Risk assessment to interpret the physiological host range of *Hydrellia egeriae*, a biocontrol agent for *Egeria densa*

Rosali Smith · Rosie Mangan · Julie A. Coetzee

Abstract *Egeria densa* Planchon (Hydrocharitaceae) is a submerged macrophyte native to South America. It forms part of a new suite of invasive aquatic plants that has benefited from open nutrient-rich freshwater systems following the successful biological control of floating aquatic plants in South Africa. The specificity of the leaf-mining fly, *Hydrellia egeriae* Rodrigues (Diptera: Ephydridae) was tested, using traditional laboratory host-specificity testing (i.e., no-choice and paired choice). Only one non-target species, *Lagarosiphon major* Deeming (Hydrocharitaceae) supported larval development during pair-choice tests. In order to avoid the rejection of a safe and potentially effective agent, continuation (i.e., multiple generations) tests were conducted to measure the ability of the non-target species to nutritionally support a population indefinitely. None of these species could sustain a viable agent population for more than three generations. Laboratory host-specificity tests are limited as they exempt certain insect-host behaviours. To enhance the interpretation of host-specificity results, a risk assessment was conducted using agent preference (i.e., choice tests) and performance (i.e., choice and continuation tests) results. The feeding and reproductive risk that *H. egeriae* poses to non-target species is below 2%. Based on these findings, permission for its release in South Africa has been obtained.

Keywords Submerged aquatic weed · Ephydridae · Continuation test · Multiple generation test

Introduction

The aquatic weed *Egeria densa* Planchon (Hydrocharitaceae) is a freshwater plant, native to Brazil and temperate and subtropical areas of Argentina and Uruguay (Cook and Urmi-König 1984). *Egeria densa* is considered a vigorous and highly invasive plant of freshwater ecosystems outside its native range, rapidly producing dense infestations and swiftly colonising previously unaffected areas (Yarrow et al. 2009; Cabrera Walsh et al. 2013; Cook and Urmi-König 1984). The successful control of aquatic invasive weeds can be difficult to achieve using traditional methods such as mechanical and chemical control, which are often only effective in the short term. The physical removal of *E. densa* from waterways using water-level drawdowns or machinery can be counter-productive, facilitating the dispersal of the weed

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through fragmentation (Gettys et al. 2014; Hussner et al. 2017). In addition, the use of herbicide control in freshwater systems is increasingly deemed unsuitable due to its negative environmental effects on non-target species (Coetzee and Hill 2012).

During a national review of invasive aquatic weeds in South Africa (Coetzee et al. 2011), *E. densa* was identified for biocontrol as part of a rapid response to its range expansion. *Hydrellia egeriae* Rodrigues (Diptera: Ephydridae) has been identified as a promising agent due to its wide distribution in the native range, as well as significant oviposition and feeding on *E. densa*. Native range host specificity tests were conducted to establish the potential safety of *H. egeriae* (Cabrera Walsh et al. 2013). The results revealed that *H. egeriae* showed a clear preference for *E. densa*. However, the fly also developed on two other species within the same family: *Egeria naias* Planchon, and *Elodea callitrichoides* Rich. Casp. Species from the genera *Egeria* and *Elodea* do not occur naturally in South Africa (Cabrera Walsh et al. 2013) and given the specificity and favourable developmental attributes of *H. egeriae*, the fly was imported into South Africa in September 2014 for quarantine host-specificity testing.

Host-specificity testing forms the foundation of any biocontrol program. Despite the high safety record of released weed biocontrol agents (Hinz et al. 2019), concern for non-target effects by regulatory authorities, the general public and some scientists have been a major driving force for extensive refinement of host-specificity methodology. Traditional laboratory host-specificity tests include starvation (no-choice), choice, multi-choice and choice minus target tests, and less frequently used continuation (i.e., multiple generation) tests and time dependent tests (Marohasy 1998; van Driesche and Murray 2004). Choice tests although somewhat limited are valuable, creating a rank order of preference of plants that should be considered hosts. In some cases, further testing is required to examine the suitability of a host to support a biocontrol agent population over the long-term. Continuation tests are not common practice in classical biocontrol and often extend for long periods of time. These tests measure the ability of the host plant to nutritionally support a population indefinitely (Buckingham and Okrah 1993; Coetzee et al. 2003; Day et al. 2016). For example, choice tests with the sap-sucking mirid, *Eccritotarsus eichhorniae* Henry (Hemiptera: Miridae), illustrated

