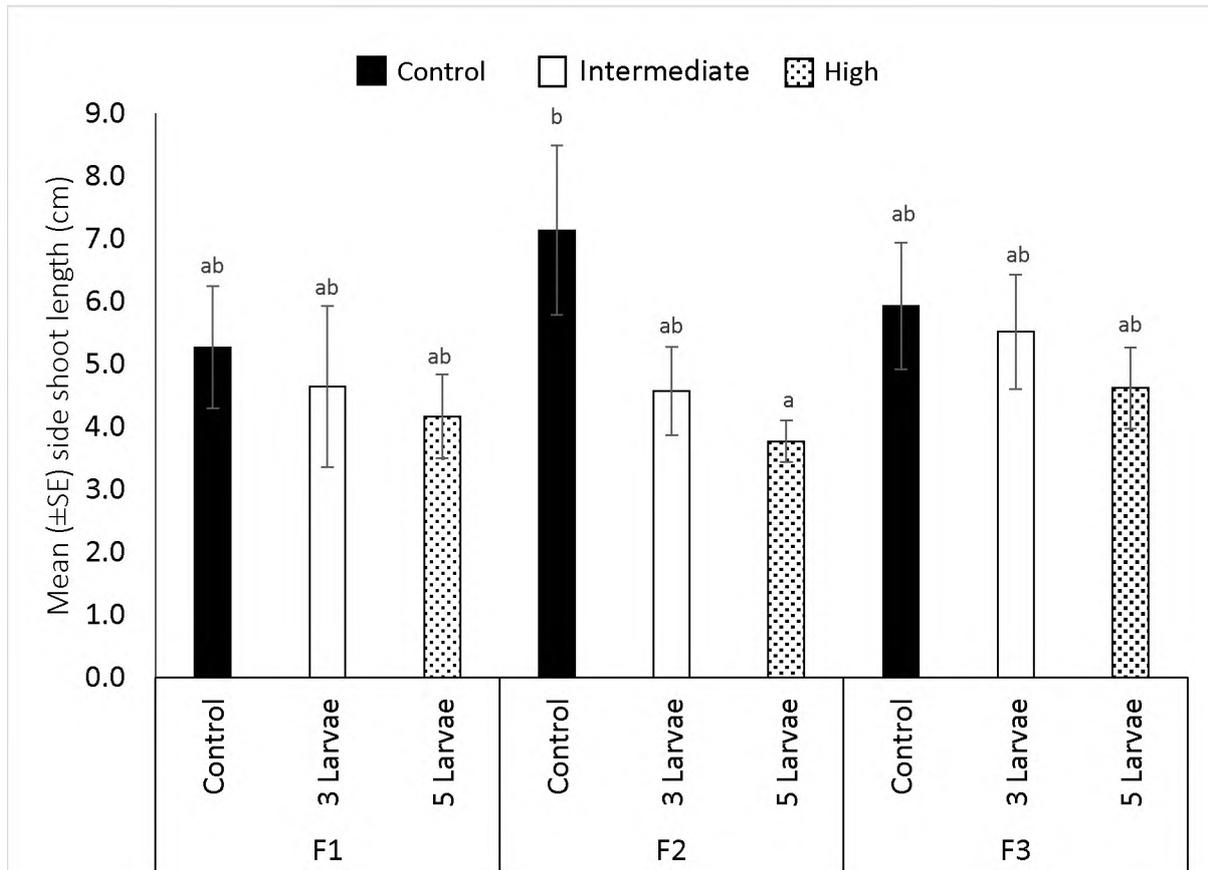


Figure 4.4: Mean ( $\pm$ SE) length of *Egeria densa* side shoots when exposed to no, intermediate and high larval density feeding for three consecutive generations. ( $Wald X^2 = 132.32$ ,  $df = 33$ ,  $P < 0.001$ ). Error bars represent S.E., different letters above means indicate statistical differences within larval treatments.

