A NATIVE WEEVIL AND AN EXOTIC PLANTHOPPER: INVESTIGATING POTENTIAL BIOLOGICAL CONTROL AGENTS FOR *NYMPHAEA MEXICANA* ZUCCARINI (NYMPHAEACEAE) AND ITS HYBRIDS IN SOUTH AFRICA

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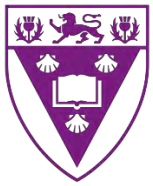

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Note to readers

Please note that this thesis has been formatted such that each chapter (barring the introduction and discussion chapter) takes the form of a scientific journal article. As such, some repetition may occur, but an attempt has been made to minimise this.

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Abstract

Nymphaea mexicana Zuccarini (Nymphaeaceae) is an invasive plant originating from southern USA and Mexico that has become problematic in South Africa, invading several water bodies around the country. Manual removal of this plant is very labour intensive and is not cost efficient or effective for long term control, while the use of herbicides is damaging to the environment and expensive. Consequently, this plant is a desirable candidate for biological control, which takes advantage of enemy release of the target weed and aims to re-establish population suppression induced by host specific natural enemies. Initiating biological control requires that several steps are followed to maximise the success of the programme, and the first few of these, including overseas surveys in the native range of the plant, have already been completed. This thesis aimed to continue biological control research for this species to take further steps at effectively managing the plant. Firstly, pre-release surveys in the invaded range are necessary to: determine what factors (including enemy release) contribute to the invasiveness of the target weed; establish a baseline of information to allow for comparison after biological control agents have been released; and identify any insect herbivores that may already be present in the country. The pre-release surveys conducted in this study revealed useful information about *N. mexicana* invasions in South Africa and provided evidence that enemy release is applicable to this case. However, these studies determined that a native weevil, *Bagous longulus* Gyllenhal (Coleoptera: Curculionidae), has expanded its host range to include the exotic *N. mexicana* at three sites, and may thus have potential for management of the species through augmentative releases.

The invasion of *N. mexicana* in South Africa is further complicated by the presence of several *Nymphaea* hybrids originating from a complex history of horticultural trade. Although previous research has shown that several hybrid groups are present in South Africa, their parentage is not known. As biological control requires the use of host specific insects adapted to overcome the unique chemical and morphological defences utilised by plant species, hybrids are notoriously difficult to manage because they possess intermediate characters inherited from parent species to which natural enemies may not have adapted. Although biological control of hybrids is challenging, other case studies have demonstrated that it is possible to find suitable agents, but the chances of success are increased if putative parents of the hybrids are known so that they can be surveyed for natural enemies. Further molecular studies including possible parents of the *Nymphaea* hybrids in South Africa were thus carried

out in this thesis to focus future surveying efforts. Two main hybrid groups were identified with genetic similarity to two tested putative *Nymphaea* parents, and this will allow further investigations of these species to improve the chances of successfully managing these hybrid groups. Some of the tested hybrids showed genetic contributions from multiple groups, some of which were unidentified, so it is necessary to prioritise the most problematic hybrids for biological control.

With more insight into the genetic makeup of the *Nymphaea* hybrids in South Africa, investigations into the host specificity of potential biological control agents can be conducted. The ideal biological control agent should have a broad enough host range to impact and survive on both *N. mexicana* and its hybrids, but without a host range so broad that it would pose risk to native South African species. Host specificity trials are thus necessary to determine the suitability of potential agents. The identification of *B. longulus* feeding on *N. mexicana* during pre-release surveys motivated further investigations to determine the natural distribution, field host range, and host specificity of *B. longulus* in experimentally controlled conditions. Further surveys were therefore conducted at native *Nymphaea* sites around South Africa in addition to host specificity trials using the native *Nymphaea nouchali* Burm. f. (Nymphaeaceae), two populations of *N. mexicana*, and a cultivated hybrid. Results from the surveys and host specificity tests suggest that *B. longulus* is widely distributed across South Africa, is specific to *Nymphaea* with no observed preference between *N. mexicana* and the native *N. nouchali,* and does not perform well on *Nymphaea* hybrids. Hence, *B. longulus* is promising for use in new association biological control through augmentative releases but is not suitable for management of hybrids.

In addition to the potential use of the South African *B. longulus,* it is necessary to conduct host specificity trials for natural enemies from the native range of *N. mexicana* that were prioritised in previous studies. *Megamelus toddi* Beamer (Hemiptera: Delphacidae) is one such species that was imported into quarantined laboratory conditions from Florida, USA. Host specificity trials were conducted using the same test plants as described for the studies on *B. longulus,* in addition to multigeneration trials to determine how long *M. toddi* could survive on non-target host plants. As with the *B. longulus* studies, no statistically significant differences in preference were observed between *N. mexicana* and *N. nouchali,* but *M. toddi* could not complete development on the test hybrid, indicating that this species is also unsuitable for the management of *Nymphaea* hybrids. Despite suboptimal plant health, *M. toddi* completed development for three generations on the native *N. nouchali*. This lack of

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The concluding chapter of this thesis discusses the aforementioned findings in a broader context by considering the driving forces of plant invasions in general and specifically for *N. mexicana* in South Africa. Case studies are also consulted to provide insight into how to proceed with managing *Nymphaea* hybrids in South Africa, while the factors governing host specificity and host range expansion are also discussed and considered in the context of *B. longulus* and *M. toddi*. Finally, after a consideration of the limitations of these studies, recommendations are made to continue the development of biological control for *N. mexicana* in South Africa.

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Chapter 1: General introduction

1.1 INVASIVE ALIEN PLANTS

Biological invasion can be defined in many ways. Some researchers argue that a species must overcome a geographic barrier or major distance to be considered invasive (Richardson et al. 2000b, Richardson & Pyšek 2006), while other researchers counter that such a definition would exclude species native to a region that naturally expand to inhabit new adjacent habitats (Thompson et al. 1995, Davis & Thompson 2000) or that become invasive due to disturbance (Blackburn et al. 2011). Similarly, some definitions state that a species must have a major impact on the affected community or ecosystem in order to be considered invasive (Davis & Thompson 2000, 2002, Inderjit 2005), while others disagree as impact can be difficult to assess and interpret (Daehler 2001, Rejmánek et al. 2002). In an attempt to develop a general definition that encompasses most of the ideas posed by other researchers, Valéry et al. (2008) proposed that a biological invasion should be defined as follows:

"A biological invasion consists of a species' acquiring a competitive advantage following the disappearance of natural obstacles to its proliferation, which allows it to spread rapidly and to conquer novel areas within recipient ecosystems in which it becomes a dominant population."

Throughout history, plants have been introduced into novel environments for horticulture, industry, and agriculture, and become established after overcoming geographic, environmental, reproductive, and dispersive barriers (Richardson et al. 2000b). The introduction and spread of invasive species have increased with improved transport and globalisation. For example, in Europe the number of alien species introduced increased by over 76% between 1970 and 2007 (Butchart et al. 2010). Biological invasions have such severe impacts that they are considered to be the second greatest global threat to biodiversity, after habitat destruction (Wilcove et al. 1998, Simberloff et al. 2013).

Of the 100 worst invaders of the world, 37% are plant species (Luque et al. 2014). Many invasive plant species create major problems economically and environmentally by outcompeting native species and disrupting native ecosystems, inhibiting recreational activity, transforming agricultural land, and consuming water resources (Mack et al. 2000, Pejchar & Mooney 2009, Vilà et al. 2011).

For example, plant invasions have been estimated to reduce livestock production value in South Africa by R340 million per year (O'Connor & van Wilgen 2020), while in south-eastern France, common ragweed *Ambrosia artemisiifolia* L. (Asteraceae) was estimated to impact human health through allergenic effects to the value of €9.7–14.0 million annually between 2008 and 2015 (Mouttet et al. 2018). Similarly, severe impacts on the environment and water quality have been recorded in South Africa (Moran et al. 2005, Chamier et al. 2012), and invasions in aquatic environments are linked to decreases in macrophytes, zooplankton, and fish, as well as other strong and consistent ecological impacts on a global scale (Gallardo et al. 2016). Overall, the effects of invasive alien plant species are far reaching across a range of contexts and are impacted by other factors such as climate change (Kumar Rai & Singh 2020). These costs and negative impacts illustrate the importance of developing sustainable and effective methods to manage alien invasive plant populations.

1.2 MANAGING INVASIVE ALIEN PLANTS USING BIOLOGICAL CONTROL

Invasion processes and hypotheses

In order to manage plant invasions, it is necessary to understand the processes underlying their successful introduction, establishment, and spread. These processes are dependent on various factors including plant traits, propagule pressure, genetic bottlenecks, and the invasibility of the environment. The factors that encourage the success of plant invasions have been covered by several authors and allow researchers to make important decisions around the best means of controlling problematic species at each stage of invasion (Williamson & Fitter 1996b, Williamson 2006, Theoharides & Dukes 2007, Ward et al. 2008). There is a plethora of hypotheses and terminologies used by different researchers to explain and categorise invasions, however, and these inconsistencies complicate the integration of data from different systems. To remedy this problem, Blackburn et al. (2011) developed a unified framework that recognizes the division of the invasion process into stages, during which there are barriers that must be overcome by the species or population in question to progress to the next stage. Additionally, it is recognized that different terminology can be used to describe a species depending on which invasion stage it is in, and varying management strategies should be applied at different stages.

As a continuation of this idea of using unified frameworks to study invasions, Blackburn et al. (2014) further suggest that it is necessary to classify species based on their

level of impact in order to focus resources towards controlling the most problematic species. Such a classification system categorizes invasive species impact as either minimal, minor, moderate, major, or massive, depending on the effects, or potential effects, on the environment in terms of: competition, predation, hybridization, transmission of diseases to native species, parasitism, poisoning/toxicity, biofouling, grazing/herbivory/browsing, chemical, physical or structural impact on ecosystems; and interaction with other alien species. These are based on the mechanisms of impact that are used in the International Union of Conservation of Nature (IUCN) Global Invasive Species Database to code species and include categories to encompass species for which information is lacking, including 'No Alien Populations', 'Data Deficient', and 'Not Evaluated'. By providing standardized frameworks and classification schemes, invasion biologists may be better able to compare systems and provide recommendations to mitigate the impacts caused by species at each stage of invasion.

Varying management strategies are appropriate to control invasions at each stage in the invasion process (Theoharides & Dukes 2007). For example, at the transport stage of invasion, management strategies include regulation of nursery trade and screening of seed stock, promotion of native species in landscape design, and education of the public. To target the colonisation stage, climate modelling techniques can be used to predict potential ranges of alien invasive species, while habitat fragmentation can be reduced, and mechanical and chemical control of smaller populations started immediately. Before invasive alien plants establish, native ecosystems should be strengthened, biological control investigated, human disturbance reduced, and existing control measures continued. Finally, the spread of established species might be prevented by minimising disturbance corridors in natural landscapes, promoting the growth of competitive native species especially at the edge of disturbance corridors, isolating source populations of alien species, eliminating or reducing transport vectors for reproducing populations, and prioritising sites that are susceptible to invasion (Theoharides & Dukes 2007). Notably, many authors agree that preventing introductions of alien species is more cost effective than managing invasions after they have been introduced (Leung et al. 2002, Puth & Post 2005, Wittenberg & Cock 2005, Simberloff 2006, Simberloff et al. 2013).

Several hypotheses attempt to explain how and why species manage to invade and are so successful in introduced regions, but in many cases, there are studies that both support and refute the hypotheses (Williamson & Fitter 1996a, Richardson & Pyšek 2006, Theoharides &

Dukes 2007). Enders et al. (2018) attempted to formulate a network of invasion hypotheses based on researchers' knowledge and understanding of them. The three best-known and most central hypotheses according to this study were the propagule pressure hypothesis (Lockwood et al. 2005), the enemy release hypothesis (Keane & Crawley 2002), and the disturbance hypothesis (Elton 1958) (Table 1.1). While the propagule pressure hypothesis emphasises the importance of human action in invasions, the enemy release and disturbance hypotheses represent the importance of ecological interactions with other biota and abiotic factors respectively (Enders et al. 2018). These hypotheses were also among the hypotheses that are well investigated, according to a review by Lowry et al. (2013).

Table 1.1: Summary of key hypotheses of invasion success. Extracted and adapted from Enders et al. (2018).

Hypothesis	**Summary**	**References**
Propagule pressure hypothesis (PP)	Invasion success is caused by a high propagule pressure, where propagule pressure is a measure of the number of individuals introduced per introduction event and the frequency of introduction events.	(Lockwood et al. 2005, Colautti et al. 2006)
Enemy release hypothesis (ERH)	Invasion success is caused by the absence of natural enemies in the invaded range.	(Keane & Crawley 2002, Joshi & Vrieling 2005)
Disturbance hypothesis (DS)	Highly disturbed ecosystems are more likely to have high numbers of successful invaders than in relatively undisturbed ecosystems.	(Elton 1958, Hobbs & Huenneke 1992)

Biological control and the enemy release hypothesis

Although there are various management options for invasive alien species at each stage of invasion, here I discuss management strategies for invasive alien plant populations that are already established and naturalised. Such invasive alien plants may be controlled using: 1) chemical control, in which herbicides are used to treat existing populations; 2) mechanical control, in which plants are removed manually by hand or using machinery; or 3) biological control, which uses populations of one species to control another species. Chemical and mechanical measures of control are short term solutions and can become expensive and damaging to the environment (Jurado et al. 2011, Maluleke et al. 2021). Biological control, however, is a cost efficient, long term, and environmentally sustainable solution.

There are four types of biological control: natural, conservation, classical, and augmentative (Eilenberg et al. 2001, Cock et al. 2010). Natural biological control occurs when naturally occurring organisms control pest organisms without human intervention. Conservation biological control may be described as the protection and stimulation of naturally occurring natural enemies by human actions in order to suppress pest populations. In classical biological control, researchers collect natural enemies from the origin of an invasive species and release these enemies into the invaded range to manage the pest populations. Finally, augmentative biological control involves mass-rearing of natural enemies in large numbers to immediately control crop pests with short reproduction cycles (inundative biological control) or to control pests over several generations for crops with long production cycles (seasonal inoculative biological control) (Cock et al. 2010, van Lenteren et al. 2018). Classical biological control was the first type of biological control to be practised deliberately and widely, and may be associated with various hypotheses, but most notably the enemy release hypothesis (Maron & Vilà 2001, Keane & Crawley 2002, Torchin et al. 2003, Liu & Stiling 2006, Lowry et al. 2013). Essentially, classical biological control aims to reestablish the population control exerted on plant populations by natural enemies in the native range, thereby reverting the effects of enemy release.

Support for the enemy release hypothesis may be contradictory, depending on how it is investigated and at what scale (Maron & Vilà 2001, Keane & Crawley 2002, Colautti et al. 2004, Vilà et al. 2005). However, an alternative way to approach the enemy release hypothesis (ERH) is to use the hierarchy-of-hypotheses (HoH) approach (Jeschke et al. 2012, Heger et al. 2013). This approach aims to solve the problems associated with assessing the

usefulness of a hypothesis: 1) that ecological hypotheses are usually broad and imprecise; 2) that the wording of a hypothesis varies and is interpreted differently by different authors (Heger et al. 2013); and 3) that accumulated empirical evidence in ecology does not often allow simple conclusions for a broad hypothesis, as some case studies support a given hypothesis while others do not (Heger & Jeschke 2014). The HoH approach does this by replacing single hypotheses by hierarchies of hypotheses, in which broad and unspecific formulations are at the top level and branch into increasingly specific sub-hypotheses at each lower level. This allows broad conceptual ideas as well as specific hypotheses to be integrated into one common framework.

Heger & Jeschke (2014) investigated the ERH using the HoH approach, and found that when they considered the broadly formulated hypothesis (that the absence of enemies in the exotic range is a cause of invasion success), there was almost as much evidence to support this hypothesis (36%) as to question it (43%). However, at the sub-hypotheses level, empirical evidence strongly supported some sub-hypotheses, while others did not receive much support. For example, over 50% of the studies that investigated levels of natural enemies on alien plants in the native range compared natural enemy levels in the invaded range, found support for the hypothesis that there is more natural enemy infestation on aliens in the native range. Support was also found for studies that compared alien species to native species. Similarly, more than 50% of studies that investigated levels of damage by enemies supported the sub-hypothesis that aliens in their native range had higher levels of damage than in the invaded range, but over 50% of the studies that investigated invasive vs. non-invasive aliens refuted this sub-hypothesis. These findings show the importance of maintaining consistency between studies where possible, and in being specific with the hypotheses that are being tested. Overall, we can conclude that several sub-hypotheses of the broad ERH are useful, but that other formulations should be more critically evaluated (Heger & Jeschke 2014). Furthermore, habitat differences and varying taxonomic groups might show contrasting support for hypotheses, as well as focus on contrasting functional groups (generalists vs. specialists).

Success of classical biological control

Although it is likely that there are various factors other than enemy release that affect invasion success, classical biological control (which will hereafter be referred to as "biological control" or "biocontrol"), by means of introducing host specific natural enemies into the invaded range, has had considerable success in managing invasive alien plants (Forno

& Julien 2000, McFadyen 2000, van Driesche et al. 2010, van Lenteren et al. 2018). It should be noted that the success of biological control agents is dependent on how "success" is defined and evaluated. Success may be measured by ecological, economic, and social impacts of an invasive alien plant with biological control compared to without it, and may focus on an individual biological control agent, the target weed, or ecosystem responses (Morin et al. 2009). Defintions of success can be vague and open to interpretation (Hill et al. 2020), but more recent classifications consider parameters such as density, biomass, area, and rate of spread of invasive alien plants before and after biocontrol, wherein each goal is specific to the target species (Moran et al. 2021). Ideally, biological control agents will be host specific, establish and proliferate in the invaded range, inflict high levels of damage and reduce populations of the target weed, and be able to survive in the climatic conditions of the invaded range (Harris 1973, 1991).