an oviposition preference for its host plant, *Eichhornia crassipes* (Martius) Solms-Laubach (Pontederiaceae) compared to its family member *Pontederia cordata* L. The number of progeny that developed on *E. crassipes* was 13 times higher than for *P. cordata*. However, nymphs did not show a clear preference for *E. crassipes*, and continuation tests indicated that *P. cordata* was suitable to maintain a viable population over five generations (Tipping et al. 2018). Continuation tests can also tease out some of the limitations of laboratory host-specificity testing (Marohasy 1998). Buckingham and Okrah (1993) used continuation tests to establish that the non-target species, *Potamogeton crispus* L. (Potamogetonaceae), was unable to sustain *Hydrellia pakistanae* Deonier (Diptera: Ephydridae), a biocontrol agent for *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae), for more than eight generations. Following the agent's release, there have been no records of fly damage to *P. crispus* in the field.

Spill-over may occur temporarily where biocontrol agents cause a crash in the target weed population, and continuation tests can give an indication of how long the biocontrol agent could survive on the non-target species. It is important to note that continuation tests may fail to identify impact to non-target species when both target weed and non-target species overlap geographically. Therefore, short-term spill-over events have been simulated in pre-release experiments before. When transferred to non-target species after being fed with its target weed, adult longevity and female fecundity of *Bikasha collaris* Baly. (Coleoptera: Chrysomelidae), a biocontrol agent for Chinese tallowtree (*Triadica sebifera* L.), was comparable to no-choice tests (Wheeler et al. 2017). Ultimately, all tests conducted should model the ecological context in which the agents will interact with the potential hosts (Louda et al. 2003; Briese 2005), and interpretation of results should be carefully considered to ensure they are representative of the natural host-range or field host specificity (Cullen 1990; Balciunas et al. 1996; Cruttwell McFadyen 2003, Marohasy 1998).

Extrapolating laboratory results (i.e., the fundamental host range of the agent) to its realised host range can be challenging. Factors such as small cage sizes, bypassing steps in host location and agent experience or learning may produce agent behaviour that would not occur under natural conditions (Sheppard et al. 2005). Native range host-specificity testing is useful in making such predictions, but can be limited

as it may not always include test species of the target region (Briese 2005). Risk assessment can enhance field-predictions of a potential biocontrol agent (Paynter et al. 2015). It uses the agent's host-specificity results on non-target species relative to the target weed to calculate risk scores. These scores represent the feeding and developmental risk that the agent poses to each non-target species in the field (Wan and Harris 1997). Because risk assessment scores are standardized and easier to interpret, they can also be used as a tool to better communicate laboratory results to regulatory authorities, stakeholders and the general public.

In this study, in addition to choice and no-choice tests, we also conducted continuation tests to determine if non-target species used during choice-tests are physiologically suitable to sustain agent populations in the field. We also used risk assessment to determine the risk of releasing *H. egeriae*. We present the results of host specificity tests on *H. egeriae*, together with a risk assessment pertaining to the release of *H. egeriae* in South Africa.

Materials and methods

Host plant culture

Plant material was collected throughout the years 2014 and 2015 from the Kouga River, Patensie, Eastern Cape, South Africa (S 33°44′ 54.622″; E 24°38′ 7.605″) and cultured in a flow-through system in a polytunnel at the Waainek CBC Facility in Makhanda, South Africa. Thirty shoots, 20 cm in length, were individually planted in 13.5 l round tubs (41 cm × 41 cm × 24 cm) with pond sediment and the slow release fertilizer Multicote^{TM} (Haifa) at a ratio of 0.7 g per 1 kg sediment. A 1 cm silica sand layer was placed over the sediment to minimize water clouding and algal growth. Planted tubs were placed in 600 l tanks connected to a flow-system. Plants were given a fluid nutrient stock solution every third month that consisted of calcium chloride (91.7 mg l^{-1}), magnesium sulphate (69.0 mg l^{-1}), sodium bicarbonate (58.4 mg l^{-1}) and potassium bicarbonate (15.4 mg l^{-1}) (Smart and Barko 1985). Plant material from this *E. densa* culture was used for all of the experiments in this study.