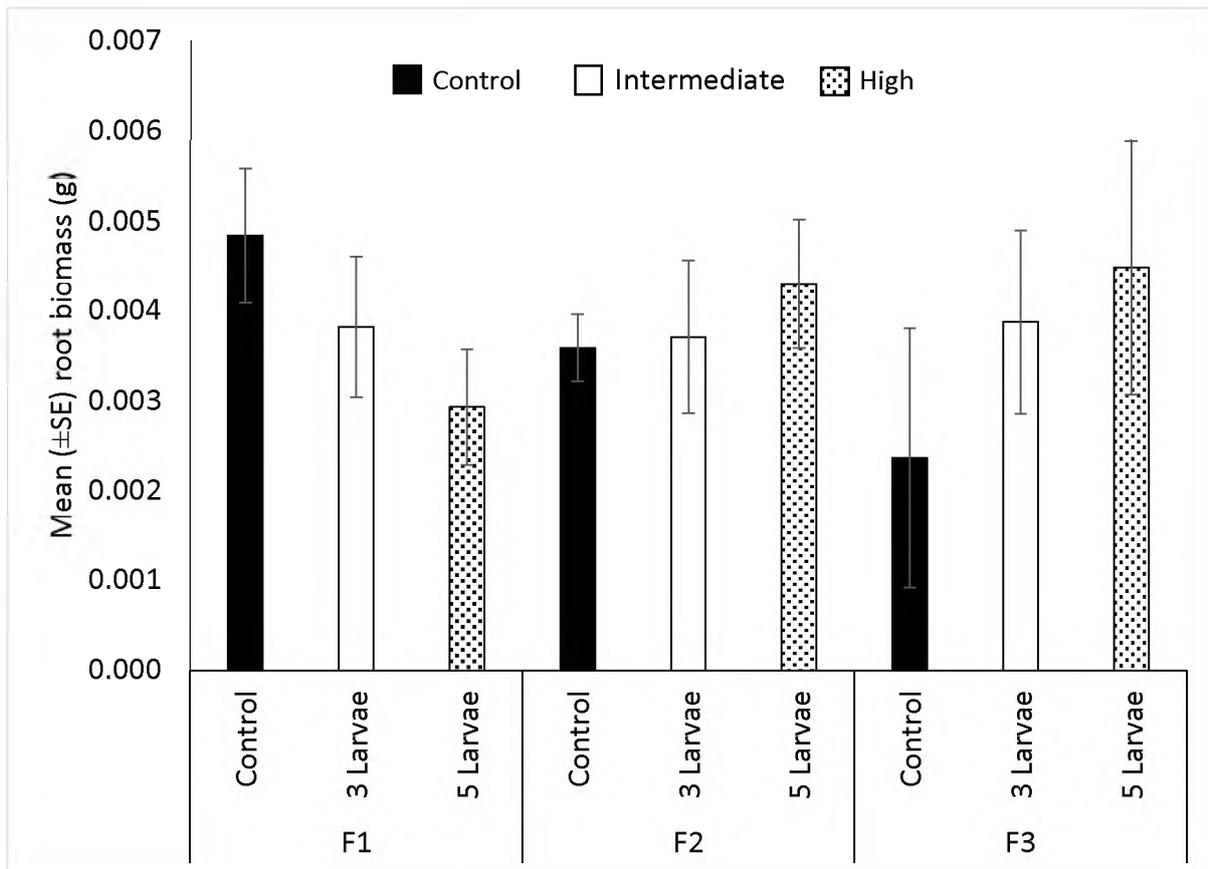


Figure 4.5: *Egeria densa* root biomass ( $\pm$ SE) for three consecutive generations with no, intermediate and high larval density feeding. ( $Wald X^2 = 0.00013$ ,  $df = 33$ ,  $P = 0.99$ ). Error bars represent S.E.

From these results, it is evident that *E. densa* has the ability to rapidly increase its biomass under laboratory conditions within a short period of time. Only high larval abundances inflicted significant damage on *E. densa* within the time frame of this study. Increased resource demand under high larval abundances resulted in side shoots that were 49% shorter than control plants after the F<sub>2</sub> of leaf-mining. However, *E. densa* compensated for herbivory during the F<sub>3</sub> of leaf-mining. The overall biomass (shoot and root) of *E. densa* and the number of side shoots produced was not significantly affected by agent feeding during this study. However, accumulative damage by intermediate abundances suggests that given sufficient time, *H. egeriae* has the capacity to reach damaging levels, and thus, may have a significant impact on the overall fitness of *E. densa*.

### 4.3 DISCUSSION

Invasive alien plants often express higher growth, reproduction and overall competitive ability in their introduced range. From this study, it is evident that *E. densa* has the ability to double its biomass every 17 days under laboratory conditions. Field surveys in KwaZulu-Natal, Western Cape and Eastern Cape provinces showed that *E. densa* canopy densities ranged between 80% and 100% at majority of the sites (Martin & Smith, unpubl. data). Weeds that form dense monoculture stands with high intra- and interspecific competition are more susceptible to herbivory (Crawley, 1989). Following herbivory, individual plant recovery depends on available resources, but in the presence of intense weed competition, resource availability is reduced, plant regrowth diminished and plants become stressed and deteriorate (Louda, 1994; van Driesche & Bellows, 1996; Charudattan *et al.*, 2008). Progressively, diminished canopy cover of invasive alien plants releases resources that benefit native vegetation regrowth and competitiveness (Doyle *et al.*, 2007). The lack of statistical differences between generational larval abundances for shoot growth, despite the overall significant impact of consecutive leaf-mining is the result of high variance within the dataset. This is attributed to the disparate shoot growth patterns for the different larval abundances. Under high larval abundances, shoot growth was negative during F<sub>2</sub> and F<sub>3</sub> leaf-mining, but positive for the control and intermediate larval treatments. Additionally, under high larval abundances, *H. egeriae* stunted *E. densa* side shoot length, but only during the F<sub>2</sub> of larval-mining. Given sufficient time, negative shoot growth and reduced branching may reduce weed canopies in the field. This was illustrated in the long-term post release evaluation of *H. pakistanae* in Lake Seminole, Florida, USA. Eight years after its first release, chronic leaf-mining reduced *H. verticillata* canopy densities and increased the growth, and subsequent abundance of native pondweeds (*Potamogeton* spp.) and water-nymphs (*Najas* sp.) in freshwater systems (Grodowitz *et al.*, 2003).