In a review by (van Driesche et al. (2010), 98% (48 out of 49) of biological control projects assisted with the protection of biodiversity, 47% (23) provided protection for products, and 25% (12) conserved ecosystem services. Of the same 49 reviewed projects, 27% (13) achieved complete biological control (in which case the pest is no longer considered important), 33% (16) achieved partial control (in which control was achieved in some areas but not in others), and 49% (24) were still in progress (in which outcomes did not yet exist at the time). Using more recent classifications of biocontrol success using the parameters described above, for 54 cases of successful agent establishment, 28% are considered as very successful (in which the invasive plants are suppressed below suitable thresholds for density, biomass, area, and rate of spread, with a categorisation of A+, A, or A-), 46% have mixed success (categorised as B+, B, or B-), and 26% are considered as least successful or failed (C+, C, or C-) (Moran et al. 2021). In yet another consideration, biological control programmes implemented against five invasive aquatic plants in South Africa have considerably reduced negative impacts caused by their presence in South African ecosystems (Hill & Coetzee 2017) (Table 1.2).

Table 1.2: Impact scores of the generic impact scoring system (GISS) (Nentwig et al. 2016). This measures 12 categories of environmental and socio-economic impacts caused by alien species, with impact intensity quantified on a scale of 0 (no detectable impact) to 5 (highest possible impact). Thus, higher scores represent greater impacts. Here scores are shown for the top five aquatic alien plants in South Africa, considering the worst-case scenario in which

there is no biological control, and the current situation in South Africa with biological control implemented. Adapted from (Hill & Coetzee 2017).

		Socio-economic and environmental impact	
Weed	**Family**	**Before biological control**	**After biological control**
Pontederia crassipes Mart. [≡ *Eichhornia crassipes* (Mart.) Solms]	Pontederiaceae	43	23
Pistia stratiotes L.	Araceae	38	6
Salvinia molesta D.Mitch.	Salviniaceae	38	6
Azolla filiculoides Lam.	Salviniaceae	40	0
Myriophyllum aquaticum (Vell.) Verdc.	Haloragaceae	38	15

Calculation of benefit-cost ratios indicates that biological control is more cost effective than chemical and mechanical control, despite initial costs to conduct field surveys and host-specificity tests (Zimmermann et al. 2004, Moran et al. 2005). For example, successful biological control of skeleton weed *Chondrilla juncea* L. (Asteraceae) resulted in a benefit-cost ratio of 112 (Marsden et al. 1980), while the benefit-cost ratio of tansy ragwort *Jacobaea vulgaris* Gaertner syn. *Senecio jacobaea* L.(Asteraceae) was 15 (Coombs et al. 1995). Beneficial benefit-cost ratios have also been recorded for other biological control programmes (Chippendale 1995, McFadyen 2000). In South Africa, it has been estimated that biological control of four aquatic weeds (*P. stratiotes, S.molesta, A.filiculoides,* and *M. aquaticum*) cost R7.8 million in contrast to R150 million to R1 billion (depending on the application method) to achieve the same level of control using chemical control (Maluleke et al. 2021). This equated to biological control avoiding probable costs of R90 to R631 of chemical control for every R1 spent using biological control agents. Similarly, global benefit

to cost ratios for biological control are estimated to range from 23:1 to 7405:1 for various combinations of impacts to agriculture and wildlands (Driesche & Heinz 2016).

In additional assessments of biological control success, out of 15 biological control agents assessed by Forno & Julien (2000), most were successful in the introduced range in the sense that they sustained populations on the target plant. However, only five of the 15 investigated agents showed successful reduction of the density and area covered by the target plant, in over 50% of the habitats infested in most of the countries where they had been released. Indeed, while many biological control programmes are successful (Caltagirone 1981, McFadyen 1998, Bellows 2001), there are several agents that fail to establish or sufficiently control invasive populations (Julien & Chan 1992, Dray et al. 2001). For example, out of 59 biological control projects against invasive weeds, 33 projects were considered successful and 26 were unsuccessful (Denoth et al. 2002). In many cases, climate incompatibility may explain the failure of agents to establish and control the target plant. A review of case histories for 199 biological control agents that failed to establish after being released 148 times against 84 pests suggests that 34.5% of the investigated failures were attributed to incompatibilities with climate (Stiling 1993). Other potential explanations for lack of success include varying genetic structure of the weed or the presence of hybrids in the invaded range (Urban et al. 2011, Paterson et al. 2016), and the presence of natural enemies of the biological control agents in the invaded range (Goeden & Louda 1976, Paynter et al. 2010). Understanding the importance of these factors allows researchers to develop better methods to select biological control agents, and to better predict the likelihood that they will be successful (van Klinken & Raghu 2006, Zalucki & van Klinken 2006, Paynter et al. 2010, 2018).

Overall, although there are several means of classifying or quantifying biocontrol success and despite issues of contrasting terminology and methodology, it is clear that biological control is effective for the management of many plant species, even if to various extents (Moran et al. 2021).

Steps to initiate biological control

For biological control to be successful, the agents should be sufficiently host specific such that they do not pose a threat to non-target species in the invaded range. An agent may be host specific as a result of complex ecological and evolutionary interactions, in which a natural enemy selects a particular host through habitat location, host location, host

acceptance, and host use, all of which are governed by olfactory, gustatory, and tactile cues (Heard 1999; Bernays and Chapman 1994). Particular plants may possess unique compositions of these cues, which act to initiate behavioural and physiological reactions in host-specific taxa to initiate feeding and oviposition, while non-host plants may possess deterrents which prevent feeding and oviposition (Bernays & Chapman 1994). It follows that plants that are more closely related to each other are more likely to possess similar morphological and biochemical traits than distantly related taxa.

To initiate biological control, it is necessary to follow steps to maximise the likelihood that selected biological control agents will establish and effectively manage invasive populations (Denoth et al. 2002, Goolsby et al. 2006b, van Klinken & Raghu 2006, Zalucki & van Klinken 2006) (Table 1.3). These steps aim to reduce costs and risks, and maximise efficacy, but will depend on the target plant and the ecological and management context in which it occurs, as well as the negative impacts that need to be mitigated through the use of biological control (van Klinken & Raghu 2006).

Table 1.3: Explanation of steps necessary to develop a biological control programme. Steps marked with asterisk are those that are covered in this thesis. Steps adapted from van Klinken & Raghu (2006).

Step	**Explanation**	**References**
Identification of target weed (including genetic matching) *	Proper identification is necessary to determine which plant species should be used to test host specificity (centrifugal phylogenetic testing), identify sites in the native range of the target plant to survey for natural enemies, and to determine the compatibility of natural enemies to act as host specific biological control agents.	(Day & Neser 2000, Briese 2003, Gaskin et al. 2011)

Exploration for potential biological control agents	Determine suitability of natural enemies as potential biological control agents by determining field host range and impact. Compare native range conditions with invaded range.	(Kuhlmann & Mason 2002, Goolsby et al. 2003, Reid et al. 2020)
Prioritisation of biological control agents	Rank potential agents based on host specificity, impact, abundance, and climate compatibility among other factors.	(van Klinken & Raghu 2006, Dhileepan et al. 2009)
Pre-release surveys in invaded range *	Establish a baseline of information about invaded sites with which to compare after biological control is established; determine the presence of accidentally introduced natural enemies from the native range, or new associations between native enemies and invasive species; determine impacts on biodiversity; compare herbivore abundance and diversity between invaded and native range.	(Goolsby et al. 2009, Paterson et al. 2011a, Canavan et al. 2014, Hokkanen & Pimentel 1984, 1989)
Host specificity testing/pre-release impact assessments *	Determine risk of potential agents impacting non-target plants, determine potential of agents to successfully manage invasive populations.	(Briese 2005, Sheppard et al. 2005, Paynter et al. 2018)

Release approval, mass-rearing, and release	Mass rear and distribute agents to invaded sites to begin management of invasive populations.	(van Lenteren & Tommasini 1999)
Monitoring, evaluation, and integrated management	Monitor establishment and success of control agents, take further steps to enhance management through integrated use of mechanical and chemical control.	(Morin et al. 2009, Miller 2018)

It is important to note that despite efforts to ensure host specificity of biological control agents, nontarget effects can occur. While early cases of nontarget attack could have been predicted from host range data, nontarget effects can occur even for host-specific biological control agents (Suckling & Sforza 2014). Nontarget effects can occur through direct impacts on nontarget plant species (Paynter et al. 2004, 2018), compensatory growth and production of secondary compounds by invasive plants as a result of herbivory by biological control agents, which in turn may result in increased competition with native species (Callaway et al. 1999, Ridenour & Callaway 2003); altered food-web interactions including parasitism and predation, and disease (Goeden & Louda 1976, Pearson & Callaway 2003, Fowler et al. 2012, Paynter et al. 2018), and other similar effects (Pearson & Callaway 2003, Paynter et al. 2018). These risks can be reduced by selecting biological control agents that are effective at reducing population densities of the target weed, thereby allowing populations to be regulated through density-dependent feedback loops (Balciunas 2004). Risks such as these highlight the importance of taking the necessary steps to maximise the likelihood of biological control success.

1.3 AQUATIC INVASIONS AND BIOLOGICAL CONTROL IN SOUTH AFRICA

Introductions of alien plant species into South Africa first began in the 1600s, at which time the Cape of Good Hope was a refurbishing stop for European ships. Numerous plant species were brought in and cultivated for horticulture, agriculture, and industry among other reasons, with many species originating from Australia and South and Central America (Zimmermann et al. 2004). Since then, many species have become highly problematic,

threatening natural resources. Althoug most of the concern in the past has been the effects of these invasive alien plants on conservation areas, natural vegetation, and agricultural productivity, the impact of aliens on water resources has become increasingly important. A study in 2000 by Le Maitre et al. (2000) estimated that the total incremental water use of invasive alien plants was 3 300 million m^3 of water per year, which could result in the loss of considerable volumes of water in valuable catchment areas. Similarly, alien invasive plants can reduce water quality through reduced stream flow, alteration of nutrient cycles and elevation of groundwater nutrient concentrations, alteration of fire regimes resulting in increased soil erosion, among other impacts (Chamier et al. 2012). Where invasions cause a reduction in aquatic plant diversity, there may even be impacts on climatic regime (Sieben et al. 2021). The reduction of water quantity and quality in an already water-scarce country illustrates the importance of managing alien plant invasions in South Africa and other countries. Research such as this resulted in the formation of the Working for Water programme in South Africa, which aims to protect water resources by clearing invasive alien plants using various control methods including biological control, while also providing employment and upliftment in poor rural communities (Görgens & van Wilgen 2004, Zimmermann et al. 2004, Working for Water 2007).

Biological control has been used in South Africa since 1913, when cochineal insects were imported to control drooping prickly pear, *Opuntia monocantha* Haworth (Cactaceae) (Zimmermann et al. 2004). Since the success of this programme, numerous new biological control programmes have been initiated against weeds such as Cactaceae, Australian *Acacia* species, and floating aquatic plants (Zimmermann et al. 2004, Moran et al. 2005, Paterson et al. 2011b). In 2004, South Africa was leading in exploration and release of agents for the biological control of *Lantana camara* L. (Verbenaceae), *Chromolaena odorata* (L.) R.M. King & Rob. (Asteraceae: Eupatorieae), and *Pontederia crassipes* (Zimmermann et al. 2004). Although terrestrial invasive alien plants have negative impacts on water quality and quantity and are important to manage, significant focus has also been placed on the control of aquatic invasive plants in South Africa. Aquatic alien plants have invaded South African water systems since the 1800s, and eutrophication resulting from urban and agricultural pollution has contributed towards the major proliferation of these invasive species (Coetzee & Hill 2012). Some of the worst invaders of South African water systems are: *P. crassipes* (water hyacinth); *Pistia stratiotes* (water lettuce); *Salvinia molesta* (salvinia/Kariba weed); *Myriophyllum aquaticum* (parrot's feather); and *Azolla filiculoides* (red water fern) (Hill

2003). These species are now being managed successfully using biological control (Hill & Coetzee 2017).

All of the above-mentioned plants are free-floating, with the exception of *M. aquaticum.* As these floating weeds are being controlled, waterways are open to invasion by submerged weeds such as *Egeria densa* Planch. (Hydrocharitaceae) (Brazilian water weed) and *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae); and emergent species including *Sagittaria platyphylla* (Engelm.) J.G.Sm. and *Sagittaria latifolia* Willd. (Alismataceae); *Lythrum salicaria* L. (Lythraceae) (purple loosestrife), *Nasturtium officinale* W.T. Aiton. (Brassicaceae) (watercress); *Iris pseudacorus* L. (Iridaceae) (yellow flag); and *Hydrocleys nymphoides* (Humb. & Bonpl. ex Willd.) Buchenau (Alismataceae) (water poppy) (Coetzee et al. 2011b, 2011a). *Salvinia minima* Baker (Salviniaceae) and *Azolla cristata* Kaulf. (Salviniaceae) (Mexican azolla) are two new floating species that are also in the early stages of invasion (Hill & Coetzee 2017), in addition to the rooted floating-leaved Mexican water lily, *Nymphaea mexicana* Zuccarini (Nymphaeaceae), which is the subject of this thesis. The likelihood of successful control is increased when invasive alien plants are targeted in the early stages of invasion, so it is important to implement biological control programmes before the ranges of these problematic plants are allowed to expand (Olckers 2004). This thesis aims to continue the initiation of a biological control programme for *N. mexicana* in South Africa, in order to manage this plant before it becomes even more problematic.

1.4 *NYMPHAEA MEXICANA*

Description and South African congeners

Nymphaea mexicana (Mexican or yellow water lily) is a hardy (cold resistant) water lily originating from south-eastern USA, and Mexico (Capperino & Schneider 1985) and placed within subgenus *Nymphaea* (Figure 1.1). This plant is characterised by bright yellow flowers with lanceolate petals, green and purple/red floating leaves with brown patterning, roots and rhizomes that remain planted in the soil, and 'brood-bodies' consisting of a cluster of root tubers and buds that produce new plants (Wiersema 1988) (Figure 1.2). In the native range, canvasback ducks *Aythya valisineria* Wilson and ring-necked ducks *Aythya collaris* Donovan feed on these tubers (Alexander 1987). New plants may also be produced from thick stolons, and the rhizomes are erect (Johnstone 1982). This biology allows *N. mexicana* to outcompete both emergent vegetation by means of the root system, and submerged plants when the floating leaves block out sunlight (Wiersema 1988). Sexual reproduction also

occurs via insect pollination. The yellow flowers are showy and have a diameter of 15-33 cm, a calyx with four elliptic to lance ovate sepals, and produce stigmatic fluid that washes pollen from visiting insects, thus facilitating pollination (Capperino & Schneider 1985). After fertilisation, green berries containing seeds are produced and grow under water from which new plants develop.

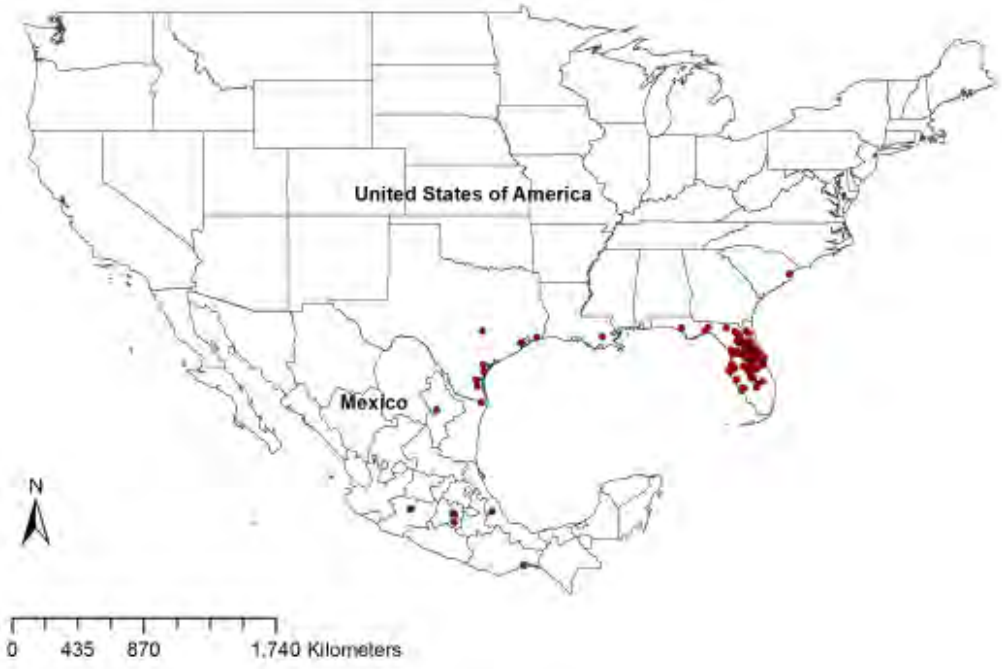

Figure 1.1: Native range of *Nymphaea mexicana*. Mapped in ArcMap (Environmental Systems Research Institute 2014) using distribution data from GBIF (GBIF.org 2019).