Insect culture

In September 2014, *H. egeriae* was imported under permit (P0063110) from the Exotic and Invasive Weeds Research (EIW) facility of the Agricultural Research Service in California, USA to the Rhodes University Quarantine Facility in South Africa. The founder culture was initiated in May 2013 from one shipment that contained individuals from four different populations in Argentina (John Herr, pers. comm.; Guillermo Cabrera Walsh pers. comm.).

Biology of *Hydrellia egeriae*

Adults are between 1.3 and 3.0 mm in size, live on the water surface and feed on fungi, yeast, nectar and small and/or trapped insects. Females oviposit eggs on protruding *E. densa* leaves and have a lifespan of about 13 days (at 22 °C). *Hydrellia egeriae* immatures are fully aquatic and undergo three instars during which they mine on the photosynthetic tissue of *E. densa* leaves. Larvae mine on average 24.5 leaves. After 16 days of feeding, the third instar undergoes a non-feeding pre-pupa stage, before pupariation within an *E. densa* leaf. Adults emerge after ten days and float to the water surface in an air bubble.

In order to start a culture of the potential control agent in South Africa, *H. egeriae* larvae were placed in transparent boxes (41 cm × 17 cm × 29 cm) equipped with a mesh window and kept in a controlled environment of 22 ± 2 °C under fluorescent lighting (Osram Gro-Lux 58 W, 3700 lumens, 1.5 m) and a 12:12 L:D photoperiod. Each box was half-filled with spring water and contained 25 *E. densa* apical stems, 15 cm to 20 cm in length, and a floating Petri dish with a yeast hydrolysate/sugar mixture (4 g Bacto^{TM} TC yeastolate per 7 g sugar per 10 ml H$_2$O) (Buckingham and Okrah 1993). Immatures were left to complete development and newly emerged adults were collected with a mouth aspirator and transferred to new boxes to allow mating, oviposition and development. Every week, one new box was set up with newly emerged adults, during which boxes were checked for inconsistencies (e.g., fungal growths) to maintain a disease-free insect culture. Water and new plant material were added as needed. All tests conducted with *H. egeriae* were conducted in the Centre for

Biological Control (CBC) quarantine facility and used individuals from this fly culture.

Host specificity

Test plants

Non-target plants for host-specificity testing were selected using the centrifugal phylogenetic method (Wapshere 1974) with modifications by Briese (2003). 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 that are related to the target plant. Species of these families and genera that are present in South Africa were selected for testing (Table 1). One species,

Myriophyllum spicatum L. (Haloragaceae), was selected on the basis of ecological similarity.

Prior to experimental set up, individual test plants were planted in 3 cm × 5 cm vials, containing sediment and a slow release fertilizer, MulticoteTM (Haifa) to a ratio of 0.7 g per 1 kg sediment. Plants were grown in 600 l tanks that are connected to a flow-system in a polytunnel at the Waainek CBC Facility, Makhanda. A fluid nutrient stock solution was added to the tanks to ensure healthy plant growth (Smart and Barko 1985). Rooted plants were used for host-specificity testing, and if not available, healthy leaves or plant fragments were used.

Three test species from the Hydrochariataceae, *Lagarosiphon ilicifolius* Obermeyer, *Lagarosiphon verticillifolius* Obermeyer and *Ottelia exserta* Ridley, could not be collected, despite extensive efforts.

Table 1 Non-target species selected for host-specificity testing of *H. egeriae* with degrees of phylogenetic separation (Briese 2005) within the Alismatales

Family	Test plant	Degrees of phylogenetic separation
Hydrocharitaceae	*Hydrilla verticillata* Royle*	2
	Najas horrida A. Brown ex Magnus	2
	Najas marina L.	2
	Vallisneria spiralis L.	2
	Lagarosiphon cordofanus Caspary	3
	Lagarosiphon ilicifolius Obermeyer	3
	Lagarosiphon major Ridley	3
	Lagarosiphon muscoides Harvey	3
	Lagarosiphon verticillifolius Obermeyer	3
	Ottelia exserta Ridley	3
Aponogetonaceae	*Aponogeton distachyos* L. filius	7
Potamogetonaceae	*Potamogeton crispus* L.	7
	Potamogeton pussilus L.	7
	Potamogeton schweinfurthii A. Bennett	7
	Potamogeton thunbergii Chamisso and Schlechtendal	7
	Stuckenia pectinata L.	7
Alismataceae	*Alisma plantago-aquatica* L.	8
	Echinodorus cordifolius (L.) Griseb	8
	Sagittaria platyphylla (Engelmann.) J.G.Smith*	8
Araceae	*Lemna* sp.	10
Haloragaceae	*Myriophyllum spicatum* L.*	Different order