Chronic herbivory is favourable and has proven to significantly suppress weed growth, survival, reproduction and successional success in both terrestrial and aquatic environments (Crawley, 1989; Louda, 1994; Grodowitz *et al.*, 2003; Hobb *et al.*, 2016; Mangan & Baars, 2016; Wang *et al.*, 2016). *Hydrellia egeriae* expressed the potential to inflict significant damage to *E. densa* at intermediate abundances. Although no significant impact for intermediate larvae abundances on any of the target weed growth parameters was observed during the study, the trend of an increase in damaged leaves over time is promising. Progressively, intermediate larval abundances attained similar damage levels as high abundances. In many cases, biocontrol agents require multiple generations of feeding before quantifiable impacts are apparent (McClay & Balciunas, 2005; Hogg *et al.*, 2016). In a multigenerational impact study (~150 days) with the phloem feeding psyllid *Arytinnis hakani* Loginova (Hemiptera: Psyllidae) for the control of *Genista monspessulana* (L.) L.A.S. Johnson (Fabaceae), only three out of 28 trees died after the first generation (~50 days), whereas 23 trees died after the third generation (Hogg *et al.*, 2016). Cumulative leaf damage may have affected *E. densa* fitness, but due to the termination of the study after the third generation, the subsequent effects of the leaf damage on plant growth was not observed. In a similar study, the effect of *H. lagarosiphon* mining on the subsequent growth of *L. major* fragments was evaluated. *Lagarosiphon major* shoots 22cm in length were exposed to one generation of low (1), and high (5) agent density mining. Shoots were subsequently planted individually in pots with lake sediment and grown for 134 days in 600L circulation tanks under ideal conditions (Mangan & Baars, 2016). After 90 days of growth, shoots of low and high larval abundances were 30 and 50% shorter than control plants. Additionally, side shoot growth, root biomass and shoot biomass of high larval abundances were 50, 55 and 70% less than that of control plants.

#### *Limitations of the study*

From this study, it is evident that resource limitation had a significant effect on *H. egeriae* survival and potentially its damage capacity. Shoots between 16 and 30cm could not sustain high abundances of larvae for multiple generations, given the reduction in survival rate after the F<sub>2</sub> of leaf-mining. In a plant resource availability study, Mangan & Baars (2016) illustrated that four was the highest number of *H. lagarosiphon* larvae that an 11cm and 22cm *L. major* shoot could support. In its native range, *H. egeriae* generally mines in the top 15 – 25cm of *E. densa* shoots, but under high abundances, plants are completely mined (Cabrera Walsh *et al.*, 2013). Because fresh plant material was not added after each generation, larvae had no opportunity to move to new feeding niches, and therefore, this quarantine-based study may underestimate the damage capacity of *H. egeriae*. Under natural conditions, larvae will have the opportunity to move to adjacent *E. densa* plants or mine on older leaves deeper within the water column. For large weed infestations, resource availability should not be a limiting factor for survival, at least during the initial phase of the agent's establishment; allowing initial agent establishment and population build-up. Despite the availability of plant material in the field, vertebrate predation has been associated with *Hydrellia* species in the past (Cabrera Walsh *et al.*, 2013; Halwart *et al.*, 2012; Letsinger *et al.*, 2013). Native range studies showed that *H. egeriae* adult emergence was reduced by 28, 51 and 62% when exposed to fish, leeches and damselflies nymphs, respectively (Cabrera Walsh *et al.*, 2013). Letsinger and colleagues (2013) found that vertebrate such as fish, frogs, birds and lizards also predate on adult *H. philippina* in the Philippines.

Under natural conditions, it is expected that *H. egeriae* will increase in population size with each generation and will also have multiple overlapping generations. Collectively, these factors increase the amount of damage incurred to *E. densa* for each generation in the field. For example, in its native range, *H. egeriae* immature densities ranged from approximately 10 to 800 immatures per 50 *E. densa* shoots (50cm in length) over a four year

period (Cabrera Walsh *et al.*, 2013). During this study, both these factors were excluded, which explains the lack of significant differences in damage incurred to *E. densa*. It is also apparent that the difference in larval densities was not significant, which contributes to a lack of significant differences between plants exposed to intermediate and high larval densities.