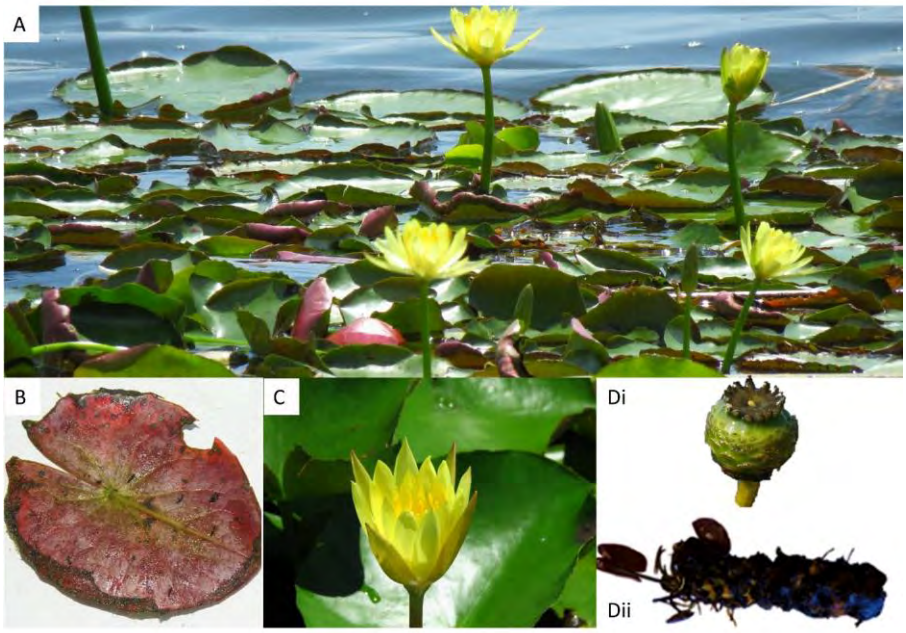

Figure 1.2: *Nymphaea mexicana.* A: Yellow flowers and floating leaves in the native range. B: Leaves are green on the top and red on the underside, with some brown/purple blotching. C: Flowers are bright yellow with lanceolate petals. Di: Fruit. Dii: Large tubers resemble bananas, with fleshy rhizomes (Photo: Prinavin Naidu).

There are no hardy water lilies native to South Africa. Instead, South African water lilies are tropical (not cold resistant) and belong to subgenera *Brachyceras* and *Lotos.* Although there is come contention about the taxonomy of South Africa water lilies (see Chapter 4), the officially accepted species include *Nymphaea lotus* L. and *Nymphaea nouchali* Burm. F, of which there are several varieties: var. *petersiana* (also treated as a synonym of *Nymphaea capensis* by Conard (1905)), var. *ovalifolia* (Conard) Verdc., var. *caerulea* (Savigny) Verdc., var. *mutandaensis* Verdc., and var. *zanzibariensis* (Casp.) Verdc. Based on current data from IUCN's (International Union for Conservation of Nature) red list, all of the native *Nymphaea* in South Africa are categorised as least concern.

Global distribution and impacts

Nymphaea mexicana has become invasive in Australia, New Zealand, India, Europe, Spain, and South Africa (Johnstone 1982, Garcia-Murillo 1993, Henderson 2010, Newfield & Champion 2010, Hussner 2012, Shah & Reshi 2012) among other countries. Many introductions occurred through the ornamental trade owing to the aesthetic appeal of the plant, and the ability of the plant to grow rapidly has facilitated its invasion in aquatic

ecosystems (Capperino & Schneider 1985). Without sufficient control, it is likely that *N. mexicana* will spread to other water bodies through dispersal of rhizomes, tubers, and seeds by water and boats or machinery (Fowkes 2016).

Nymphaea mexicana forms dense mats on water surfaces and blocks out light for submerged plants (Figure 1.3), and is capable of colonising nutrient rich waters up to 2 m deep. Hence, infestations of *N. mexicana* can reduce gas exchange, restrict water movement, increase siltation, decrease recreational value, and reduce water quality (Capperino & Schneider 1985, Hofstra et al. 2013). Oxygen levels in occupied water bodies may also be depleted after rhizomes die in response to herbicide treatments, which in turn has negative impacts on aquatic fauna. For example, oxygen depletion resulting from *N. mexicana* infestations may have caused fish deaths downstream of infestations in Goulburn River, Victoria, Australia (G-MW 2009, Hofstra et al. 2013), which in turn affects birds that prey on fish. *Nymphaea mexicana* also results in economic losses as landowners attempt to clear water bodies. These negative impacts emphasise the importance of controlling *N. mexicana* invasions in South Africa and other invaded countries as soon as possible, before populations expand further. Using the Biological Control Target Selection (BCTS) system (Canavan et al. 2021), *N. mexicana* ranked 64th out of 299 evaluated species based on scoring systems related to the impact or importance of the plant, likelihood of achieving success, and required investment.

Figure 1.3: *Nymphaea mexicana* in the invaded range (Emmarentia Dam, Johannesburg, RSA), with the leaves forming dense mats on the water surface. Photo: Prinavin Naidu.

Current control

Chemical control of *N. mexicana* invasions has been utilised in Australia using the glyphosate-containing herbicide Roundup Biactive (G-MW 2009). Low coverage of *N. mexicana* is more easily maintained using chemical control during midsummer to autumn, as the herbicidal solutes are translocated more effectively during this period, and new growth is only triggered in spring (Hofstra et al. 2013). During summer, low cover maintainance requires two applications per year as well as mechanical removal of rhizomes after the plants begin to die off. Mechanical control can also provide some effectivity, but regrowth occurs within 8-12 months after chemical and/or mechanical control (G-MW 2009, Hofstra et al. 2013).

Nymphaea mexicana in South Africa

As invasive floating macrophytes such as salvinia, water lettuce, and red water fern are being successfully controlled using biological control in South Africa, *N. mexicana* as a rooted floating aquatic plant is able to occupy the freed aquatic niches, with the potential to

become increasingly invasive. *Nymphaea mexicana* was first recorded in the Vaal River near Vanderbijlpark, Johannesburg in 1968. In 2008 it was recorded in the Westlakes and Keyser's rivers in Cape Town, and by 2016 populations had extended along the length of the Westlakes river. It is now recorded in seven of the nine provinces in South Africa (Figure 1.4), and is classified as a Category 1b invasive weed according to the National Environmental Management: Biodiversity Act (No. 10 of 2004) (NEM:BA). This category prohibits trade or planting, and the species must be controlled, and where possible, removed and destroyed.

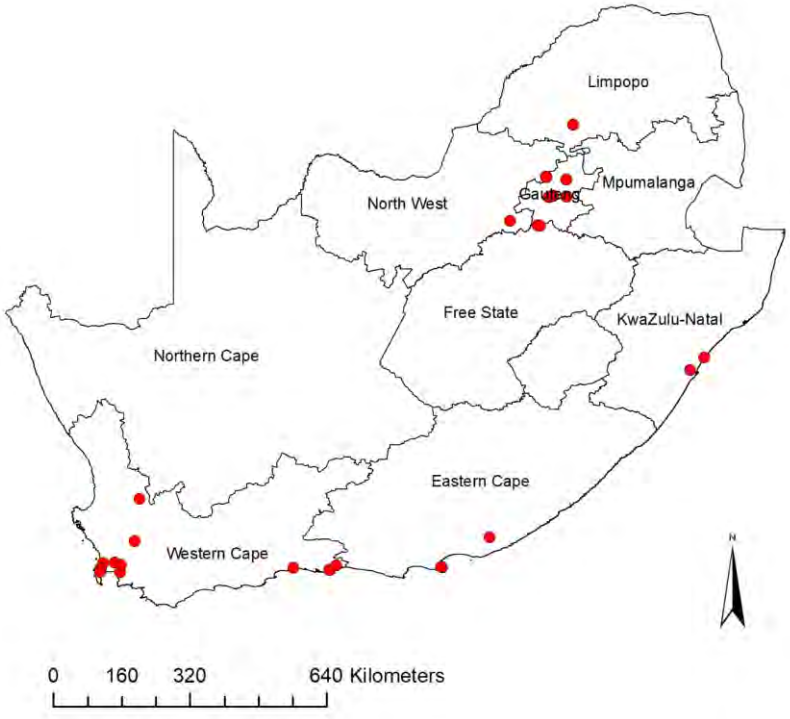

Figure 1.4: Sites in South Africa invaded by *Nymphaea mexicana*. Map created using Arcmap (Environmental Systems Research Institute 2014).

Mechanical removal of *N. mexicana* is difficult because, unlike floating weeds, the roots must be removed from submerged soils to prevent resprouting from rhizomes and stolons. As such, if left without control, *N. mexicana* has the potential to pose a greater threat than water hyacinth (Fowkes 2016). At present time, there are no registered herbicides for use against *N. mexicana* in South Africa (Fowkes 2016). Although infestations of *N. mexicana* in Westlakes, Cape Town (34°04'54.9"S 18°27'23.4"E) were originally sprayed with glyphosate, authorities resorted to manual removal after outcry from the public against the use of glyphosate (Naidu 2018). Infestations at Emmarentia Dam are also removed manually through hand-pulling of leaves by a contracted company (Naidu 2018).

As chemical and mechnical/manual control is expensive, damaging, and requires frequent reapplication, biological control is a desirable strategy to control *N. mexicana* in South Africa. There are no approved biological control agents used to control this plant in any of the countries in which is in invasive. As such, a biological control programme was initiated in 2016 by the Centre for Biological Control at Rhodes University. The biology of *N. mexicana* in South Africa was initially determined, and genetic analyses were carried out to determine the genetic structure of populations in South Africa and compare these to two populations in the native range (Naidu 2018). This was followed by further genetic work using samples from 17 populations in south-eastern USA (Reid et al. 2021), as well as field surveys in the native range to identify and prioritise potential biological control agents (Reid et al. 2020). The results from the work conducted in the initial stages of establishing a biological control programme are summarised in the sections below.

Genetic structure of *Nymphaea mexicana* in South Africa

For biological control of invasive plants to be successful, and to minimise the risk of non-target impact, it is important to utilise agents that are host specific. Biological control agents are more likely to be host specific if they are collected from populations in the native range that match genetically to populations in the invaded range (Gaskin et al. 2011). It is thus necessary to conduct molecular analyses on invasive populations in the early stages of biological control programme initiation. It was important to determine the genetic structure of *N. mexicana,* as many of the Nymphaeles hybridise readily with other *Nymphaea* species. Indeed, in some cases hybrids that were created through horticulture have naturalised in the wild and may displace indigenous 'pure' bred species (Dkhar et al. 2013, Nierbauer et al. 2014). Hybrids can be difficult to distinguish using morphological characteristics, which can become problematic in areas that are invaded. It is thus necessary to conduct molecular analyses on invasive populations in the early stages of biological control programme initiation.

Initial molecular studies using Amplified Fragment Length Polymorphisms (AFLPs) of *N. mexicana* in South Africa suggested that one out of 17 of the sampled populations matched to samples from the native range (southern USA) (Naidu 2018), while the rest were suspected to be hybrids. However, only two sites from the native range were used in this analysis, so it was necessary to repeat the study using a greater number of sites. The study was repeated using Inter-Simple Sequences Repeats (ISSRs), using 17 sites from the native range. Fourteen sites from the invaded range were included in the final analysis after the data

had been filtered and low quality samples removed. Seven out of the 14 investigated sites in the native range were genetically similar to the populations in the native range, including the same site that grouped with the native samples in the AFLP analyses (Reid 2019, Reid et al. 2021). The remaining seven populations are likely to be hybrid forms of the plant. *Nymphaea mexicana* does not appear to hybridise with the native *Nymphaea* in South Africa (Prinavin Naidu personal observation) so it is likely that other exotic species are parents of these hybrids. This knowledge is useful to target populations in the native range for biological control agents that are likely to be adapted to feeding on populations with a similar genetic structure.

Potential biological control agents for *Nymphaea mexicana*

Climate matching (using Maxent modelling) was used to prioritise regions in the native range that experienced similar climatic conditions to those in invaded sites in South Africa. Seventeen sites in Florida, Louisiana, and Texas, USA were surveyed for natural enemies of *N. mexicana* between August and October in 2018. Fifteen species were found feeding on *N. mexicana* in the native range, and some taxa were identified only to family level. Of these, two species were prioritised based on feeding damage, abundance, incidence, and observations of field host range: *Bagous americanus* LeConte (Coleoptera: Curculionidae) and *Megamelus toddi* Beamer (Hemiptera: Delphacidae) (Reid et al. 2020).

Bagous americanus is known to be hosted by *Nymphaea odorata* Aiton (Nymphaeaceae), but results from host specificity studies by Cronin et al. (1998), and preliminary host specificity tests by (Reid et al. 2020) suggest that this species is specific at least to the genus *Nymphaea.* This information suggests that it is possible that *B. americanus* may be suitably host specific for use as a control agent of *N. mexicana* in South Africa, but sufficient host specificity testing using plants from the invaded range is necessary. Little host information exists in the literature for *M. toddi,* but *Megamelus scutellaris* Berg (Hemiptera: Delphacidae) is an effective biological control agent of *P. crassipes* (Coetzee et al. 2011b, Tipping et al. 2014), suggesting that *M. toddi* may have high potential as a biological control agent. It is thus necessary to import these two species into quarantine for host specificity testing to confirm their suitability as biological control agents of *N. mexicana* in South Africa.

Potential impact of biological control

Nymphaea mexicana, like many other invasive aquatic weeds, has many negative impacts on natural ecosystems and human activities. These effects can be devastating economically,

requiring millions of dollars to remove invasive populations and mitigate negative impacts. Biological control is a long-term, eco-friendly, relatively inexpensive solution that can reduce the negative impacts caused by *N. mexicana* and other invasive weeds. Biological control also creates job opportunities through initiatives such as Working for Water, and this is especially important for developing countries with socio-economic challenges such as South Africa. Additionally, jobs are created for physically and mentally handicapped individuals from poorer communities to maintain plant and insect cultures at Waainek Research Facility at the Centre for Biological Control, Rhodes University, Makhanda, South Africa. By continuing and improving biological control efforts, these schemes will be allowed to grow and continue to provide environmental and socio-economic benefits.

1.5 THESIS OUTLINES AND AIMS

The first steps required to initiate a biological control programme for *N. mexicana* have been carried out (Naidu 2018, Reid 2019, Reid et al. 2020). This thesis aims to continue the next stages required to establish biological control of this species in South Africa. Firstly, it is necessary to conduct surveys in the invaded range of an invasive alien plant to determine whether or not potential biological control agents are already established in the invaded range, and to better understand the factors contributing to the success of the alien species (Keane & Crawley 2002, Dudley et al. 2006, Theoharides & Dukes 2007). These surveys should occur before biological control agents are introduced. Similarly, pre-introductory surveys can provide a baseline of information to compare weed coverage before and after biological control agents are released, thereby allowing researchers to measure biological control success. Pre-introductory surveys of *N. mexicana* in South Africa are therefore detailed in Chapter 2.

Success of biological control programmes may be hindered by taxonomic uncertainties surrounding the invasive target plant (Urban et al. 2011, Williams et al. 2014, Simelane et al. 2021). Hence, it is important to maximise taxonomic understanding of the invasive plant in the early stages of biological control programme development, especially where hybridisation occurs. Preliminary studies have determined that multiple *Nymphaea* hybrids exist in South Africa, but the parentage of these hybrids is not known. Chapter 3 thus aims to determine the likely parents of the sampled hybrid populations in South Africa. This knowledge will allow an expansion of the populations that can be surveyed for potential biological control agents to manage problematic *Nymphaea* hybrids.

In some cases, native insects may be found to develop new associations with invasive flora and may act as potential biological control agents without the need to import potential agents from the native range. One such South African species is identified in Chapter 2, and Chapter 4 details surveys for this species across South Africa to determine its distribution and field host range, as a means to determine its suitability for augmentative control of *N. mexicana* in South Africa. Additionally, Chapter 4 compares feeding damage by this species on *N. mexicana,* a *Nymphaea* hybrid, and the native *Nymphaea* species, while also investigating preliminary releases of the insect to manage *N. mexicana* populations in the field.

Surveys in the native range of *N. mexicana* resulted in the discovery and prioritisation of two potential biological control agents: *B. americanus* and *M. toddi.* Initial efforts to import and rear *B. americanus* in quarantine conditions were unsuccessful, and the native insect discovered in Chapter 2 fills a similar ecological niche as *B. americanus* on *N. mexicana*. Instead, *M. toddi* was imported into quarantine, and Chapter 5 details preliminary host specificity trials of this potential agent on the same test plants as described in Chapter 4. These studies allow for a comparison between the native insect and the exotic *M. toddi* as potential biological control agents for *N. mexicana*.

Finally, Chapter 6 provides an interpretive overview and synthesis of the preluding chapters, discusses the implications of the results obtained, and considers other factors to be considered to continue efforts to manage *N. mexicana* and other invasive alien plants.

Chapter 2: Pre-release faunal surveys on *Nymphaea mexicana* in South Africa*

*At the time of submission, an altered version of this chapter was accepted for publication in African Entomology.

2.1 INTRODUCTION

Surveys for insect fauna in the invaded range of an invasive alien plant, before biological control agents are introduced, are useful to ascertain whether or not enemy release is likely to contribute to the plant's invasiveness (Keane & Crawley 2002) and to determine whether potential biological control agents are already established in the invaded range (Dudley et al. 2006). This information is important to make decisions about measures to manage invasive alien plant populations and may save considerable time and money that would otherwise be invested in importing and testing potential biological control agents from the native range. For example, in South Africa, pre-introductory surveys for herbivores of *Sesbania punicea* (Cav.) Benth. (Papilionaceae) revealed that *Trichapion lativentre* (Bèguin-Billecocq) (Curculionidae), one of four weevils that was identified in the native range and imported into quarantine for host specificity testing, was already established in the country as a result of an earlier unintentional introduction (Hoffmann & Moran 1991). Thereafter, *T. lativentre* was used in an augmentative biological control programme for *S. punicea*, and the weevil became widespread and resulted in considerable control of the plant in three years (Hoffmann & Moran 1991). Similarly, in California, USA, surveys revealed that the potential biological control agent *Tetramesa romana* Walker (Eurytomidae) was already established on the invasive alien *Arundo donax* L. (Poaceae) (Dudley et al. 2008). *Tetramesa romana* was also found on *A. donax* in South Africa, as well as another specialist herbivore that had been accidentally introduced from the native range, and an additional specialist native herbivore with a closely related host plant (Canavan et al. 2014). Pre-introductory surveys can therefore provide useful information and should be prioritised especially for plants that have a long history in the invaded range and/or have been introduced multiple times.