Test species were selected on the basis of phylogenetic relatedness (Briese 2003). Test species ordered according to increasing degrees of phylogenetic separation and listed alphabetically within each degree of phylogenetic separation

*Indicate exotic plant species

Lagarosiphon ilicifolius is from southern Africa (Mozambique, Namibia and Botswana) and exportation of these species into South Africa was problematic. *Lagarosiphon verticillifolius* and *O. exserta* could not be collected due to an extensive drought in 2015 and 2016 that resulted in low water levels in the restricted rivers and dams where they occur. These species are also geographically isolated and rarely found. Nonetheless, test species within the Hydrocharitaceae were well represented, including species from four genera that are more commonly found in South Africa.

Hydrellia egeriae individuals for testing

A combination of first instars (< 24 h old) and eggs were used for host-specificity tests. To obtain individuals, ten pairs of newly emerged adults were placed in a transparent box (41 cm × 17 cm × 29 cm), half-filled with 10 l spring water, 25 *E. densa* apical shoot tips and a yeast hydrolysate/sugar mixture (4 g Bacto[TM] TC yeastolate per 7 g sugar per 10 ml H_2O) provided on a floating feeding station. Adults were allowed to mate and oviposit and leaves with eggs were harvested and placed in a Petri dish containing spring water. Five neonate larvae/eggs were transferred to test plants by excising the leaf material around it, and pinning the excised leaf with the larva/egg, onto the test plant. Eggs were checked for larval emergence after initiation of the replicate.

No-choice larval feeding

Test plants were individually placed in 600 ml containers (24 cm × 7.5 cm) filled with spring water. An excised *E. densa* leaf containing first instar/eggs was pinned to leaves on the test plants with minuten pins. Containers were enclosed with netting, held in place by an elastic band to prevent any *H. egeriae* adults from escaping. One replicate consisted of sufficient test plant material for feeding and development and five *H. egeriae* larvae/eggs. After 30 days, replicates were checked for larval mining and pupariation. Larval mining was determined by stereo microscope observation and recorded. The leaf area mined (¼, ½, ¾ or 1) as well as the total number of leaves for the test species were recorded in order to calculate the percentage of the test plant damaged. Survival was

measured as the number of *H. egeriae* individuals that pupated on the test plant.

Paired choice larval feeding

Egeria densa and a test species were placed together in a 1.5 l container with spring water. Stems of the tests species were intertwined with each other. Excised *E. densa* leaves with first instars/eggs, were attached to a 1 cm × 1 cm piece of condensed sponge with a minuten pin and placed in the middle of the container to drift in the water over the test species. The sponge allowed for buoyancy while the instars/eggs were suspended just below the water surface, allowing them to choose their feeding site. The number of damaged leaves was recorded as well as the number puparia for each test plant.

Continuation test

Test species that supported agent development during paired choice tests were subjected to continuation tests. Thirty apical shoots of the test species were placed in a transparent culture container (41 cm × 17 cm × 29 cm) filled with spring water. To initiate the test, a total of 100 *H. egeriae* first instars/eggs on excised *E. densa* leaves were pinned to shoots of the test species and left to feed and develop. After 30 days, the container of each test species was checked for adult emergence every second day, during which the adults were removed with a mouth aspirator and placed into a new culture container containing the test species from which they emerged. Food (4 g Bacto[TM] TC yeastolate per 7 g sugar per 10 ml H_2O) for adults was provided on a Petri dish. The continuation test for the target weed was conducted until F_3, and for non-target species, until the population died out.

Risk assessment

Potential non-target effects (i.e., feeding and reproductive risk) posed by releasing *H. egeriae* were calculated using the agent's feeding and survival result for each non-target species relative to the target weed, *E. densa* (Wan and Harris 1997). The following criteria were used: plant preference, plant acceptability, larval survival and number of F_1 adults. The feeding risk for each non-target species was calculated

as the product of the plant preference (i.e., mean percentage feeding on a non-target species relative to its host plant during choice tests) and plant acceptability (i.e., mean number of mined leaves during no-choice tests relative to its host plant). Similarly, the reproductive risk was calculated by multiplying the relative survival of *H. egeriae* on non-target species during no-choice tests to its host plant and the mean number of F_1 adults that emerged from non-target species during continuation tests. Zero values were replaced with 0.001 to facilitate calculation of risk scores. Standard errors ($\pm$ SE) for preference and performance scores were calculated using $\sqrt{\frac{p(1-p)}{n}}$, where p represents the risk score and n the total number of *H. egeriae* individuals used for the respective test plant during each host-specificity test.