*Hydrellia* leaf-mining inflicts direct damage to its target weed (Baloch *et al.*, 1976; Doyle *et al.*, 2002; Martin *et al.*, 2013; Cabrera Walsh *et al.*, 2013; Mangan & Baars, 2016), but also induces secondary infection. In a bioassay, Shabana *et al.* (2003) investigated the pathogenic effect of fungi within the introduced range of *H. verticillata*. Species were collected directly from the target weed or from the soil and water in close proximity to the weed. This included F71PJ *Acremonium* sp., F531 *Cylindrocarpon* sp., F542, *Botrytis* sp., and F964 *Fusarium culmorum* [Wm. G. Sm.] Sacc. Plants were initially exposed to *H. pakistanae* herbivory and subsequently inoculated with the fungal species. Results showed that fungal attack increased the level of plant damage by 1.6, 2.8 and 3.0 fold. For example, *F. culmorum* increased the percentage shoot damage from 23.75% and 35% to 76% and 97.5% for *H. verticillata* shoots 9cm and 12cm in length (Shabana *et al.*, 2003). Therefore, *E. densa* damage in the field could realistically be enhanced by secondary infection.

Although *H. egeriae* significantly reduced the length of side shoots under high agent abundances, further investigation is necessary to determine the viability of mined plants from all damage levels. Post-release evaluation of *H. pakistanae* in the USA indicated that leaf-mining increases fragmentation of *H. verticillata* (Owens *et al.*, 2008), while *H. lagarosiphon* reduces *L. major* buoyancy (Mangan & Baars, 2016), which may enhance anchorage of settling target weed fragments. However, impact studies show that mining by both *Hydrellia* spp. reduces the vigour and colonization potential of damaged propagules (Owens *et al.*, 2008; Mangan & Baars, 2016). *Hydrilla verticillata* shoots that were replanted and grown for 30 days, following *H. pakistanae* exposure, exhibited significant reductions in root growth,

anchorage, shoot length, side shoot production and biomass (Owens *et al.*, 2008). Under high damage levels (70% to 100% of a 20cm shoot), shoots produced no roots, experienced negative shoot growth and an 83% reduction in biomass, compared to undamaged plants. For intermediate damage levels (40% to 60%), there was a two-fold reduction in settling and a 68% reduction in biomass. Additionally, side shoot production of intermediate and high damaged shoots was reduced by 75% (Owens *et al.*, 2008).

For successful biocontrol, agents should inflict significant damage to their target weed during vulnerable life stages (Morin *et al.*, 2009). Knowledge of the phenology of the target weed is important for effective weed management as it allow practitioners to execute control actions when plant carbohydrate storage is at its lowest (Madsen & Owens, 1998). Pennington & Systma (2009) investigated *E. densa* total non-structural carbohydrate (TNC) patterns at two sites in California, USA. *Egeria densa* TNC concentrations were lowest early in the growing season and highest during autumn for one site. However, the same pattern in TNC was not evident from the second site as lowest TNC concentrations were recorded during late spring to early summer, and high concentrations during late summer until spring. This suggests that *E. densa* exerts high phenotypic plasticity, despite its low genetic diversity (Pennington & Systma, 2009), and that TNC patterns are less predictable. *Hydrellia egeriae* population growth parameters are predicted to increase with warmer temperatures (Chapter 2). This suggests that the level of *H. egeriae* damage should be greater during warmer seasons when *E. densa* growth is optimal (Haramoto & Ikusima, 1988; Yarrow *et al.*, 2009). Nonetheless, temperatures above 35°C may result in *Hydrellia* larval mortalities (Grodowitz *et al.*, 2003; Cuda *et al.*, 2008).

Considering the multi-dimensional impacts expressed by other *Hydrellia* biocontrol agents on their host plants, this study clearly provides preliminary information regarding the damage capacity of *H. egeriae*. To fully understand the scale of *H. egeriae* herbivory on its

host plant, impact studies have to incorporate characteristic such as a growing population for each generation, multiple overlapping generations, conduct impact studies for longer ecological periods, include secondary infections, and investigate the fitness and settling behaviour of damaged plant fragments.

This study illustrated that *H. egeriae* has the potential to inflict significant damage to vital plant parameters. However, there are many factors that could influence the establishment and performance of *H. egeriae* in the field (e.g., parasitism, fish predation), and therefore, it is necessary to consider such factors to determine the overall potential of *H. egeriae* as a biocontrol agent for *E. densa* in South Africa.