According to the Enemy Release Hypothesis (ERH), alien plants may become invasive due to reduced herbivory as a result of the lack of natural enemies in the invaded range (Keane & Crawley 2002). This is closely linked to the Evolution of Increased Competitive Ability (EICA) hypothesis, which suggests that invasive alien plants shift resource allocation from defence to growth and reproduction when they experience reduced

herbivory (Blossey & Notzold 1995). Although there is contrasting evidence for these hypotheses, in which there may be more complex and varied explanations for invasion success in different species (Colautti et al. 2004, Joshi & Vrieling 2005), understanding the drivers for invasions are important to determine the best means of managing problematic populations. Hence, pre-release surveys are necessary to confirm whether enemy release contributes to invasion success, and to improve researchers' understanding of exotic plant invasions (Canavan et al. 2014).

Pre-release surveys are also important to determine the native insect assemblages present on invasive alien plant populations. In some cases, new associations between native insects and invasive plants develop, and may create potential for the management of problematic populations without the need to import foreign insects into the invaded range. One such example occurred in northern USA, where Eurasian watermilfoil *Myriophyllum spicatum* L. (Haloragaceae) is invasive. A native weevil, *Euhrychiopsis lecontei* Dietz (Coleoptera: Curculionidae), was found associated with declining populations of *M. spicatum* at some lakes in northern USA, where full lifecycles were recorded (Creed & Sheldon 1991, Newman & Biesboer 2000). Subsequent experiments showed a reduction in watermilfoil growth as a result of adult weevil feeding on the stems of leaves of the plants (Creed & Sheldon 1993). Additionally, *E. lecontei* preferred *M. spicatum* over a native milfoil, *Myriophyllum sibiricum* Komarov (= *Myriophyllum* ex- *albescens* Fernald) for feeding and oviposition (Creed & Sheldon 1993) and also had higher survival rates on hybrid (Northern X Eurasian) watermilfoil compared to the native species (Roley & Newman 2006). Presently, *E. lecontei* is successfully being used a biocontrol agent for *M. spicatum* in the US (Smith 2010, Thorstenson 2011). Examples such as this, although not necessarily common, demonstrate the potential for host range expansion of native insects to include exotic plants. As the native hosts of indigenous insects may not always occur at invaded sites, pre-release surveys are necessary across a broad range to determine the insect assemblages present on invasive plants.

In addition to providing useful information about the insect assemblages that occur on invasive plants in the introduced range, pre-introductory surveys are beneficial for determining the impacts of invasive plant species on native ecosystems. Acquiring such information allows biological control researchers to determine the success of biological control agents by comparing factors such as area coverage, invasive plant density, and native plant diversity, before and after the release of biological control agents (Diop & Hill 2009a,

2009b). This is also important to determine threshold population abundances under which invasive plants should be kept to restore native species richness (Gooden et al. 2009, Panetta & Gooden 2017), and to understand whether or not additional biological control agents or other forms of management are necessary to ensure that invaded habitats are effectively restored (Prior et al. 2018). Factors such as nutrient levels and shading can also be recorded to develop an understanding of interacting drivers of invasions, as well as possible constraints to biological control. Moreover, pre-introductory surveys may generate data that can be used to compare invasive alien plants in their native and invaded ranges.

Pre-introductory faunal surveys of *N. mexicana* have not previously been conducted in South Africa. Hence, in this chapter, pre-release surveys of sites across South Africa invaded by *N. mexicana* are described and discussed, with the aim of establishing baseline information for this species and determining the insect assemblages present. Surveys in the native range of this plant have already been conducted (Reid et al. 2020), so it is possible to compare the results of these surveys in the invaded range to those carried out in the native range. This will determine the role of enemy release in *N. mexicana* invasions, and to collect valuable information to assist in the development of a biological control programme for this species, as well as to make data available for comparison after the release of future biological control agents to establish their effectiveness. Hence, this chapter aims to: 1) obtain baseline information about invaded sites to assist the development of a biological control programme for the species in South Africa, 2) test the hypothesis that enemy release will apply to *N. mexicana* in South Africa, 3) test the hypothesis that plant size will be greater in the invaded range compared to the native range.

2.2 METHODS

Site descriptions

Initial surveys were conducted in Gauteng in March 2020 but were then halted due to the enforcement of level 5 lockdown in South Africa due to COVID-19. The surveys resumed in the Eastern Cape and Western Cape provinces in September 2020, and were repeated in February 2021, whereas the Gauteng surveys were repeated in December 2020/January 2021. Thirteen sites comprising both *N. mexicana* and hybrids were sampled each twice to account for possible seasonal differences in insect assemblages. Sites were selected to represent varying degrees of disturbance (four were in nature reserves, the remainder were either

artificial ponds or recreational dams in estates or parks). At some sites, second samplings were not possible as the plants had been removed manually or with herbicides.

After the repeated sampling of these sites, one site revealed the presence of a native weevil, *Bagous longulus* Gyllenhal (Coleoptera: Curculionidae) feeding on *N. mexicana.* In light of this result, additional sites across the country were included for once off sampling to increase the likelihood of finding additional herbivores including the weevil, and to incorporate more sites with varying genetic structure and growing alongside native *Nymphaea* species (Figure 2.1).

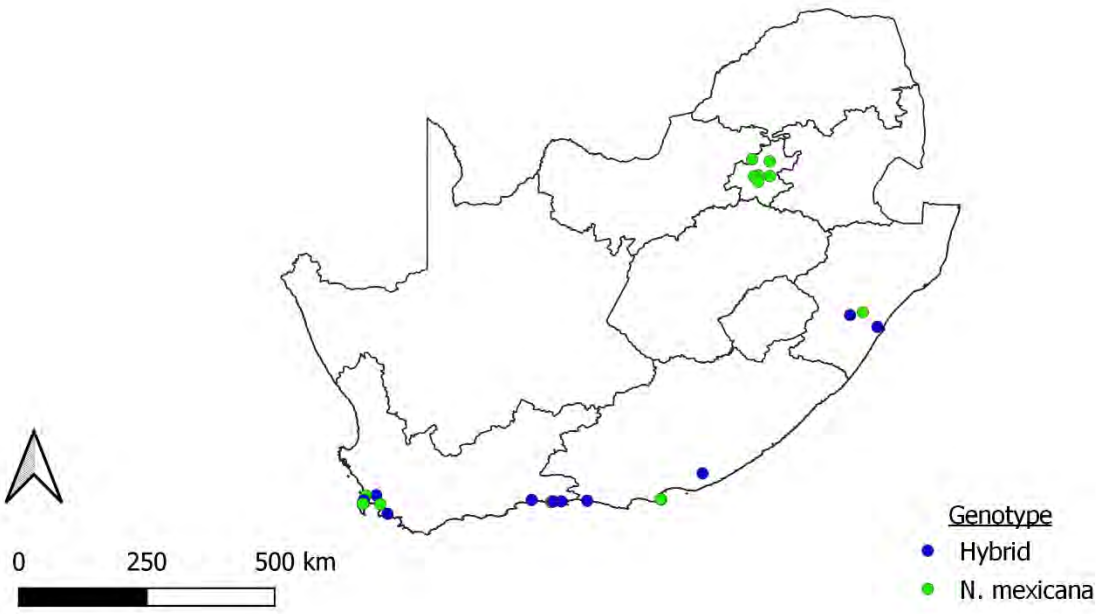

Figure 2.2.1: Sites invaded by *Nymphaea mexicana* and *Nymphaea* hybrids included in pre-release surveys. Genotype indicated as hybrid (blue) or *N. mexicana* (green). Map produced using QGIS (QGIS Geographic Information System 2021).

Water nutrient measurements

A Sanxin PC5 pH/conductivity multimeter (Shanghai San-Xin Instrumentation Inc.) was used to take readings of water parameters at each site including pH, temperature, total dissolved solids, electrical conductivity, and salinity, all replicated five times. Four 50 mL plastic containers were used to collect water at the sites that were sampled twice within the year as well as two additional sites to obtain nitrate and phosphate concentration measurements. These water samples were analysed in the laboratory at the Institute for Water Research at Rhodes University, Makhanda, Eastern Cape. Water samples were

analysed for orthophosphate-phosphorous (PO_4-P) using a photometric phosphate Spectroquant® concentration test kit (HC399495) and a Biotek microplate reader, utilising calibration curves to calculate phosphate in mg L^{-1}. Nitrate-nitrogen (NO_3-N) was analysed according to summarised protocols from Ondrus (1996) and APHA (American Public Health Association) (1998) (Nelson Odume and Khaya Mgaba, pers. comm.).

The nitrate analysis was conducted by adding 25 mL of sample to 25 mL of distilled water in 250 mL Erlenmeyer flasks, including a blank sample. 1 mL of 1:4 HCl and 1 mL sulfanilic acid were added, followed by 1.5g Zinc and NaCl mixed powder. The samples were then shaken thoroughly until the powder dissolved, 1 mL of buffer-colour reagent was added, before adding 1 mL sodium acetate ($NaCH_3COO$) to each sample and mixing. The samples were left for five minutes to allow for colour development, and three replicates of 250 µL of each sample as well as the blank were added to a 96-well microplate and read at 540 nm in a Biotek microplate reader. The concentrations of nitrate were calculated in mg L^{-1} by entering the blanked readings obtained from the microplate reader into the equation obtained from a calibration curve prepared before samples were processed. If negative values were present, they were set to half of the lowest concentration value obtained to allow for statistical analyses (N. Odume and K. Mgaba pers. comm.).

Sites where nitrate and phosphate levels were tested were classified as oligotrophic (< 500 µg N/l, < 5 µg P/l), mesotrophic (500 – 2 500 µg N/l, 5 - 25 µg P/l), or eutrophic (2 500 – 10 000 µg N/l, 25 – 250 µg P/l) according to the South Africa Water Quality Guidelines for Aquatic Ecosystems (DWAF 1996).

Plant measurements

At each site, *N. mexicana* plant cover was estimated across the visible water body using a DAFOR abundance scale (Dominant: 75-100%, Abundant: 50-75%, Frequent: 25-50%, Occasional: 5-25%, Rare: 1-5%) (Kent & Coker 1992). To obtain plant coverage percentage estimates, two to three researchers (the principal investigator, and the field assistant(s)) independently estimated the coverage of *N. mexicana* on the visible open water body. The means of these estimates were calculated to reduce sampler bias. If other *Nymphaea* species were present, their percentage coverage was also estimated, and the presence of other emergent vegetation and plant species were recorded. Leaf size can be used as an indication of resource allocation to growth and is thus useful to allow comparison between sites and develop an understanding of effects of herbivory (as a source of stress

resulting in declining plant size) and nutrients on populations (Poorter et al. 2012). Although crowding may affect leaf size, preliminary observations in the field determined that leaf size was still comparatively large even when the plants grew in high densities. Hence, *Nymphaea mexicana* leaf length (cm) was used as an indicator of leaf size and was measured using a ruler or tape measure from five random leaves at each site.

Insect assemblages

Leaves and stems were investigated for feeding damage for approximately 30 minutes. During this time, estimates of leaf damage were made by visual observation, and plants were monitored for insect feeding behaviour and mode of damage. If an insect was associated with *N. mexicana* damage, it was recorded. Where *N. mexicana* was surrounded by other vegetation, the surrounding plants were also searched for insects.

Site descriptions and statistical analyses

Descriptions of each site, including estimates of percent coverage by *N. mexicana* and notes on levels of damage, were compiled to form a baseline of information with which to compare the extent of invasion at each site pre- and post- biocontrol agent release. Percent coverage and leaf size was used as a metric to compare the level of invasion between sites. The species richness of herbivorous insects found damaging *N. mexicana* was calculated for each site, and incidental species were excluded. Individual based rarefaction species accumulation curves, using Chao 2, ICE (incidence coverage estimator), and Jack1 estimators, were generated for the insects collected at each site using EstimateS version 8.0 (Colwell 2006). Different estimators may produce varying accuracy, so multiple indicators were used in this case to estimate species richness based on results from other studies (Martı´nez-Sanz et al. 2010, González-Oreja et al. 2010). Species accumulation curves were generated for sampling occasions for all sites.

All subsequent statistical analyses were carried out in R version 4.1.0 (R Core Team 2021) with model diagnostics performed using the DHARMa package (Hartig 2021) and figures made using ggplot2 (Wickham 2016). As *Bagous longulus* was found feeding and reproducing on *N. mexicana* at three sites and was also found at a few hybrid sites, *B. longulus* presence was included in some of the analyses as a categorical predictor.

To evaluate the impact of water chemistry variables on leaf length, a general additive model (GAM) was used from the mgcv package (Wood 2010). Leaf length (cm) was

modelled as a function of pH, electrical conductivity (µS), total dissolved solids (ppm), salinity (ppt) and temperature (ºC). Site was included as a smoothed penalised random effect to account for non-linear variation between sites not measured by the other effects. A GAM was selected to account for non-linear trends between leaf length and the water chemistry variables. A Wald's test was used to test the significance of each factor.

To assess the effect of nitrate and phosphate concentrations (µg/L) on leaf length (cm), a generalised linear mixed model was used to test leaf length as a response variable with nitrate and phosphate as predictor variables, and site as a random effect to account for variation. Significance was assessed using a Likelihood-Ratio Test (LRT).

As an investigation of the effects of *B. longulus* presence and plant genotype on leaf length (cm), a linear mixed effects model was run using site as a random effect to account for variation, and *B. longulus* presence and plant genotype as factors. Models including different combinations of the fixed effects were tested against a null model to assess the best model fit using ANOVA. Assessment of statistical significance for the best model was achieved using an LRT.

To assess whether leaf length differed significantly between the native and introduced range of *N. mexicana,* a linear mixed effects model was used with length (cm) measurements as the response variable, range as a fixed effect, and site as a random effect, assessing significance with an LRT. Data from the native range surveys were collected in Reid et al. (2020).

Finally, the three sites where *B. longulus* was reproducing on *N. mexicana* were compared in terms of percentage plant cover and leaf length at each of the sites. These comparisons did not involve statistical tests because of the small number of sites. Instead, general descriptive differences were noted.

2.3 RESULTS

Site descriptions

Twenty-four *N. mexicana* sites were surveyed, of which 10 also had hybrids. At most of the sites, there were low levels of damage, and if there was leaf damage, this was usually caused by generalist herbivores such as snails, aphids, or natural decay. The species richness at the sites was low, even after a second sampling, with a mean species richness of 1.7 ± 1.1 SD across sites. Florida Lake in Gauteng (26°10'36.7"S 27°54'28.8"E) had the highest

species richness of five species, but nevertheless damage levels were low at this site. Most of the species encountered were generalists, such as Aphididae, snails, Erebidae, or crambid moths such as *Parapoynx* sp. Hübner (Lepidoptera: Crambidae).

Many of the sites had high levels of estimated percentage coverage of *N. mexicana* and its hybrids, and a few - Yellowwood Dam in Somerset West (34°05'38.8"S 18°51'54.3"E), Maynardville Park in Cape Town (34°00'19.1"S 18°27'51.5"E), Intaka Island, Century City in Cape Town (33°53'16.1"S 18°30'46.1"E), Moreleta Kloof Nature Reserve in Pretoria (25°48'50.0"S 28°17'05.3"E) - were managed periodically by means of manual or chemical control. The mean estimated percentage coverage of the water bodies sampled (after taking the means of both sampling sessions for the sites that were surveyed twice) was 41.17 % ± 32.36 SD. Based on the percentage coverage using the DAFOR classification, of the 24 sites surveyed, eight were classified as occasional, four as frequent, seven as abundant, and four as dominant. These included sites that were managed periodically using chemical or manual control, as well as those that had higher levels of damage by herbivores. A detailed description of the conditions at each site is given in Table 2.1.

Table 2.1: Detailed site descriptions of waterbodies invaded by *Nymphaea mexicana* in South Africa (March 2020 – March 2022), including information about damage to plants by herbivores. Coverage estimates were taken from at least two independent researcher observations in the field and averaged to reduce bias. Some sites were sampled twice to account for possible seasonal effects.

Site/Code	**Province**	**Survey date**	**Genotype**	**Site description**	**Mean coverage estimate %**	**Herbivore species richness**	**Notes on damage**
				Sites sampled twice			
Emmarentia Dam	Gauteng	15 Mar 2020	*N. mexicana*	No shade, *Pontederia cordata* L. (Pontederiaceae) growth around banks. > 1 m depth.	70	3	Some snail damage, a few tearing marks possibly from birds.
		23 Dec 2020			80	3	Minimal leaf damage.
Benoni Lake, Homestead	Gauteng	16 Mar 2020	*N. mexicana*	Knee depth near banks, up to 6m deep. Reports of sewage flow into the dam.	8 (of visible dam area)	2	Lots of yellowing (presumably from aphids), some minor chewing damage.

				Reports of *Nymphaea nouchali* Burm. f. (Nymphaeaceae) occurrence previously. *Pontederia crassipes* Mart. (Pontederiaceae) present.			
		22 Dec 2020			4	3	Low damage overall, but some scraping/chewing damage from Erebidae (Lepidoptera) larvae.
Florida Lake	Gauteng	16 Mar 2020	*N. mexicana*	Partly shaded, dense growth along banks. 0.4-0.8 m depth along banks. Littered water.	15	5	Chewing damage from green Lepidoptera larvae, some damage from snails.

		23 Dec 2020			10	5	Green and brown Lepidoptera larvae present. Many aphids.
Cosmos Lake, Pecanwood Estate	North West	17 Mar 2020	*N. mexicana*	Shaded along bank deepest point 2-3m, most growth in middle of dam. Landowners arrange manual removal along banks.	75	1	Low levels of damage, any present likely from snails.
		28 Dec 2020			50	1	Very low levels of damage, only snails recorded.
Moreleta Kloof Nature Reserve	Gauteng	17 Mar 2020	*N. mexicana*	Interdispersed with heavy growth of *Typha* sp. L. (Typhaceae) and *N. mexicana*. Approximately 0.7 m deep. Some	35 (remainder occupied by *Typha* sp.)	0	Very healthy leaves, no damage apart from normal decay of old leaves.

				shading along edges, reports of sewage flow from upland. Landowners manage plants through manual removal.			
		28 Dec 2020			3 (of visible water body, remainder occupied by *Typha* sp.)	1	Snails present, very low levels of damage.
Klipriviersberg Nature Reserve	Gauteng	18 Mar 2020	Unknown	Small pond at reserve entrance, slight shading, approximately 0.5 m depth. Dense surrounding cover (*Cyperus* sp. L. (Cyperaceae) and Poaceae).	75	1	Some damage from snails, and some yellowing. Otherwise, minimal.