Statistical analysis

All statistical analyses were conducted in the R environment version 3.2.3 (R Core Team 2014). The distribution of larval damage and survival for no-choice and choice feeding tests was tested for normality using the Shapiro–Wilk test. Due to the uneven distribution of all the dependent variables, a non-parametric Kruskal–Wallis test was used to determine statistical difference between test plants for larval feeding and survival during no-choice tests. The post-hoc Kruskal-Dunn test was used to identify significant differences ($P < 0.05$) between test plants

during no-choice tests. A Wilcoxon rank sum test was used to determine statistical differences between plants during paired choice tests.

Results

No-choice larval feeding

In total, 19 plant species in six families were tested. *Hydrellia egeriae* expressed significant preference for its host plant, *E. densa*. Larvae produced over three times more damage to *E. densa* leaves than any of the non-target species (Kruskal–Wallis test, $\chi^2 = 59.98$; df = 5; $P < 0.001$) (Table 2). During the no-choice tests, *H. egeriae* mined only closely related species within the Hydrocharitaceae. These included *L. major, L. muscoides, L. cordofanus, H. verticillata* and *V. spiralis*. *Egeria densa* supported over five times more *H. egeriae* survival to adulthood compared to non-target species (Kruskal–Wallis test, $\chi^2 = 71.82$; df = 5; $P < 0.001$) with a percentage of $82.22 \pm 4.04\%$ (Table 2). Non-target species that supported larval development were *L. major, L. muscoides* and *V. spiralis* with survival percentages of $12.00 \pm 4.42\%$, $6.67 \pm 5.12\%$ and $3.53 \pm 0.16\%$, respectively. Only species that supported agent survival during no-choice tests were subjected to choice larval feeding tests. Furthermore, 13 of the 19 non-target species tested under no-choice conditions incurred no larval mining

Table 2 Test species that incurred *H. egeriae* damage ($\pm$ SE %) and that supported agent development ($\pm$ SE %) during no-choice feeding tests

Test plant	*n*	% Feeding[a]	Relative damage[b]	% Survival[c]	Relative survival[d]
Hydrocharitaceae					
Egeria densa	135	25.19 ± 1.60a	1.00	82.22 ± 4.04a	1.00
Hydrilla verticillata	55	0.83 ± 0.17b	0.03	0	–
Lagarosiphon cordofanus	60	0.23 ± 0.17b	0.01	0	–
Lagarosiphon major	50	4.76 ± 1.56b	0.19	12.00 ± 4.42b	0.14
Lagarosiphon muscoides	60	2.32 ± 0.66b	0.09	6.67 ± 5.12b	0.08
Vallisneria spiralis	85	7.69 ± 2.61b	0.31	3.53 ± 1.91b	0.04

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

[a] Number of mined leaves/total number of leaves $\times$ 100

[b] Relative damage determined using the mean percentage of damaged leaves per test species in proportion to that on the target weed

[c] Number of puparia/5 $\times$ 100

[d] Relative survival determined using the mean survival on the test species in proportion to that on the target weed

and supported no agent development. Two of these species, *Najas horrida* and *N. marina*, are within the Hydrocharitaceae, the remainder belong to less closely related families that include the Potamogetonaceae, Alismataceae, Araceae, Aponogetonaceae and Haloragaceae (Table 1).

Paired choice larval feeding

During paired choice tests, *H. egeriae* preferred *E. densa* for feeding eight times more than the non-target species *L. major* (Wilcoxon rank sum test, $W = 174$; $P < 0.001$), *L. muscoides* (Wilcoxon rank sum test, $W = 35$; $P = 0.002$) and *V. spiralis* (Wilcoxon rank sum test, $W = 16$; $P = 0.02$) (Table 3). Larval survival followed the same trend with *L. major* (Wilcoxon rank sum test, $W = 422.5$; $P < 0.001$), *L. muscoides* (Wilcoxon rank sum test, $W = 49$; $P < 0.001$) and *V. spiralis* (Wilcoxon rank sum test, $W = 25$; $P = 0.007$) as significant inferior options for pupation. The percentage of *H. egeriae* that pupated in *E. densa* was over 13 times more than the non-target species *L. major*. Additionally, *H. egeriae* did not pupate in *L. muscoides* or *V. spiralis*.