## Chapter 5: General Discussion

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### 5.1 INTRODUCTION

This thesis presents the findings of a pre-release assessment of the potential of *H. egeriae* as a biocontrol agent for *E. densa* in South Africa. The objectives of this study were to establish the life history, host-specificity and impact of *H. egeriae* on *E. densa*. Results illustrated that *H. egeriae* has high female fecundity and the ability to reproduce rapidly, which are both favourable attributes for mass-rearing and rapid population growth during field releases (Chapter 2). Subsequently, host-specificity trials illustrated that *H. egeriae* has a host range restricted to *E. densa*, and poses little feeding and reproductive risks to non-target species in the field (Chapter 3). Finally, impact trials showed that *H. egeriae* has the potential to incur significant damage to *E. densa*, and more experiments are required to establish the impact of a growing population with multiple overlapping generations to *E. densa* growth and vigour (Chapter 4). The next step in this biocontrol programme is to apply for permission to release the agent, and if granted, to mass-rear healthy agent populations for field release (Zimmermann *et al.*, 2013).

Pre-release assessment of a biocontrol agent reduces the risk of undesirable outcomes following its release, and also predicts the efficacy of the agent in the field (Harris, 1991; McClay & Balciunas, 2005; Morin *et al.*, 2009). Predictions are made from laboratory tests and interpreted in view of native range studies. However, the absolute success of biocontrol depends on effective mass-rearing and field releases of the agent. It also relies on the many biotic and abiotic factors that drive populations within the recipient community and the ability of the biocontrol agent to function within these conditions (Morin *et al.*, 2009; Martin, 2013; Seastedt, 2014). Therefore, a review of all the factors that may influence the success of

the biocontrol of *E. densa* in South Africa, should permission to release *H. egeriae* be granted, are discussed.

## 5.2 MASS REARING CONSIDERATIONS

The aim of mass-rearing is to produce high numbers of high quality agents for field-releases within a hygienic, disease-free environment (Julien & White, 1999; Freedman *et al.*, 2001; Harms & Grodowitz, 2009). Mass-rearing should also be cost effective (Leppla & Ashley, 1989). It requires proper equipment, high maintenance and high quality plants (Julien & White, 1999). One of the challenges for mass-rearing is to maintain the genetic integrity of the culture, e.g. culturing insects that are adapted to laboratory conditions often makes them unsuitable for field conditions (Julien & White, 1999; Harms & Grodowitz, 2009).

Mass-rearing techniques for *H. egeriae* will be based on those used in the USA for *H. pakistanae* and *H. balciunasi* (Harms & Grodowitz, 2009). Initially, *Hydrellia* biocontrol agents were mass-reared in a greenhouse that was labour intensive and time-consuming. Larvae were held in 3L containers with *H. verticillata* sprigs that were replaced weekly, emerging adults had to be removed from immature containers every day and placed into oviposition chambers. Eggs were counted weekly and transferred to immature containers. Additionally, separate containers were used for each generation. Costs to rear one fly were \$0.05. Later, field mass-rearing was used, whereby individuals were reared in 0.0405 hectare ponds, with growing plants (Harms & Grodowitz, 2009). The uses of actively growing plants for mass-rearing are particularly beneficial for insects that are external leaf-feeders, sap-feeding insects and leaf miners. This is because plant material lasts longer, resulting in decreased immature handling and labour (Julien & White; 1999). Pond rearing for *H. pakistanae* and *H. balciunasi* increased the number of individuals reared at a substantially lower cost of \$0.02 per fly. For example greenhouse mass-rearing yielded approximately

630 000 individuals over a 3 year period, whereas approximately 12 million individuals were field-reared in 1 year (Harms & Grodowitz; 2009).

For this programme, mass-rearing will occur in the flow through systems in a temperature controlled polytunnel located at the Waainek Research Facility. Flow through systems creates favourable conditions for healthy, fast growing *E. densa* on which flies will be reared. Production of *H. egeriae* will be counted on a monthly basis, allowing maximum numbers of agent releases without decimating the population. Specifically, 10 *E. densa* shoots (15cm) will be randomly collected from each rearing tank and the number of larvae and puparia per shoot will be recorded (Freedman, pers. comm.).

Because *Hydrellia* spp. adults are polyphagous and female fecundity is directly affected by nutrition, adult diet during mass-rearing is important (Wheeler, 1996). Generally, *Hydrellia* spp. is reared on a traditional diet composed of yeast/hydrolysate diet (Chapter 2; Freedman *et al.*, 2001). Mangan *et al.* (2015) reported that *H. lagarosiphon* population growth parameters were significantly improved with an insect derived diet, compared to the traditional diet. For example, the net reproductive rate showed a threefold increase from 11.5 to 33.1 and the population doubling time decreased by 30% from 16.2 days to 11.4 days with the addition of *D. melanogaster* to the culture's diet. Using these insect derived nutritional diets could improve culturing techniques for *H. egeriae* significantly and will benefit mass rearing efforts, potentially saving time and reducing associated costs (Mangan *et al.*, 2015).