				Myriophyllum aquaticum (Vell.) Verdc. (Haloragaceae) also present.			
		22 Dec 2020		Pond had been drained, no *N. mexicana* present.	0		
King's Beach Skate Park, Port Elizabeth	Eastern Cape	18 Sept 2020	*N. mexicana*	Open artificial pond, some intermittent shading along the banks. 0.5-1 m depth. Worker employed to pull leaves occasionally.	30	2	*Bagous longulus* Gyllenhal (Coleoptera: Curculionidae) found, chewing damage (shot holes in leaves) and mining.
		10 Jan 2021			20	2	High levels of damage, holes from chewing (*B. longulus* and snails) and mines.

Tsitsikamma National Park	Eastern Cape	18 Sept 2020	Hybrid	Shading around edges, another pond 200 m along trail with *Nymphaea nouchali* and *Bagous longulus*. 1 m depth.	50	0	No insect damage obvious, some fungal damage and natural rotting of old leaves.
		9 Jan 2021			95	3	Scale insects (possibly Coccidae or Pseudococcidae) present in abundance on some leaves (especially those shaded). Some yellowing, but minimal damage overall.
Horse Fields Along N2, George	Western Cape	18 Sept 2020	Hybrid	Various waterlilies present (pink and white hybrids, as well as native *N. nouchali*). *N.*	6	1	*B. longulus* present, some chewing damage but no mining.

				mexicana hybrid with greatest coverage. *Pontederia cordata* present along banks. Turbid water, no shade.			
		25 Feb 2021			35	2	Some damage from Crambid moths, *B. longulus* present but minimal damage on any of the hybrids.
Westlakes, Muizenberg, Cape Town	Western Cape	19 Sept 2020	*N. mexicana*	Under bridge, many invasives around including *Pistia stratiotes* L. (Araceae), *Nasturtium officinale* W.T.Aiton (Brassicaceae),	3	0	No noticeable herbivores.

				Azolla filiculoides Lamarck. (Salviniaceae), *Myriophyllum aquaticum,* *Sagittaria* sp. L. (Alismataceae). Site is managed using mechanical/manual removal.			
		26 Feb 2021			60	2	Evidence of some Lepidoptera feeding and snail damage, but low damage overall.
Intaka Island, Century City, Cape Town	Western Cape	19 Sept 2020	*N. mexicana*	Wetland under balcony, no shade, surrounded by *Typha*. Other invasives present, including *Azolla*	90	1	Low levels of damage, only some snail damage and yellowing.

				filiculoides and *Ceratophyllum demersum* L. (Ceratophyllaceae). Under 1 m depth. Reported treatment using chemical and manual control.			
		27 Feb 2021			55	0	Some leaf spots, otherwise low damage.
Maynardville Park, Cape Town	Western Cape	20 Sept 2020	Unknown	Pond with some shading in the middle, littered and tepid water. Plants managed using manual/chemical control.	1	2	Minimal damage, some Chironomid larvae and snails.
		27 Feb 2021			0 – recent treatment/removal.		

Yellowwood Dam, Somerset West	Western Cape	20 Sept 2020	*N. mexicana*	Dam in residential area, some shading around banks but high coverage of *N. mexicana*. Evidence of manual/mechanical removal.	95	2	Some snails and Chironomid larvae, lots of snail eggs.
		26 Feb 2021			40	2	Little/no damage. Aphids present.
Sites sampled once							
Boardwalk pond, Port Elizabeth	Eastern Cape	10 Jan 2021	*N. mexicana*	Pond leading to larger section, minimal shading along edges, too deep to wade. Other waterlilies present, including *N. nouchali,* possibly *N. odorata*, but *N.*	90	2	Little damage, only some yellowing significant. Aphids and snails present.

				mexicana coverage most prominent. *Pond has since been drained for renovation of the Boardwalk.			
Knysna Estate	Western Cape	25 Feb 2021	*N. mexicana*	Dam in residential estate, runoff reported from fertilized gardens uphill, lots of algae present. 1 m depth along edges, steep increase in depth towards middle. Native *N. nouchali* present in small numbers along edges.	60	2	Evidence of Crambid moth larval damage. *Bagous longulus* present in high abundance, relatively few larval mines.
Bellevue Wine Estate	Western Cape	27 Feb 2021	Hybrid?	Pond on roadside coming into estate.	20 (remainder *Salvinia molesta*)	0	No herbivores found. No noticeable damage.

				Nymphaea mexicana present in non-shaded sections. *Salvinia molesta* D.Mitch (Salviniaceae) present in abundance. Polluted water, seepage from pipe protruding from under road.			
Radway Green, Manley Flats	Eastern Cape	10 Mar 2021	Hybrid	Pond on side of farm road, lots of algae present, *Typha* abundant along banks.	10	2	Low damage overall, but Coccidae/Pseudococcidae present on underside of some leaves.
Kleinmond Golf Course	Western Cape	26 Feb 2021	Hybrid	Pond on golf course, native *Nymphaea* present in greater	20	2	*Bagous longulus* present in higher abundances on native *Nymphaea*, some mining in hybrid, 1-2

				abundance than hybrid. Unshaded. *Pontederia cordata* present along banks.			early instar larvae found in hybrid stems, compared with more abundant later instar larvae in native *Nymphaea.*
Haga Haga Farm	Eastern Cape	12 Feb 2021	Hybrid	Farm dam with some shading along banks, native *N. nouchali* present alongside hybrid.	60	1	*Bagous longulus* present, damaging and reproducing on native *Nymphaea,* only a few exploratory feeding on hybrids, with only early instar larvae not reaching petiole.
Masescha Lodge, Plettenberg Bay	Western Cape	8 May 2021	Hybrid	Small, shallow dam in garden, without shade. *Nymphaea nouchali* present in similar numbers.	35	2	Some snail damage, and exploratory feeding by *B. longulus.* No *B. longulus* larvae found in hybrid stems, multiple larvae found in native *Nymphaea* stems.

Pledge Nature Reserve, Knysna	Western Cape	8 May 2021	Hybrid	Two small ponds surrounded and shaded by trees and Cyperaceae. Reports of occasional sewage overflow from upland areas during heavy rains. 1 m depth along banks to more than 2 m. Native *Nymphaea* present.	30	1	Some fungal spotting and yellowing.
Belvidere pond, Knysna	Western Cape	8 May 2021	*N. mexicana*	Shallow pond in middle of park, shaded by trees. *Azolla filiculoides* present, as well as *Typha* along banks.	90	1	Lots of aphids, some yellowing, similar to Yellowwood dam in Somerset West.

New Germany Nature Reserve	KwaZulu-Natal	7 March 2022	Hybrid	Open dam, some shading at edge at southern edge.	96	0	Some evidence of sap sucking, no herbivores seen.
Fountainhill Estate	KwaZulu-Natal	8 March 2022	*N. mexicana*	Waterway either side of bridge in full sun, mixed with native *Nymphaea*.	13	1	Damage from *Bagous longulus*, larvae found in abundance in stems, suggesting reproduction. *B. longulus* also on native *Nymphaea*.
Sakabula Golf Club	KwaZulu-Natal	8 March 2022	*N. mexicana*	Dam, water from groundwater supply. Overflow from adjacent properties, knee depth.	37	1	Some yellowing from aphids.

At three of the sites (King's Beach Skate Park: 33°58'26.4"S 25°38'39.8"E, Knysna Estate: 34°01'30.4"S 23°00'44.6"E, and Fountainhill Estate: 29°27'49.1"S 30°32'25.4"E), higher levels of leaf damage were observed compared to the other surveyed sites. Multiple feeding holes were present in the leaves, in addition to signs of mining. On closer inspection, *Bagous longulus* (previously known as *Pseudobagous longulus* Gyllenhal (Coleoptera: Curculionidae)) was collected in at these sites, and all lifecycle stages were present on *N. mexicana.* At King's Beach Skate Park and Knysna Estate, *N. mexicana* leaf growth density was less dense and with smaller leaf sizes compared to all other surveyed sites where *B. longulus* was not present (Figure 2.2). At Fountainhill Estate in KZN, coverage by *N. mexicana* was only 13% compared with 56% by *Nymphaea nouchali* Burm. f. (Nymphaeaceae), but leaf size did not differ considerably compared to the other sites. *Bagous longulus* is native to South Africa and is typically recorded on native South African *Nymphaea* such as *N. nouchali* (R. Stals pers. comm.). At sites where native *Nymphaea* spp. were growing with *N. mexicana* hybrids, lower levels of feeding damage were observed on the hybrids, and only early larval instars were found in the hybrid leaves.

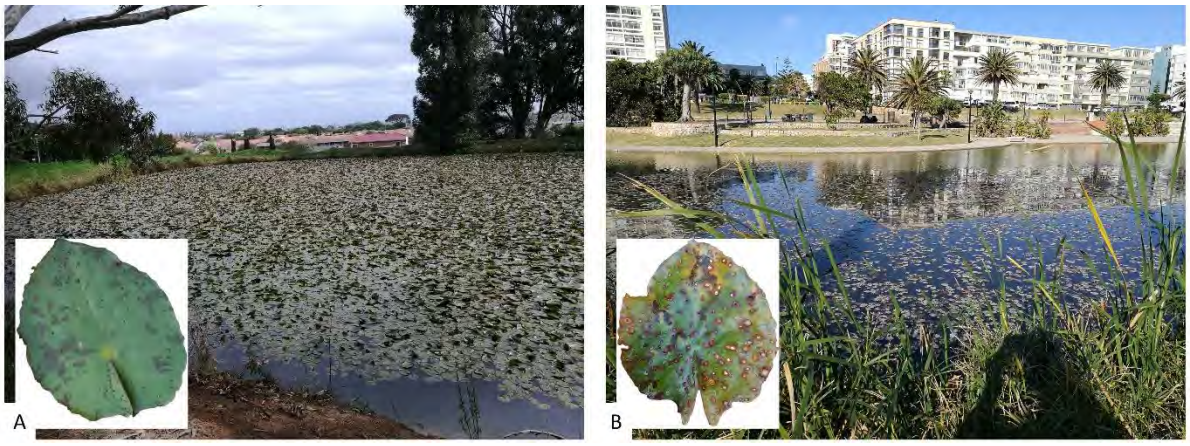

Figure 2.2: Comparison of coverage and leaf damage levels of *Nymphaea mexicana* at two invaded sites in South Africa. A: High coverage and low damage levels at Yellowwood Dam, Somerset West, Western Cape, where *Bagous longulus* was not present. B: Lower coverage and higher damage levels at King's Beach Skate Park in Port Elizabeth/ Gqeberha where *B. longulus* was present.

Species accumulation curves

The indicators used to generate curves suggested that herbivore communities on *N. mexicana* were adequately sampled in South Africa. This was indicated by the S(est), ICE, Chao 2 and Jack 1 curves reaching asymptotes. The Chao 2 upper 95% confidence interval curve suggested that about 16 additional species could be present (Figure 2.3).

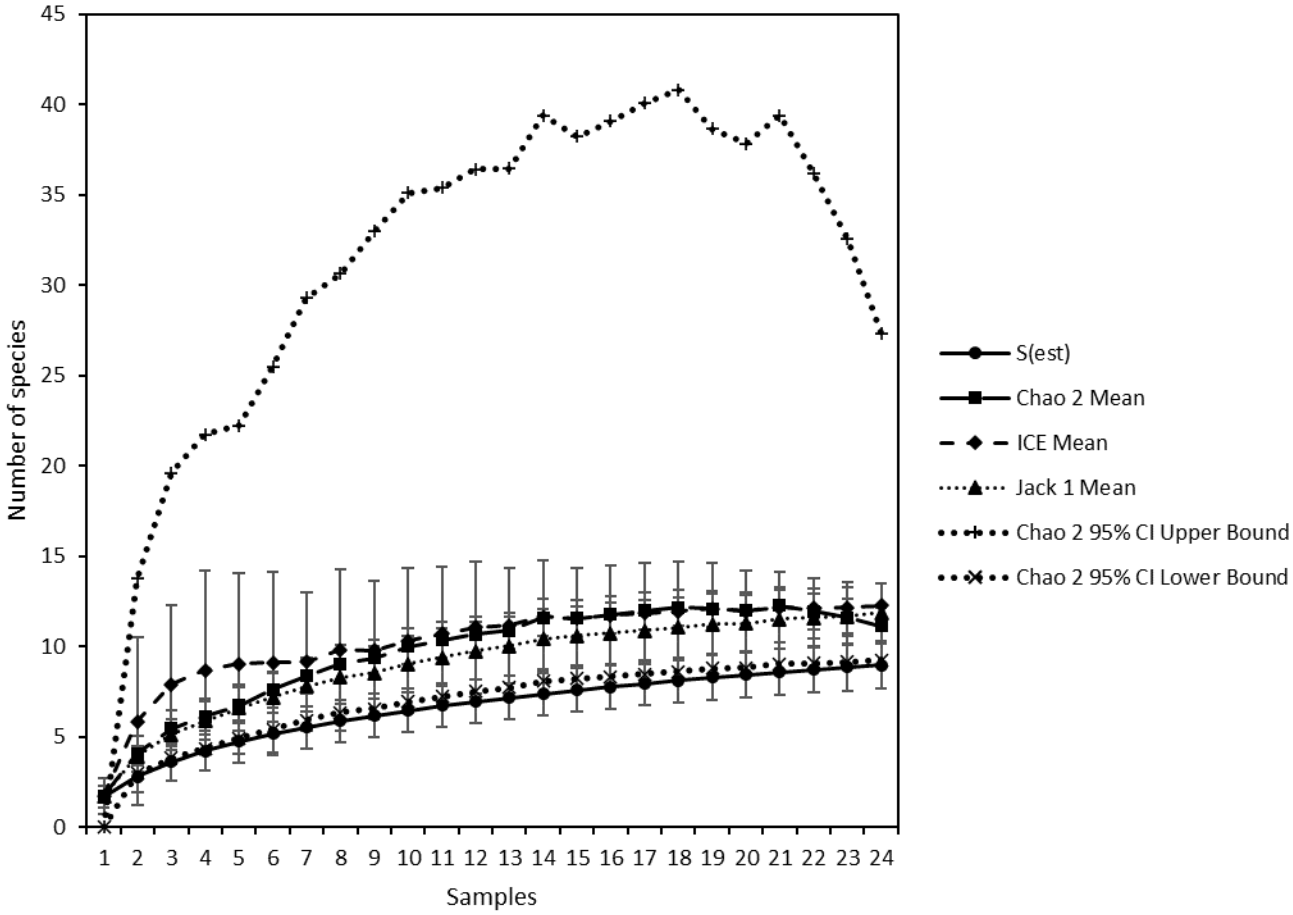

Figure 2.3: Species accumulation curves for *Nymphaea mexicana* sites sampled across South Africa.

Water parameters

The water parameters varied considerably between sites, indicating that *N. mexicana* can survive in a range of conditions. The mean (± SD) pH was 7.2 (± 0.8), with a broad range of electrical conductivity (509.4 ± 517.6 µS), total dissolved solids (361.4 ± 369.3 ppm), and salinity measurements (241.1 ± 271.2 ppt) (Table 2.2). For the sites where phosphate concentrations were tested, eight were classified as oligotrophic, six as mesotrophic, and one as

eutrophic. For nitrate concentrations, seven were classified as oligotrophic, six as mesotrophic, and two as eutrophic. Intaka Island in Cape Town had the highest nitrate concentration (mean ± SD) (7080 ± 3510 µg/L), followed by Westlakes in Cape Town (34°04'52.8"S 18°27'19.4"E) (2090 ± 2220 µg/L) and Florida Lake in Gauteng (2000 ± 222 µg/L), whereas Florida Lake also had the highest phosphate levels (79.1 ± 6.26 µg/L), followed by Yellowwood Dam in Somerset West (21.5 ± 18.4 µg/L), and Intaka Island (12.7 ± 9.45 µg/L).

Table 2.2: Mean (± S.D.), minimum, and maximum values for water parameters measured at sites invaded by *Nymphaea mexicana* or *Nymphaea* hybrids across South Africa.

Parameter	**Unit**	**Mean**	**Standard deviation**	**Minimum**	**Maximum**
pH	-	7.2	0.8	5.3	9.1
Electrical conductivity	µS	509.4	517.6	66.4	2070
Total dissolved solids	ppm	361.4	369.3	46.3	1460
Salinity	ppt	241.1	271.2	0.03	1030
Temperature	ºC	24.0	4.4	16	35.5

Leaf size

The mean leaf length of *N. mexicana* in the sites surveyed across the country was 19.0 cm ± SD 4.2. Most of the sites had similar leaf sizes, but two sites, namely King's Beach Skate Park (9.8 cm ± 2.4) and Knysna Estate (9.1 cm ± 1.2), had considerably smaller leaf sizes. As a general observed trend, *N. mexicana* sites where *B. longulus* was absent had longer leaves on average (mean ± SD) (19.8 ± 2.7 cm) compared to *N. mexicana* sites where *B. longulus* was

present (13.8 ± 6.6 cm) (Figure 2.4). This did not apply at hybrid sites, where mean leaf length was similar whether *B. longulus* was absent (20.1 ± 2.9 cm) or present (21.4 ± 3.4 cm).