Continuation test

The only test plant that could sustain a growing agent population was *E. densa* (Table 4). The mean number of *H. egeriae* instars that survived to F_1 was 75.5 ± 4.5, which produced an F_2 population of 217.5 ± 25.5 individuals. *Lagarosiphon major* was the only test plant that supported a viable population during the founder population, with 6.75 ± 3.9 adults developing unto adulthood. However, population growth was negative with no viable adults produced in the first generation.

Risk assessment

Despite some feeding and development on non-target species during no-choice, choice and continuation tests, risk assessment scores illustrated that the non-target risk posed by *H. egeriae* is very low. Relative to the target species, the feeding and reproductive risk of non-target species, *L. major,* is ten time less compared to *E. densa* (Table 5). Additionally, feeding and reproductive risk scores for *L. muscoides* and *V. spiralis* did not exceed 0.03%.

Table 3 Number of mined leaves ($\pm$ SE) and percentage survival ($\pm$ SE %) of 1st instars during paired-choice tests

Test plant	n	Number of mined leaves		Percentage (%) survival[a]		Relative survival[b]
		E. densa	Non-target	*E. densa*	Non-target	
Lagarosiphon major	105	58.92 ± 10.27a	7.25 ± 3.13b	61.90 ± 7.16a	4.55 ± 2.67b	0.07
Lagarosiphon muscoides	35	82.80 ± 5.44a	1.80 ± 0.37b	68.57 ± 5.95a	0.00 ± 0.00b	0.00
Vallisneria spiralis	25	56.25 ± 3.77a	2.50 ± 0.96b	71.33 ± 8.67a	0.00 ± 0.00b	0.00

Means ($\pm$ SE) within columns followed by the same letter are not significantly different ($P < 0.05$, Wilcoxon rank sum test). Test species listed alphabetically

[a]Number of puparia/5×100

[b]Relative survival determined using the mean survival on the test species in proportion to that on the target weed

Table 4 The mean number ($\pm$ SE) and range of *H. egeriae* adults reared on test species during multi-generation continuation tests

Test plant	n	F_1	Range	F_2	Range
Egeria densa	2	75.5 ± 4.5	71–80	217.5 ± 25.5	192–243
Lagarosiphon major	4	6.75 ± 3.9	0–18	0.75 ± 0.48	0–2
Lagarosiphon muscoides	1	0	0	0	0
Vallisneria spiralis	2	0	0	0	0

n: one replicate consisted of 100 individuals (eggs or 1st instars). Test species listed alphabetically

Table 5 Risk assessment of non-target attack by *H. egeriae*, using its relative preference (± SE) for, and relative performance (± SE) on test species during no-choice, choice and continuation tests

Test species	Plant preference[a]	Plant acceptability[b]	Feeding risk (%)[c]	Larval survival[d]	Number of F$_1$ adults[e]	Reproductive risk (%)[f]
Egeria densa	1.00	1.00	100	1.00	1.00	100
Lagarosiphon major	0.07 ± 0.02	0.19 ± 0.06	1.33	0.14 ± 0.05	0.09 ± 0.02	1.26
Lagarosiphon muscoides	0.00 ± 0.00	0.09 ± 0.04	0.01	0.08 ± 0.04	0.00 ± 0.00	0.01
Vallisneria spiralis	0.00 ± 0.00	0.31 ± 0.05	0.03	0.04 ± 0.02	0.00 ± 0.00	0.00

Test species listed alphabetically

[a] Agent feeding on test species relative to target plant during choice tests (Table 3)

[b] Agent feeding on test species relative to its target plant during no-choice tests (Table 2)

[c] Product of suitability indices for preference[a] and performance[b]

[d] Survival of agent on test species relative to its host plant during no-choice tests (Table 2)

[e] Number of adults (F$_1$) that emerged from non-target species relative to the target weed from multi-generation tests (Table 4)

[f] Product of suitability indices for larval survival[d] and generational turnover