### 5.3 RELEASES

#### Pre-screening bottlenecks

Many studies describe the importance of a diverse genetic pool in a biocontrol agent as this may limit the agent's ability to establish within the novel environment (Franks *et al.*, 2011; Taylor *et al.*, 2011; Fauvergue *et al.*, 2012; Zepeda-Paulo *et al.*, 2016). Generally, pre-

screening analysis requires a minimum of three years (Kluge, 2000; van Driesche *et al.*, 2010), during which agents are subjected to a series of bottlenecks that includes agent selection in its native range, mortality during importation, and inbreeding during cultivation in quarantine (Franks *et al.*, 2011; Taylor *et al.*, 2011; Zepeda-Paulo *et al.*, 2016). In some cases, agent populations are kept in quarantine for up to twelve years (Bownes *et al.*, 2010), which can result in multiple bottlenecks that reduce population diversity (Leberg & Firmin, 2008). For example, *Eccritotarsus catarinensis* Carvalho (Hemiptera: Miridae) from Brazil, a biocontrol agent for *E. crassipes*, was reduced to one gravid female during culturing in quarantine (Taylor *et al.*, 2011). Studies on the genetic diversity of biocontrol agents for weeds have shown that once released, agents may increase their genetic diversity due to the availability of abundant resources (Nei *et al.*, 1975), as in the case of *E. catarinensis*, where the genetic variation of the introduced population was significantly higher than that of the quarantine population, despite the initial bottleneck.

*Hydrellia egeriae* was imported into quarantine at Rhodes University, Grahamstown, from the Exotic and Invasive Weeds Research (EIW) facility of the Agricultural Research Service in California, USA. The founder culture was initiated in May, 2013 from one shipment comprised of individuals from four different populations in Argentina (John Herr, pers. comm.; Guillermo Cabrera Walsh, pers. comm.). Ultimately, *H. egeriae* in South Africa has been in culture in quarantine for almost four years, which could have an effect on its genetic diversity and subsequent performance, but this remains to be tested.

#### Release effort

The level of effort (number of releases, size of releases) during releases is central to the success of biocontrol programmes (Zepeda-Paulo *et al.*, 2016). This was illustrated in the biocontrol of *H. verticillata* in the USA. In a review on the success of two *Hydrellia* biocontrol agents, Grodowitz *et al.* (2003) concluded that effective mass-rearing was critical

for the establishment success of the agents. For example, due to the difficulty in mass-rearing *H. balciunasi*, fewer field releases were made, which resulted in reduced field establishment. Overall, less than 300 000 *H. balciunasi* individuals were released compared to over 3 million *H. pakistanae* individuals, which established with great success in the field. Results from Chapter 2 illustrated that *H. egeriae* is able to build up large populations within a short period of time, which is typical for ephydrid species. Additionally, population growth parameters are predicted to be optimal at temperatures between 25°C and 30°C, suggesting that mass-rearing will be successful under the same temperature range, and that high numbers of individuals can be reared efficiently, allowing for extensive agent releases.

#### Release protocol

Successful establishment of agents relies on the interplay of 1) stochastic demography: the likelihood of changes in mortality and birth rates, 2) Allee effects: specific density-dependent mechanisms that reduce agent fitness and 3) stochastic environment events: for example a severe storm following a release event (Grevstad, 1999; Memmott *et al.*, 2005; Fowler *et al.*, 2008). Grevstad (1999) illustrated that multiple small releases increase the probability of establishment within variable environmental conditions (environmental stochasticity); if the environment conditions are stable, but an Allee effect is present, fewer release events with larger numbers of individuals are preferable. It is also important to consider the life stages of the agent released, since a more heterogenic agent population will reduce its susceptibility to the above mentioned factors (Fowler *et al.*, 2008).

Large agent numbers and fewer release events were used for the release of *H. pakistanae* in the USA. For example, one release event consisted of releasing between 23 000 to 176 000 immatures (eggs, instars and puparia), depending on the size of the water body (Freedman, pers. comm.). In another example, more than 1 million immatures of *H. pakistanae* were released at sites in Florida and Texas (Grodowitz *et al.*, 2003) over two

growing seasons. Should permission to release *H. egeriae* be granted, it is likely that large agent numbers will be released, as frequently as possible to facilitate establishment in the field.

## 5.4 INITIAL ESTABLISHMENT

### Abiotic considerations

#### *Temperature*

Chapter 2 reported that *H. egeriae* tolerates a wide range of temperatures. Dense stands of submerged aquatic plants often reach high surface temperature, due to the attenuation of radiation (Herb & Stefan, 2004). Grodowitz *et al.* (2003) found that temperatures above 35°C resulted in *Hydrellia* larval mortalities. They also found that *H. verticillata* canopies reached temperatures between 35°C and 40°C during summer. Considering that larvae of *Hydrellia* species mostly mine nitrogen rich leaves in the crown of their host plant, lethal temperatures during summer may limit *H. egeriae* performance (see Cuda *et al.*, 2008), but this remains to be tested in the field.