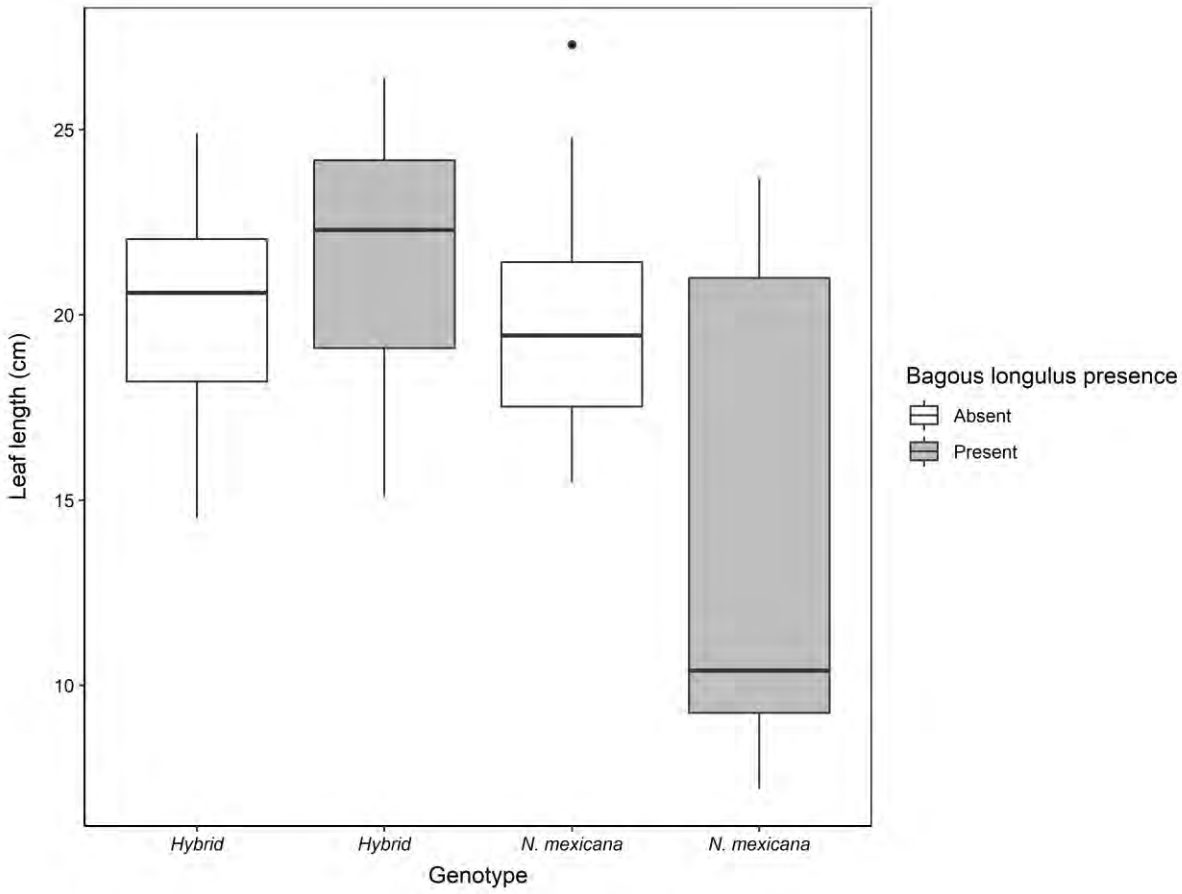

Figure 2.4: Leaf lengths of different *Nymphaea mexicana* genotypes (*N. mexicana* vs. hybrid) at invaded sites across South Africa. Boxes represent upper and lower quartiles and median values; whiskers represent highest and lowest values at 1.5 times the interquartile range. Outliers are marked as separate dots. Boxplots are coloured according to presence or absence of the weevil *Bagous longulus*.

Analyses of the effects of water parameters on leaf length using the GAM model revealed that leaf length did vary significantly by site ($F = 23.1500$, d.f. = 18.00, $P < 0.001$). There was also a significant effect of pH on leaf length ($F = 5.0500$, d.f. = 1, $P < 0.05$), with leaf length decreasing with increasing pH, but the remaining water parameter factors were not significant (all P-values < 0.05). This indicates that electrical conductivity, total dissolved solids, temperature, and salinity, did not significantly affect leaf length. Similarly, leaf length was not

affected by differences in nitrate ($\chi^2 = 0.0183$, $P = 0.8923$) and phosphate ($\chi^2 = 1.4698$, $P = 0.2254$) concentrations between sites (Figure 2.5).

For investigations of the effect of *B. longulus* presence and genotype on leaf length, a linear mixed effects model including an interaction between *B. longulus* presence and genotype was a significantly better fit than a null model and models with *B. longulus* presence and genotype included separately ($\chi^2 = 4.9332$, d.f. = 1, $P < 0.05$). Hence, *B. longulus* and genotype had a statistically significant interaction effect on *N. mexicana* plant length ($\chi^2 = 4.5016$, d.f. = 1, $P < 0.05$).

When the leaf lengths of *N. mexicana* in South Africa were compared with the leaf lengths in the native range (from Reid et al. 2020), leaf lengths in the invaded range (mean ± SD) (19.0 cm ± 4.19) were significantly greater ($\chi^2 = 36.8170$, d.f. = 1, $p << 0.001$) than those in the native range (12.1 cm ± 3.17). This reflected a difference in length of 36%. Most of the sites in the native range had smaller leaf sizes than those from the invaded range, except for Knysna Estate and King's Beach Skate Park (Figure 2.6). Additionally, leaves from Lake Okeechobee in the native range were bigger on average and more similar to leaf sizes from other sites in the invaded range. However, Fountainhill Estate where *B. longulus* was also present had similar leaf sizes to *N. mexicana* and hybrid sites where *B. longulus* was not present (Figure 2.6).

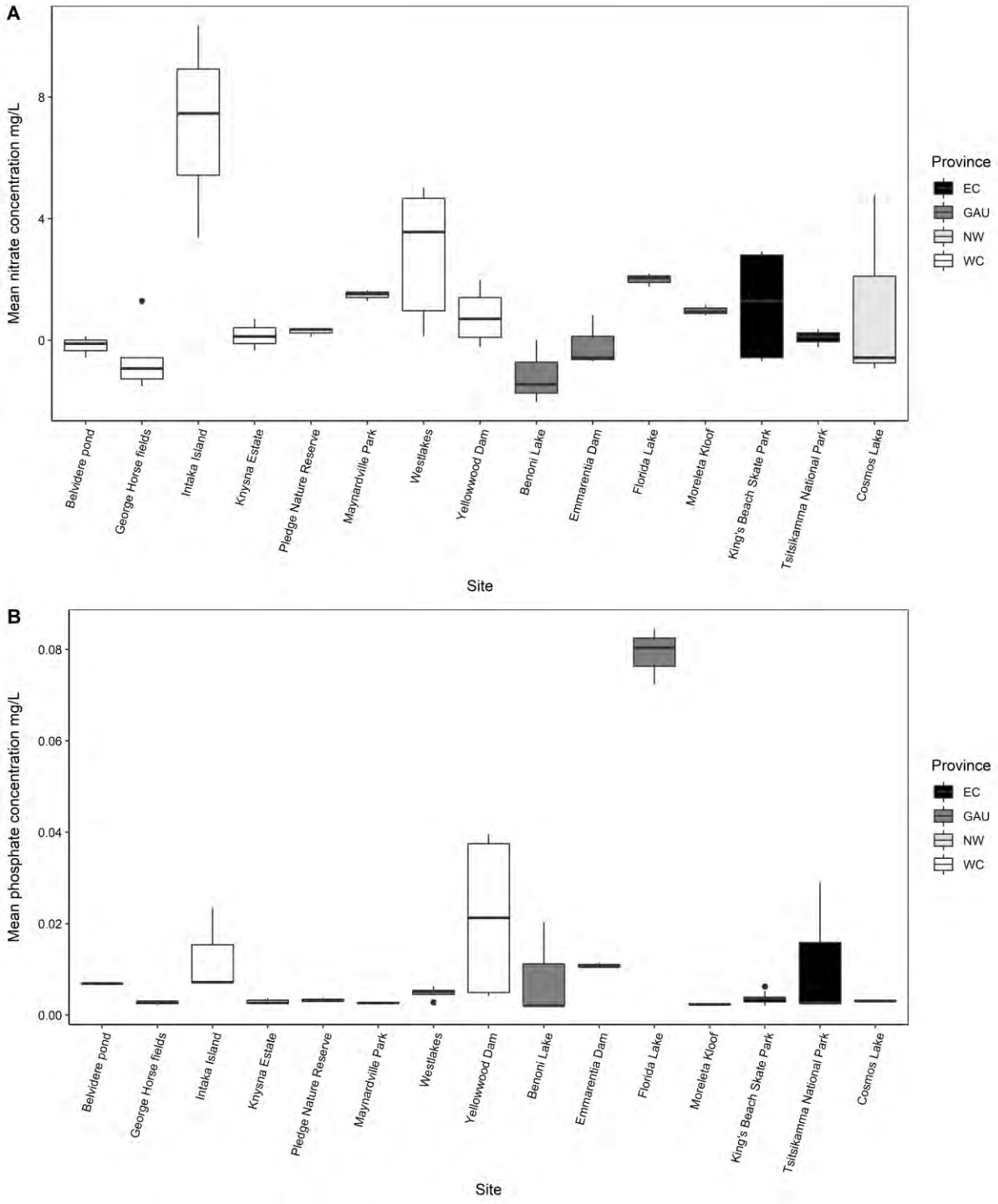

Figure 2.5: Nitrate (A) and phosphate (B) concentrations (mg/L) at various sites invaded by *Nymphaea mexicana* in South Africa. Boxes represent upper and lower quartiles and median values; whiskers represent highest and lowest values at 1.5 times the interquartile range. Outliers

are marked as separate dots. Sites are arranged by province, where EC = Eastern Cape, GAU = Gauteng, WC = Western Cape, NW = North West.

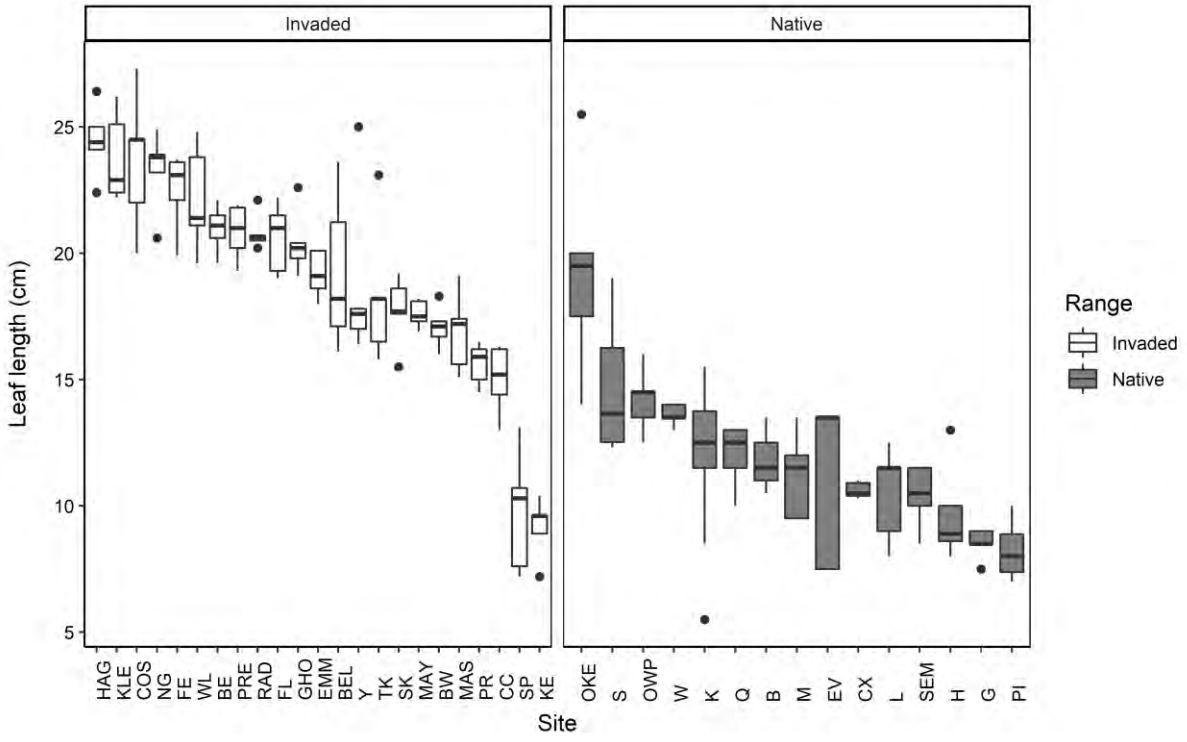

Figure 2.6: Ordered leaf lengths of *Nymphaea mexicana* in its invaded (South Africa) and native range (southern USA). Boxes represent upper and lower quartiles and median values; whiskers represent highest and lowest values at 1.5 times the interquartile range. Outliers are marked as separate dots. Name codes for each site are given in Reid et al. (2021). For those not given in Reid et al. (2021), Y = Yellowwood Dam, Western Cape; FL= Florida Lake, Gauteng; COS = Cosmos Close, North West; KLE = Kleinmond Golf Club, Western Cape; MAS = Masescha Lodge, Western Cape; NG = New Germany Nature Reserve, KZN; FE = Fountainhill Estate, KwaZulu-Natal; SK = Sakabula Golf Club, KwaZulu-Natal.

To understand differences in leaf length between the three sites where *B. longulus* was present, plots of the data indicated lower pH and electrical conductivity at Fountainhill Estate and Knysna Etate compared to King's Beach Skate Park (Figure 2.7). At sites where *B. longulus* was present and surviving on *N. mexicana,* the greatest leaf length was recorded at Fountainhill Estate (22.5 ± 1.6 cm) and the highest percentage cover by *N. mexicana* at Knysna Estate (60.7%), followed by King's Beach Skate Park (22.5%) and lastly Fountainhill Estate (12.5%).

Furthermore, Fountainhill Estate had 56% coverage by the native *N. nouchali* compared to only 4 % at Knysna Estate and 0% at King's Beach Skate Park.

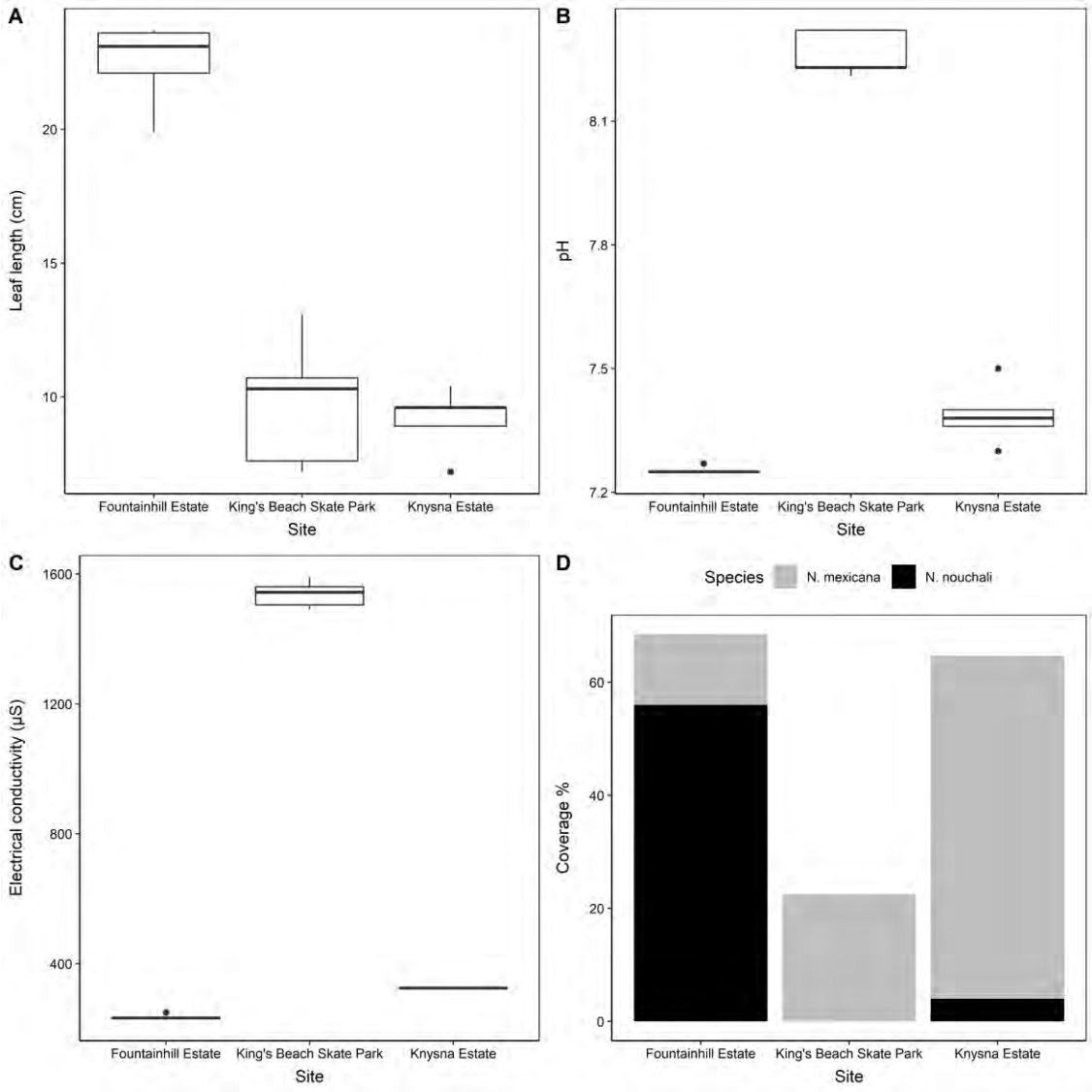

Figure 2.7: Leaf length (A), pH (B), electrical conductivity (C) and plant coverage (D) at three sites where *Bagous longulus* was feeding and reproducing on *N. mexicana* in South Africa. Total dissolved solids and salinity measurements were also taken, but because these are correlated with electrical conductivity, only EC is presented.