Discussion

Results from this quarantine-based study supports results from 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 tests (Cabrera Walsh et al. 2013). Out of 19 non-target plant species tested, *H. egeriae* only mined five non-target species, all within the Hydrocharitaceae, and completed development on three of these non-target species. Under field conditions, starved larvae isolated from their host plant may cause temporary damage to *L. major*, *L. muscoides*, *L. cordofanus*, *H. verticillata* and *V. spiralis*. This may occur if *H. egeriae* disperses to new areas where the target weed is not available or where agent damage drastically reduced *E. densa* populations. As illustrated by the continuation tests, only one non-target species, *L. major* will be able to support a viable agent population. In a review on the efficiency of using relative performance scores to predict non-target effects, Paynter et al. (2015) found that non-target effects (e.g., spill-over, full utilization) were evident for risk scores above 0.20 (20%). Based on the risk assessment, *L. major* is the only non-target species on which *H. egeriae* poses a major feeding and reproductive risk with scores below 1.34%. In the field, *H. egeriae* would also have to compete with native *Hydrellia* species that feed on native *Lagarosiphon*

species, for example, *Hydrellia lagarosiphon* Deeming (Diptera: Ephydridae), a widely distributed, host-specific, herbivore of *L. major* (Martin et al. 2013). Hybridization of biocontrol agents with related species has been recorded in four cases (Havill et al. 2012), and is an undesirable non-target effect. Yet, using an extensive systematic and ecological study of the genus *Hydrellia*, Deonier (1971) never encountered interbreeding of *Hydrellia* species, under either laboratory, or natural conditions. This suggests that hybridization of *H. egeriae* and *H. lagarosiphon* or any native *Hydrellia* species in the field is highly unlikely.

Specialist herbivores often use closely related species due to similar morphological and chemical traits (Futuyma and Agrawal 2009). 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, whereas *Hydrilla* and *Vallisneria* are located within a sister clade. The genus *Lagarosiphon* is from the Afrotropics. Species within the genus are morphologically similar to *E. densa* (Chen et al. 2012). 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. Feeding and development on the further related *V. spiralis* support the hypothesis that

no-choice tests can produce false-positives due to small cage sizes and interference with natural host finding behaviour (van Driesche and Murray 2004; Sheppard et al. 2005). In its native range, open field choice tests indicated that *H. egeriae* only colonized *E. densa*, and no leaf-mining or adults were recorded in *V. spiralis* (Cabrera Walsh et al. 2013).

Although the test plant list from this study is not phylogenetically complex, risk assessment scores have proven valuable in such cases. For example, biocontrol agents for the invasive weeds *Solanum mauritianum* Scopoli (Solanaceae) and *Tithonia diversifolia* (Hemsl.) A. Gray (Asteraceae) showed considerable preference and performance on non-target species during host-specificity testing, but had inferior feeding and reproductive risk scores compared to the target weed (Olckers 2000; Mphephu et al. 2017).

Concerted efforts should be made to fine tune testing methodology using the latest information and concepts, and drawing on past experiences to avoid repeating failures. No-choice and choice tests will continue to be the mainstay of laboratory host-specificity testing, have been used to adequately predict agent safety (Paynter et al. 2015) and further utilized in risk assessments (Olckers 2000; Mphephu et al. 2017). Although less frequently used, continuation tests add strength to host-specificity test results (Buckingham and Okrah 1993; Coetzee et al. 2003; Tipping et al. 2018), and as shown here, can be used in risk assessment to predict the reproductive risk of a biocontrol agent. Based on the findings from this study, permission for the release of *H. egeriae* in South Africa has been obtained.

Compliance with ethical standards

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Rosali Smith is a PhD candidate continuing the work from her master's degree on the biocontrol of *Egeria densa* in South Africa. This involves the development of a release strategy, monitoring the performance of the agent in the field and investigating factors that enhance or weakens biocontrol efforts against *E. densa*.

Rosie Mangan is an entomologist working to make pest control more sustainable. She studies factors regulating insect abundance, distribution, and population growth, as well as consequences of intensive pesticide use, the prevalence of insecticide resistance genes, and the development of strategies to reduce reliance on chemical pesticides.

Julie A. Coetzee research focuses on the biocontrol of invasive aquatic plants, with a recent shift in focus from floating to submerged and emergent aquatic plants. As excellent control of floating species has been gained, this new suite of species has taken advantage of these new habitats, threatening indigenous aquatic flora and fauna.