### Biotic considerations

#### *Plant quality*

Plant quality is a major determinant of agent establishment success (Price, 2000). Native range studies in a hypertrophic water body showed that *E. densa* growth was driven primarily by water level and secondly by nutrient availability (Mazzeo *et al.*, 2003). South Africa has some of the most eutrophic waterbodies in the world, promoting the proliferation of aquatic plant growth (Coetzee & Hill, 2012). This suggests that *E. densa* growth and quality will not be limited in South Africa's nutrient enriched water systems. Wheeler & Center (1996) investigated the effect of *H. verticillata* leaf quality on the growth and development of the biocontrol agent *H. pakistanae*, and showed that lower quality plants increased the mortality and development time of larvae as well as reduced the biomass of

female adults. Consequently, healthy *E. densa* plants in nutrient enriched freshwater systems should provide sufficient nutrition for healthy *H. egeriae* populations.

#### *Ecology of E. densa*

As mentioned in Chapter 2 and 4, knowledge of the phenology of the target weed in its exotic range is essential. Successful exploitation of the host plant by the agent requires that the insects feed and reproduce when their target host is in a suitable physiological state to support their development (Madsen & Owens, 1998; Sheppard *et al.*, 2005; Fowler *et al.*, 2008; Morin *et al.*, 2009). Although such information is not available yet from field infestations of *E. densa* in South Africa, some generalizations can be made from past studies on the ecology of *E. densa*. For example, previous studies have found that *E. densa* does not exhibit bimodal biomass patterns in subtropical and tropical regions, but grows actively year-round with its highest biomass in summer (Feijoó *et al.*, 1996; Mazzeo *et al.*, 2003). Observations from the field in South Africa show that *E. densa* is present year-round, even in cool climate zones, such as the Western Cape (pers.obs.). However, Pennington & Systma (2009) illustrate that *E. densa* exerts high phenotypic plasticity, despite its low genetic diversity (Chapter 4). Thus, *E. densa* phenology may differ within each climate region in South Africa. Nonetheless, agent releases in summer are optimal, as warmer temperatures favour rapid population growth of *H. egeriae* (Chapter 2), and consequently establishment. Furthermore, *H. egeriae* populations will need to reach sufficient numbers in the field, capable of adequately reducing the fitness of *E. densa* to counteract the impact of a predicted decreasing fly population during the winter months in South Africa.

#### *Parasitism*

As discussed in Chapter 2, parasitism may be a limiting factor for a biocontrol agent in its recipient community (Morin *et al.*, 2009), since it has been associated with the failure of biocontrol agents to suppress target weed populations (Paynter *et al.*, 2010). During pre-

release surveys in South Africa, native *Hydrellia* species and their parasitoids were recorded from aquatic plants that co-occur with *E. densa* (Martin & Smith, unpubl. data, Baars *et al.*, 2010) that may facilitate *H. egeriae* parasitism. Despite conflicting views on the efficacy of *H. pakistanae* as a biocontrol agent for *H. verticillata* in the USA (Forno & Julien, 2000; Grodowitz *et al.*, 2004), the agent has illustrated widespread established and distribution despite parasitism (Grodowitz *et al.*, 2009; Coon *et al.*, 2014). Post-release surveys have also indicated that despite attack from native parasitoids, parasitism does not always affect biocontrol agent populations (Goeden & Louda, 1976; Hill & Hulley, 1995). Susceptibility to attack is influenced by the level of agent concealment, its taxon and mobility; where sedentary agents were more prone to parasitism (Hill & Hulley, 1995). Biocontrol agents that were susceptible to parasitism included species from the families Lepidoptera, Diptera and Hymenoptera, whereas less susceptibility to parasitism was recorded for species from the family Coleoptera.

Parasitoids of the native *H. lagarosiphon* (*A. lagarosiphonae*, *C. luteostigma* and *C. nigristigma*) will be field-collected and exposed to *H. egeriae* in a lab-based experiment to establish the parasitism rate and its effect on the population dynamics of the biocontrol agent.

## 5.5 LONG TERM SUCCESS

### Disturbance regime

Long-term success of a control agent following establishment depends on a multitude of factors. One factor to consider is the disturbance regime within the recipient community. In its native range, flooding had a negative effect on the persistence of both *E. densa* and *H. egeriae* (Cabrera Walsh *et al.*, 2013). Long periods of flooding pulled *E. densa* plants underwater, making them inaccessible to *H. egeriae* gravid females. However, there are two *H. egeriae* traits that may mitigate the negative effects of flooding. Firstly, larvae are

endophagous and feed within *E. densa* leaves, which suggests that long periods of submersion will not affect *H. egeriae* larvae to a large extent. Similarly, even if plants are dislodged during flooding, larvae will be distributed along with their host plant. Additionally, female adults of *Hydrellia* species do not exert strict host preference under high egg load, and may oviposit on other plants or objects under these conditions. Considering the host-specificity of *H. egeriae* larvae (Chapter 3), it is expected that larvae will track their host plant for feeding upon eclosion. Heavy rain (see D.L. Deonier, pers. comm. in Buckingham 1994), hail and rapid water movement may wash away adults, and it is expected that developing larvae will replace diminished adults.