2.4 DISCUSSION

Most of the sites invaded by *N. mexicana* in South Africa had dense growth, large leaves, and low levels of damage by herbivores. Generally, few herbivorous species were encountered,

and those that were present were generalists, low in abundance, and exerted little damage. This contrasts to greater levels of herbivory and reduced growth density and leaf sizes of *N. mexicana* in its native range (Reid et al. 2020). These data are similar to other studies that have compared introduced plant sizes and herbivore load in their native and invaded ranges (Memmott et al. 2000, Paynter et al. 2003, Jakobs et al. 2004). This provides evidence that enemy release, at least in part, contributes to the success of *N. mexicana* in South Africa. It should be noted, however, that other factors likely play a significant role (Hierro et al. 2005, Vasquez & Meyer 2011, Fleming & Dibble 2015) and further studies may be necessary to determine the other factors driving *N. mexicana* invasions. Nevertheless, the observations obtained during this study suggest that it is likely that introducing specialist herbivores for biological control would result in a reduction in leaf size and growth density for this species in South Africa.

Two *N. mexicana* sites had comparatively sparse leaf growth, smaller leaf sizes, and higher levels of damage. These differences were attributed to the presence of *B. longulus*, a weevil that occurs on native *Nymphaea* in South Africa (Riaan Stals, SA National Collection of Insects, Ditsong National Museum of Natural History, Iziko South African Museum, iNaturalist). This weevil was found in abundance at these sites, and all life stages were present on *N. mexicana*. The leaves had multiple feeding scars from adult chewing, and larval mines leading to the petiole, where late larval instars, pupae, and teneral adults were found. The abundance of *B. longulus* on *N. mexicana* at these sites and the high damage levels incurred on the plants suggests that a host expansion of a native insect onto this invasive alien plant has occurred. The leaf sizes of the sites at which *B. longulus* was present were comparable to the leaf sizes of sites in the native range. This could act as further evidence of the contribution of enemy release to *N. mexicana* invasions in South Africa and indicates promise for the possibility of using *B. longulus* as an augmentative biological control agent.

An additional site in KwaZulu-Natal also showed evidence of *B. longulus* damage and reproduction on *N. mexicana*, but the leaf sizes were more similar to sites where *B. longulus* populations were not sustained on exotic *Nymphaea*. There are several possible explanations for this: 1) Although the plants were initially identified morphologically as *N. mexicana*, it is possible that some hybridisation has occurred and that they are genetically different to the *N. mexicana* at the two other sites where *B. longulus* was present (see Chapter 3); 2) higher year-

round temperatures at this site compared to the other sites allows greater plant growth despite herbivory by *B. longulus*; 3) differences in water chemistry between the sites resulted in different leaf sizes; 4) a much greater density of *N. nouchali* was present at this site compared to the other two sites (where there was 0% or 4% coverage of *N. nouchali*), resulting in a preference in *B. longulus* feeding on *N. nouchali*; 5) lack of integrated control at Fountainhill Estate (at both King's Beach Skate Park and Knysna Estate leaves were periodically removed by residents/groundskeepers in addition to the feeding by *B. longulus*). The latter reason seems to be the most likely explanation in this case: mechanical and chemical removal occurred at two other sites where *B. longulus* was not present, but leaf sizes were still large at these sites during each sampling event, reiterating the effect of *B. longulus*. Nevertheless, further investigation is necessary to confirm this. Notwithstanding, although leaf size was larger at Fountainhill Estate compared to the other sites, coverage was still low. This may be the result of competition effects from *N. nouchali*, but further investigation could disentangle this.

The varying genetic composition of *N. mexicana* in South Africa complicates the interpretation of data collected during these surveys. It is likely that multiple hybrids of *N. mexicana* exist in South Africa, and these differ morphologically to "pure" *N. mexicana* (Reid et al. 2021) (see also Chapter 3). *Bagous longulus* was prevalent at three sites that were invaded by "pure" *N. mexicana* but was also found at other sites where native *Nymphaea spp.* were growing in sympatry with *N. mexicana* hybrids. At these sites, minimal damage levels were recorded on the hybrid leaves: some early larval instars were recorded mining the leaves, but no later instars were present in the hybrids, despite full life cycles of *B. longulus* being present on the native *Nymphaea*. This suggests that *B. longulus* can survive and reproduce on *N. mexicana*, but not on these hybrids. Hybrid plants may display elevated, reduced, or unchanged resistance to herbivory (Fritz et al. 1994, 1999, Whitham et al. 1994) as a result of differences in chemical or morphological defences against herbivory (Fritz et al. 1999, Cheng et al. 2011). This could explain the lack of *B. longulus* damage on these hybrids, in contrast to the improved performance of the native weevil, *E. lecontei* on hybrid watermilfoil in the USA (Roley & Newman 2006). However, the watermilfoil hybrid was a cross between the invasive *M. spicatum* and the native *M. sibiricum*, whereas the hybrids in this study are crosses of exotic *Nymphaea* (see Chapter 3), meaning that the milfoil hybrids are more genetically similar to the native species than the *Nymphaea* hybrids are to the native host of *B. longulus* (*N. nouchali*). Biological control of these

Nymphaea hybrids could therefore be challenging, and unlikely using augmentative control of *B. longulus.* The inability of *B. longulus* to survive on *N. mexicana* hybrids should, however, be confirmed with further experimentation (addressed in Chapter 4).

Differences in water quality and level of eutrophication between sites could also affect leaf size. Indeed, eutrophication has been an important factor contributing to the growth of invasive alien aquatic plants in South Africa (Coetzee & Hill 2012, Hill & Coetzee 2017). However, although statistical models suggested that the water parameters investigated did not have a significant effect on *N. mexicana* leaf size (aside from pH), two sites were considered eutrophic, and a reduction in pollution in these water bodies could reduce *N. mexicana* density. When considering all of the sites sampled once, *B. longulus* presence also did not have a significant impact on leaf size. The lack of impact of *B. longulus* on *N. mexicana* hybrids and the relatively small number of sites where *B. longulus* did reproduce and cause high damage to *N. mexicana* (only three sites) could have masked this effect in the statistical tests. Nevertheless, regardless of statistical significance, it is clear that *B. longulus* has developed a new association with *N. mexicana* and that it has potential to suppress 'pure' *N. mexicana* plant growth.

Here I have collected useful information about sites invaded by *N. mexicana* in South Africa that can be used in post-release monitoring programmes to determine the success of biological control, thus fulfilling the first aim of this chapter. I have also determined that it is likely that enemy release contributes to the invasion of *N. mexicana* in South Africa (aim 2), and that mean *N. mexicana* plant size in South Africa is greater than in its native range in USA (aim 3). Furthermore, a decline in invasive *N. mexicana* populations has been associated with the presence of a native weevil, similar to the new association of *E. lecontei* in the US (Newman & Biesboer 2000). Indeed, host range expansion of native insects to exotic plants may be more common than expected (Jahner et al. 2011, Branco et al. 2015, Sunny et al. 2015, Castagneyrol et al. 2016, Okamoto et al. 2020), but it is important to further investigate novel associations and the mechanisms underlying them, in order to understand and safely make use of these interactions for management of invasive alien plants. This study highlights the importance of conducting pre-introductory surveys in the invaded range of an alien plant. It is necessary to continue monitoring of the sites that have shown decline of *N. mexicana* where *B. longulus* is

present, and to conduct further research to understand the potential of *B. longulus* for use in augmentative biological control. This further research is outlined in the chapters that follow.

Chapter 3: Investigating genetic contributions from putative parents to *Nymphaea* hybrids in South Africa using molecular markers

3.1 INTRODUCTION

The genus *Nymphaea* may be considered the most diverse and widespread representative of the family Nymphaeaceae and the order Nymphaeales, with about 50 species distributed almost worldwide. There are five subgenera within the genus, including: *Anecphya* comprising seven to 10 species and restricted to Australia and New Guinea, *Brachyceras* with 14-16 species with a pantropical range, *Lotos* with two to three species in the paleotropics, *Hydrocallis* with approximately 14 species in the Neotropics, and *Nymphaea* with six (previously eight) species distributed across the temperate regions in the Northern Hemisphere (Conard 1905, Borsch et al. 2007).

Hybridisation between *Nymphaea* species became popular in the horticultural trade when Joseph Bory Latour-Marliac started breeding hybrids from hardy water lilies in the 1800s, and now there is a vast abundance of *Nymphaea* hybrids available to hobbyists and horticulturalists. Indeed, a list of *Nymphaea* names compiled by Kit Knotts (including officially recognised names, synonyms, and cultivars) until October 2006 included almost 1900 listings (http://www.victoria-adventure.org/waterlilies/names/names_main.html). Generally, all known hybrids were created by crossing species within the same subgenus, until the first cross between *Nymphaea* subgenera was recorded in 2004 (Les et al. 2004). As crosses between subgenera are very difficult to achieve, most modern *Nymphaea* hybrids are variations of crosses within subgenus, and many are remnants of the original hardy water lily cultivars initiated by Latour-Marliac, which include the following species as parents: European white water lily (*Nymphaea alba* L.), the North American fragrant water lily (*Nymphaea odorata* Aiton), the pygmy water lily (*Nymphaea tetragona* Georgi) and the yellow water lily (*Nymphaea mexicana* Zuccarini) (Nierbauer et al. 2014, Sheldon 2017). *Nymphaea candida* J. Presl, is also listed under subgenus *Nymphaea* in addition to *Nymphaea leibergii* Morong, which is closely related to *N. tetragona* (previously considered synonymous with the species). *Nymphaea pygmaea* (Salisb.) W.T.Aiton has also been listed but is now considered a synonym of *N. tetragona.*

Hybridisation between *Nymphaea* species also occurs naturally in the wild and may explain the origin of some members of *Nymphaea* typically considered distinct species (Dkhar et

al. 2011, Jacobs & Hellquist 2011, Borsch et al. 2014). For example, *Nymphaea loriana* Wiersema, Hellq. & Borsch, an endemic species in Canada, originated as a hybrid cross between *N. leibergii* and *N. odorata* (Borsch et al. 2014), whereas *Nymphaea alba* var. *rubra* Lönnr. originated as a cross between *N. alba* and *N. odorata* (Dkhar et al. 2011). Although hybrids of *Nymphaea* are usually sterile, they often exhibit more vigorous vegetative growth compared to the parental species. In some cases, it may also be difficult to differentiate between parent species and hybrids because the hybrids have intermediate forms of parental features (Conard 1905, Les et al. 2004). This difficulty in distinguishing between hybrids and parent species through morphological features can be problematic in biological control programmes, because there can be confusion about the compatibility of biological control agents and hybrid populations (Urban et al. 2011).

In biological control, suitable agents are selected based on their impact on the target plant, as well as their host specificity. Generally, host specificity is reliant on close evolutionary relationships between the target plant and the natural enemy, such that slight variations in genetic makeup can affect the ability of an agent to survive and reproduce on genotypes different to that of its original host (Goolsby et al. 2006a, Rosenthal & Berenbaum 2012). Hence, biological control of hybrids may be challenging, but the chances of success may be improved by surveying the putative parents of the hybrids for potential agents. Similarly, understanding the genetic structure and parentage of hybrids presents the possibility of selecting agents that are oligophagous across species within a certain series or subgrouping. For example, several species in the genus *Prosopis* L. (Fabaceae) are invasive in South Africa and interbreed to form hybrids. Further investigation has shown that all *Prosopis* species that are invasive, barring one, belong to section Algarobia, series Chilensis (Catalano et al. 2008, Zachariades et al. 2011a). Hence, prospects for biological control are improved, as an oligophagous agent that can feed on multiple species within this series would be appropriate without threatening South Africa species which are distantly related to the invasive species (Catalano et al. 2008, Zachariades et al. 2011a).

In South Africa, several *N. mexicana* sites have been detected, and are easily identifiable by the presence of bright yellow flowers, erect rhizomes, and brown blotches on the leaves. However, plants at other sites were not so easily identified, as they possessed features that did not fit exactly into one species description (Table 3.1). Usually, these intermediate traits were

observable as unusual flower colour and petal shape as well as leaf blotching. For example, plants from several sites had pale yellow or cream coloured petals, or rounded petal shapes in contrast to pointed or slightly blunted petal tips of *N. mexicana.* Other plants had pure white flowers, but unusual leaf characteristics including blotching on the leaves. As a result of these intermediate traits, it was suspected that these plants were exotic hybrids, and this was supported by evidence from ISSR and AFLP analyses (Reid et al. 2021). Additionally, the degree to which certain hybrid populations are invasive also seems to vary: some populations seem to be isolated, while others cover whole dams or ponds in a similar way to *N. mexicana* sites. It is thus important to develop an understanding of the genetic similarity of these hybrids to putative parent lineages so that they can be prioritised based on their invasiveness and to expand surveys for potential biological control agents. Currently, although *N. mexicana* is listed as a Category 1b species according to the National Environmental Management: Biodiversity Act (No. 10 of 2004) (NEM:BA), there are no clear legislations for the management of hybrids. The separation of South African *Nymphaea* and invasive *Nymphaea* into different subgenera means that hybridisation between the exotic and native plants is highly unlikely, thus somewhat simplifying potential biological control efforts.

ISSRs have been widely used in the identification of plant hybrids and their parents (Ruas et al. 2003, Goldman 2008, Bianco et al. 2011, Jade et al. 2021) and have also been used for molecular studies investigating genetic relationships in *Nymphaea* (Woods et al. 2005b, Chaveerach et al. 2011, Poczai et al. 2011). Similarly, ITS regions have also been used to resolve relationships among *Nymphaea* species (Woods et al. 2005a, Borsch et al. 2007, Löhne et al. 2008, Volkova et al. 2010, Dkhar et al. 2012) and have also been used to determine hybridisation and putative parents (Dkhar et al. 2013, Borsch et al. 2014). Henceforth, in this chapter, molecular markers are used to determine the putative parents or genetic contributions to *Nymphaea* hybrids in South Africa in order to build on previous genetic work conducted on invasive *N. mexicana* (Reid et al. 2021). This could reveal important information that will be helpful for prospective biological control efforts for *N. mexicana* and *Nymphaea* hybrids.

Table 3.1: Morphological descriptions of *Nymphaea* species in subgenus *Nymphaea* as putative parents of suspected hybrids according to Conard (1905) and Borsch et al. (2014).

Species	Flowers	Sepals	Leaves	Rhizome	Other
N. odorata	Fragrant with 17-43 white petals with pointed tips, deep yellow carpellary appendages, flowers 6-19 cm.	Purplish green/purple.	Orbicular, usually purplish on underside but may be green, leathery texture, open or closed sinus.	Horizontal, few stout branches, phyllotaxy 2 on 5.	Petioles even, greenish/purplish in colour.
N. alba	White, odourless, 10-12 cm across; ovary covered in stamens to the top, filaments of innermost stamens narrower than anthers.	Line of insertion is rounded, not prominent.	Red when young, nearly orbicular, 10-30 cm. Lowest pair of veins straight and diverging.	Horizontal, leaves crowded on rhizome, 13 on 34 phyllotaxy.	Spherical axile process and fruit.
N. tetragona	Small, white, 2.5-5 cm across; few floral parts, receptacle tetragonal, purple stigmatic disks.		Small, ovate, 2.5-12 cm in diameter; top part green with brown blotches in younger leaves, underside dull red. Open sinus, acute lobes.	Erect, projecting leaf scars.	Two main air canals in petiole.
N. candida	White and odourless, 6.4 to 7.6 cm across. Filaments broader than anthers, ovary without stamens immediately below stigmas, contracted.	Inserted at angle, project downwards.	On underside of leaf, lowest pair of veins on lobes curve towards each other.	Horizontal, stout, surface consists of large leaf-cushions. Leaves crowded on rhizome, 13 on 34 phyllotaxy.	Ovoid fruits.
N. leibergii	White petals 8-15 in number, carpellary appendages pale yellow, small flowers 3-7.5 cm in length.		3-9 cm long, 2-15 cm wide, purplish underside	Erect.	

N. mexicana	Bright yellow, 6 to 13 cm in diameter. Elliptic, ovate or lanceolate, acute or obtuse petals. Receptacle with four swollen ridges. Brown blotches on upper surface, underside purplish. Aerial leaves may be green on underside.	Elliptic, ovate or lanceolate, acute or obtuse sepals.	Orbicular to ovate, may be narrowly peltate. Edges nearly entire or denticulate-wavy. 10-18 cm across.	Rhizome erect, densely covered between leaf bases with long hairs, sides with large projecting, leaf cushions. Upper part of rhizome sprouts naked stolons, from which brood bodies form.	Petiole inserted nearer to lobe than apex.

3.2 METHODS

Sampling and DNA extraction

Samples were collected from various natural and artificial waterbodies across invaded sites in South Africa (Table 3.2). Samples were reused from analyses in Reid et al. (2021), with additional samples collected during pre-release surveys detailed in Chapter 2. Five to ten leaf segments were collected from plants approximately 5 m apart, wrapped in paper towel or stored in envelopes and stored in sealed plastic Ziploc bags with approximately 30 g of silica gel, which was changed as needed to ensure desiccation of the leaf material. One to two dried leaf samples of putative parents were also sent by Dr Cornelia Löhne from the Botanical Gardens, Bonn University, Germany. It was challenging to acquire additional samples for DNA extraction, so only one to two replicates were available, and *N. leibergii* was not included in DNA extractions. DNA was extracted using plant extraction kits from QIAGEN (QIAGEN Inc.), and Invitrogen™ (PureLink™ Total DNA purification kit) after the samples were ground using liquid nitrogen with a mortar and pestle. The extracted DNA was stored at -20^{o}C for later use, before being amplified by polymerase chain reaction (PCR).

Table 3.2: Site details for *Nymphaea* DNA samples used in ISSR analyses. Samples from sites marked with an asterisk (*) were collected in Naidu (2018). Select samples were also included in ITS analyses (see Table 3.3). Sample codes are used to represent samples in analysis outputs. Coordinates were unavailable for one site due to poor GPS reception and difficulty pinpointing the site using satellite maps.