#### Human-mediated disturbance

Human mediated-disturbance within the recipient community may undermine biocontrol efforts. For example, mechanical removal after the release of *H. egeriae* will have devastating effects on the biocontrol programme. Thus, before an agent is released, practitioners contact landowners or persons of authority for their permission to release the biocontrol agent, but also to ask for their participation to refrain from using other control methods (mechanical or chemical) for the duration of the programme. For example, Center *et al.* (1999) found that *E. crassipes* sites treated with herbicides harboured smaller biocontrol agent populations (*N. eichhorniae* and *N. bruchi*) than sites with biocontrol management only, due to a decrease in *E. crassipes* abundance. However, weevil reproduction rates were higher for sites treated with herbicides, due to improved plant quality. This suggests that other control methods can affect target weed and biocontrol agent abundances, and that any benefits gained from IPM should be investigated first and then employed by trained personnel to obtain the best results.

### Competition with native species

The aim of biocontrol is to reduce the growth and reproduction of an invasive species until it is no longer problematic. However, the release of resources with successful biocontrol should also enhance the regrowth of native species. Knowledge of the native species community structure that occurs within the invaded area is necessary to establish the effectiveness of a biocontrol programme (Morin *et al.*, 2009). Aquatic species that co-occur with *E. densa* in South Africa are listed in Chapter 1, and it is anticipated that the successful suppression of *E. densa* will favour the regrowth and increase of these species. Considering the presence of other submerged aquatic weeds in South Africa (Chapter 1), it may be necessary to manually redistribute native species (Seastedt, 2014) to strengthen the resilience of freshwater systems to plant invasion. Holling (1973) defines resilience as “a measure of the ability of these systems to absorb changes of state variables, driving variables, and parameters, and still persist...resilience is the property of the system and persistence or probability of extinction is the result.” Levine *et al.* (2004) adds that system resilience is the ability to limit the increase and distribution of an established invasive species. System resilience is enhanced by high native species richness, but also by the ability of natives to successfully outcompete exotic species (Chadwell *et al.*, 2008). For example, Chadwell *et al.* (2008) found that the native *V. americana* reduced the ability of *H. verticillata* propagules to establish in the Otter Point Cree National Estuarine Research Reserve, Maryland, USA through the draw-down of nutrients in the water column. Recent studies have shown that *E. densa* is a superior competitor over the indigenous *L. major* (E. Strange, unpubl. PhD thesis), but these interactions have not been evaluated in the presence of herbivory. Any reduction in competitive ability of *E. densa* should favour the regeneration of indigenous submerged species in invaded systems.

### Climate change

Climate change is a global concern for environmental conservation. It intensifies droughts and floods, causes severe storms and changes seasonal and rainfall patterns that ultimately drive macrophyte assemblages (Martin, 2013) and elevates CO<sub>2</sub> concentrations (Seastedt, 2014). Over a longer time scale, climate change may alter the many factors that drive freshwater assemblages (Scheffer & Carpenter, 2003). Changes in seasonal patterns may alter the phenology of the target weed, or increase temperature and flooding frequency which may be detrimental to *H. egeriae* populations. In addition, Stiling *et al.* (1999) found that under elevated CO<sub>2</sub> concentrations, leaf nitrogen content of *Quercus* spp. (Fagaceae) trees were reduced from 1.2% to 0.3% and altered the feeding behaviour of leaf-miners species, thus making them more susceptible to parasitism. Therefore, long-term post-release evaluation will be crucial to evaluate the response of *E. densa* to elevated temperatures and CO<sub>2</sub> concentrations as well as the adaptation and performance of *H. egeriae* to such responses.

### **5.6 CONCLUSION**

Results from this thesis strongly suggest that *H. egeriae* is a suitable biocontrol agent for *E. densa* in South Africa, based on its impact to the weed and its host specificity, which should result in permission being granted for its release. Despite the development of a suitable agent, *H. purcelli*, for the biocontrol of *H. verticillata* in South Africa, the limited distribution of the weed has precluded the release of the agent. Therefore, *H. egeriae* may be the first biocontrol agent released against a submerged aquatic weed in South Africa and a catalyst for the further development of research in this field. Additionally, no biocontrol agent has been released against *E. densa* anywhere else in the world, and therefore, results from this thesis will be beneficial for countries in which *E. densa* has also become invasive,

such as New Zealand (Paynter, pers.comm.). This will create many opportunities for research collaborations between South Africa and other countries.

In conclusion, freshwater systems in South Africa stand to benefit from reductions in submerged invasive weed populations. Nevertheless, the management of submerged aquatic weeds must address the totality of factors, such as eutrophication and human-mediated invasions that underestimate biotic resistance of freshwaters. Therefore, the long-term success of this programme will require a holistic ecosystem approach whereby these underlying factors are addressed and reduced.

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