Province/State	**Locality/Species**	**Latitude**	**Longitude**	**Sample code**
Western Cape	Muizenberg, Westlakes	-34.0813	18.4554	WL
	Neil Ellis, Stellenbosch	-33.9249	18.8912	NE
	Intaka Island, Century City	-33.8878	18.5128	CC
	Kluitjieskraal	-33.0622	20.3562	KK
	Maynardville Wynberg	-34.0053	18.4643	MAYN
	George Horse fields	-33.9945	22.5262	GEO/GHOR
	Bellevue Wine Estate, Stellenbosch	-33.8791	18.7630	BELL
	Knysna Estate	-34.0251	23.0124	KE
	Belvidere, Knysna	-34.0468	22.9982	BELV
	Pledge Nature Reserve, Knysna	-34.0325	23.0445	PR
	Cottage dam farm, Kromrivier	-32.5417	19.2811	KR
Eastern Cape	Boardwalk, Port Elizabeth/Gqeberha	-33.9830	25.6574	BW

	King's Beach Skate Park, Gqeberha	-33.9741	25.6447	SP
	Private Farm near Haga Haga	-	-	HAG
	Private Farm near Plettenberg Bay	-34.0446	23.2920	N2P
	Radway Green, Makhanda	-33.3525	26.6599	RAD
	Tsitsikamma National Park, Storm's River	-34.0114	23.8680	TK
Gauteng	Benoni Homestead Lake	-26.1705	28.2890	BE
	Morelata Park, Pretoria	-25.8139	28.2848	PRET
	Emmarentia, Randburg	-26.1501	28.0058	EMM
	Louw Geldenhuys drive	-26.1461	28.0036	LG
North West	Potchefstroom NWU Botanical gardens	-26.6823	27.0950	POT
	Nymphaea marliacea chromatella obtained from AquaFlora nursery, grown at Rhodes University, Makhanda			MC
	Nymphaea marliacea albida obtained from AquaFlora nursery, grown at Rhodes University, Makhanda			MA
KwaZulu-Natal	Paradise Valley	-29.8321	30.8922	D
Florida, USA	Lake Kissimmee 1*	27.9651	-81.3278	K
	Lake Kissimmee 2	27.9792	-81.2743	K

	Lake Lawne	28.5579	-81.4381	L
	Lake Apopka	28.6722	-81.6748	AP
	Lake George	29.2828	-81.5408	G
	Lake Okeechobee	26.9329	-81.0503	OKE
	Lake Seminole	27.8414	-82.7740	SEM
	Lake Maggiore	27.7373	-82.6475	M
	Pine Island lodge	29.3119	-81.5458	PI
	Emeralda marsh near lake Griffin	28.9039	-81.8087	EM
	Orlando Wetlands Park	28.5824	-81.0022	OWP
	Everglades	26.3205	-80.3300	EV
Louisiana, USA	Cote Blanche Crossing	29.7774	-91.7155	CX
	Lake Boeuf	29.9111	-90.7117	B
	Salvador WMA	29.7657	-90.2930	S
Texas, USA	Canal roadside Harlingen	26.1903	-97.6636	H
	Lewisville Research Facility*	33.0524	-96.9373	TX
	Big lake, Welder wildlife refuge	28.1216	-97.3650	W
	Quinta urban park	26.1767	-98.2298	Q

Botanical Gardens of Bonn University, Bonn, Germany	*Nymphaea candida*	50.7252	7.0933	CAN
	Nymphaea alba	As above		ALB2
	Nymphaea odorata var. minima	As above		ALBOD1, ODMIN1
	Nymphaea tetragona	As above		TETRA

ISSR PCR protocol and analyses

PCRs were run with two primers, chosen for the best amplification patterns after initial tests with eight different primers (Reid 2019): HB15 manufactured by Applied Biosystems Inc., U.K. (Paterson et al. 2009, Barker et al. 2015) and UBC-852 manufactured by Integrated DNA Technologies, WhiteSci Whitehead Scientific (Pty) Ltd., RSA (Poczai et al. 2011). 6-FAM fluorescent dye was used by the manufacturers to label both primers at the 5' end. However, final analyses were only included for HB15, as too many samples had to be excluded in UBC-852 due to low amplification.

The reaction mixture for the samples prepared in addition to those already amplified by Reid et al. (2021) consisted of 0.4 µM of HB15 primer, 5 µL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 1.5 µL plant DNA, and 3.1 µL denucleated water. The lower volumes were used to reduce costs after previous runs demonstrated that 10 µL were sufficient for the reactions. The PCR protocol followed an initial denaturation step at 94 °C for 2 minutes, 35 cycles of 94 °C for 30 s, 45 s at 44 °C, and 90 s at 72 °C (Paterson et al. 2009). The final extension occurred at 72 °C for 20 min.

The PCR products were sent to Central Analytical Facilities (CAF) at Stellenbosch University, Stellenbosch, South Africa, where capillary electrophoresis using an ABI 3130 genetic analyser was used to visualise banding patterns. Each sample was replicated twice to ensure that the PCR reactions were replicable. A binary matrix was generated to show the absence or presence of bands, and the resultant electropherograms were analysed in GeneMarker® ver. 2.7.4 (SoftGenetics LLC.). In GeneMarker®, a minimum peak height of 20 was used, stutter peak filter and AFLP normalisation were left unchecked, smoothing was selected, and "fail < 1 check < 1 pass" was set for the minimum peak score, and all other settings were left at default (Holland et al. 2008). Scoring parameters were selected based on results from preliminary tests, which showed that these parameters had lower Jaccard's error rates and the greatest number of peaks (Bonin et al. 2004, Pompanon et al. 2005, Holland et al. 2008, Reid 2019, Reid et al. 2021). Thereafter, the datasets were scored using RawGeno ver. 2 (Arrigo et al. 2009), which is an automated application to score DNA fragments run through R ver. 4.1.2 (R Core Team 2021). In RawGeno, bin width minimum was set at 1 and maximum at 1.5, as fewer errors and better resolutions were found using these settings in other plants (Holland et al. 2008).

Resultant binary matrices were exported as tab-delimited text files, edited in Microsoft Excel®, and consolidated using BINMAT: For Fragment Analysis Data, a downloadable R package (Clarke van Steenderen: https://cran.r-project.org/web/packages/BinMat/). The first 80 base pairs were excluded from the analyses as most of the samples shared peaks at these sites, and consolidated samples with fewer than 15 total peaks were also removed, as these samples were considered to have failed to amplify and appeared as outliers on preliminary test plots.

The Jaccard's similarity index is well suited to ISSR data, because shared absence is excluded as a character (Sokal & Sneath 1963), whereas network analyses, in contrast to NJ, MP, and Bayesian analyses, consider intraspecific and population level phenomena (Posada & Crandall 2001). Hence, Jaccard's distances were used to construct a NeighbourNet phylogenetic network in SplitsTree4 ver. 4.12.3 with 1000 bootstrap replications for node support (Huson & Bryant 2006).

STRUCTURE 2.3.4 (Pritchard et al. 2000) utilises a Bayesian clustering algorithm to assign individuals to genetic clusters (K) using estimates of the probability of assignment (Q) based on similarities in genotypes. These probabilities were estimated for values of K from 1 to 10 by running five iterations of 50 000 burnins, followed by 100 000 Monte Carlo Markov Chain (MCMC) reps. Other settings were left to default, and the admixture model was used (Falush et al. 2003). The optimal number of K-clusters, determined using the ad hoc ΔK statistic (Evanno et al. 2005) and the Puechmaille Method of K selection (Puechmaille 2016), was obtained from StructureSelector (Li & Liu 2018). CLUMPAK (Kopelman et al. 2015) was used to generate graphical outputs of STRUCTURE results for selected K values.

ITS protocol and analyses

Based on the results from the ISSR analyses, 25 samples were selected to represent each major grouping and thus determine the consistency between ISSR and ITS analyses. The sequences used in the ITS analyses were selected to represent each of the genetic groups produced from the ISSR analyses, with a few samples randomly selected from sites that clustered together in each group and including the putative parent samples included in the ISSR analyses. Additional sequences were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) to supplement samples with few replicates, include variation, and include species for which leaf samples were not obtained for new DNA extracts (such as *N. leibergii*) (Table 3.3). Most of the

sequences were in subgenus *Nymphaea*, but native South African *Nymphaea* in subgenus *Brachyceras* and *Lotos* were also included in addition to other closely related genera as outgroups. ITS 4 and ITS 5 primers were used to amplify and sequence the ITS regions (ITS1 + 5.8S + ITS2) for these selected samples (White et al. (1990) cited in Woods et al. 2005a)), and only the forward strands were sequenced. PCR reaction mixtures consisted of 0.5 µL forward primer, 0.5 µL reverse primer, 12.5 µL iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 5 µL of sample DNA, and 6.5 µL ddH_2O to make a reaction mixture of 25 µL. Amplification by PCR was carried out using the following protocol: 35 cycles of denaturation at 97°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 45 s, followed by a final extension at 72 °C for 7 min, according to Löhne (2006).

To confirm the successful amplification of DNA, PCR products were visualised using gel electrophoresis, prepared using 100 mL TBE buffer (1X), 10 µL ethidium bromide, and 1 g Seakem™ agarose (1%). 5 µL of each DNA sample was mixed with 2 µL of loading buffer (Thermo Scientific 6x TriTrack DNA Loading Dye) to visualise the samples, whereas 2 µL of DNA ladder (Thermo Scientific GeneRuler 100bp) was used as a standard. A Bio-Rad Powerpac™ was used to run gels for 90 V at 40 minutes, and bands were visualised and photographed using a Bio-Rad Geldoc™ molecular imager system and Image Lab™ software respectively. Successfully amplified PCR products were then couriered to Macrogen Inc. (Netherlands) for purification and sequencing. Final sequences were submitted to Genbank via the online submission portal available at https://submit.ncbi.nlm.nih.gov/.

Chromas v2.6.6 was used to trim chromatograms (Technelysium Pty Ltd.) at the beginning and end of each sequence, where signals were erratic and severely overlapping. Chromatograms were checked manually to look for double peaks indicating polymorphisms, but overall there were few or no polymorphisms, and the presence or absence of polymorphisms was not consistent with observations of intermediate morphology. For example, even samples taken from known hybrids supplied by the nursery (i.e. *Nymphaea marliacea chromatella*) did not indicate obvious polymorphisms. Hence, sequences were used as exported from Chromas using default settings in the following analyses.

Sequence alignment was carried out using online MAFFT v7 software (Katoh & Standley 2013), which is considered more accurate and consistent than other alignment methods, such as

ClustalQ, Muscle, T-coffee, and others (Morrison 2006). Sequences were viewed and edited in MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar et al. 2018) and sequence characteristics were investigated both in MEGA X and PAST 4.0 (Hammer et al. 1999). An appropriate evolutionary model (TrNef + G) was selected using jModelTest v 2.1.10 (Guindon & Gascuel 2003, Darriba et al. 2012), and a Bayesian Inference phylogeny (using random starting trees and four chains, with 20 million generations sampled every 1000 generations) was created using MrBayes v3.2.6 (Huelsenbeck & Ronquist 2001, Ronquist et al. 2012), both on the CIPRES Science Gateway portal (Miller et al. 2011) (http://www.phylo.org/). A Maximum Likelihood phylogeny (with 1000 Ultrafast bootstraps) was also created using the online IQ-TREE v 1.6.12 web server (IQTREE Web Server: Fast and accurate phylogenetic trees under maximum likelihood (univie.ac.at)) (Trifinopoulos et al. 2016). These trees were viewed and edited in FigTree v1.4.3 and Microsoft Office PowerPoint®.

Table 3.3: Details for samples used in ITS analyses, including sequences downloaded from GenBank and those obtained during laboratory procedures in this study. Sample codes indicate labelling of each sample in analyses output figures. South African samples are labelled as species or hybrids based on results from this study as well as morphological features.

Family	**Species**	**GenBank accession No.**	**Sample code**	**Reference/ Source**
Nymphaeaceae	*Nymphaea alba* var. *rubra* Lo¨nnroth	GU222364, GU222358	Nyarclo23, Nyarclo13	Dkhar et al. (2011)
Nymphaeaceae	*Nymphaea* x *marliacea* Latour-Marliac	GQ468657, FJ597739	NymM1R, NymM2, NymM1R_2	Dkhar et al. (2011)
Nymphaeaceae	*Nymphaea leibergii* Morong	HG518089, MG237104, MG236300	NY574, CCDB-18302-B11, CCDB-18302H12	Borsch et al. (2014), Kuzmina et al. (2017)
Nymphaeaceae	*Nymphaea tetragona* Georgi	FJ597745	E2	Dkhar et al. (2011)

Nymphaeaceae	*Nymphaea tetragona*	AY707899	Partial	Liu et al. (2005)
Nymphaeaceae	*Nymphaea tetragona*	EU428057	740-02	Volkova et al. (2010)
Nymphaeaceae	*Nymphaea pygmaea* (Salisb.) W.T.Aiton (syn. *N. tetragona*)	HG518076	NY234	Borsch et al. (2014)
Nymphaeaceae	*Nymphaea odorata* Aiton	AY771857, AY771858	KN3, KN4	Woods et al. (2005a)
Nymphaeaceae	*Nymphaea odorata odorata* Britton & Brown	AY771846	KN33	Woods et al. (2005a)
Nymphaeaceae	*Nymphaea odorata tuberosa* (Paine) Wiersema & Hellquist	AY771853	KN28	Woods et al. (2005a)
Nymphaeaceae	*Nymphaea candida* J. Presl	EU428058, EU428047, EU428042, EU428041	741, 720-03, 718-01	Volkova et al. (2010)
Nymphaeaceae	*Nymphaea caerulea* Savigny	AY707897	-	Liu et al. (2005)
Nymphaeaceae	*Nymphaea capensis* Thunb	AY707898	-	Liu et al. (2005)
Nymphaeaceae	*Nymphaea lotus* Linn. f. *thermalis* (DC.) Tuzson	FM242153	thermalis/NY 003	Borsch et al. (2008)
	Nymphaea elegans Hook	FJ026602, FJ026601	NY006, NY370	Löhne et al. (2008)
Nymphaeaceae	*Barclaya longifolia* Wall.	FM242140	NY 376	Borsch et al. (2008)
Nymphaeaceae	*Nuphar japonica* DC.	AH006166	-	Padgett et al. (1999)
Cabombaceae	*Cabomba caroliniana* A. Gray	AY620424	-	Liu et al. (2005)
Nymphaeaceae	*Nymphaea* hybrid	OM614894, OM614893	TK2, TK4	Tsitsikamma, RSA

Nymphaeaceae	*Nymphaea* hybrid	OM614906, OM614905	HAG1, HAG2	Haga Haga, RSA
Nymphaeaceae	*Nymphaea* hybrid	OM614902, OM614901	KR76, KR79	Kromrivier, RSA
Nymphaeaceae	*Nymphaea* hybrid	OM614907	GEO48	George, RSA
Nymphaeaceae	*Nymphaea odorata* var. *minor* Sims	OM614898, OM614913	Odmin1, Alb1	Botanical Gardens of Bonn University, Germany. IPEN nos.: xx-0-BONN-40990/40988
Nymphaeaceae	*Nymphaea alba* L.	OM614912	Alb2	As above. xx-0-BONN-12498.
Nymphaeaceae	*Nymphaea tetragona*	OM614895, OM614896	Tetr1, Tetr2	As above. xx-0-BHU-US-0000-619, xx-0-BONN-6324
Nymphaeaceae	*Nymphaea candida*	OM614908	Can1	Adjara, Ispani mire in Kobuleti, collected by Izolda Matchutadze, 2003. GE-0-BONN-22203
Nymphaeaceae	*Nymphaea* hybrid	OM614917, OM614911	Bell69, Bell71	Bellevue wine estate, Stellenbosch, RSA
Nymphaeaceae	*Nymphaea* hybrid	OM614897	Rad1	Radway Green, Makhanda, RSA
Nymphaeaceae	*Nymphaea marliacea chromatella* Latour Marliac	OM614900, OM614899	MC1, MC2	AquaFlora Nursery, grown at Rhodes University, Makhanda, RSA

Nymphaeaceae	*Nymphaea mexicana*	OM614904, OM614903	KE1, KE2	Knysna Lifestyle Estate, Knysna, RSA
Nymphaeaceae	*Nymphaea mexicana*	OM614916	Geo45	Dam along N2, George, RSA
Nymphaeaceae	*Nymphaea mexicana*	OM614910, OM614909	Belv1, Belv2	Belvidere estate, Knysna, RSA
Nymphaeaceae	*Nymphaea mexicana*	OM614918, OM614914	S3, S5	Lake Salvador, Louisiana, USA
Nymphaeaceae	*Nymphaea mexicana*	OM614915	NM1	Westlakes, Cape Town, RSA

Summary including morphology

The most likely putative parents for samples collected from each site were determined by comparing results from all the analyses detailed above. The major groupings obtained in the other analyses served as an indicator of the most closely related putative parents, in addition to information about admixture from STRUCTURE. Where available, photographs of the flowers of species and samples included in the analyses were obtained during field collections (Reid et al. 2021) and online via Waterlily Galleries Index (victoria-adventure.org). These images were used to correlate genetic structure with flower morphologies of samples from selected sites, based on petal shape and flower colour.

3.3 RESULTS

ISSR SplitsTree and STRUCTURE

Four major groups were identified in the SplitsTree analysis, with some substructuring within these groups (Figure 3.1). One group clustered all the pure *N. mexicana* samples, including those from USA and SA. The South African samples formed a subcluster within this group, whereas samples from some sites in the US also clustered slightly separately from the others. The first hybrid group consisted of two subgroups: a group containing *N. marliaceae albida, N. alba,* and samples from Louw Geldenhuys (LG), Plettenberg Bay (N2P), Pledge Reserve in Knysna (PR); and a group containing *N. marliaceae chromatella* and samples from Radway Green (RAD), Potchefstroom (POT), and Bellevue (BELL). The second hybrid group also consisted of two subgroups, one with

N. odorata var. *minor* and hybrid samples from Durban (D), George (GEO and GHOR), Bellevue (Bell) and Kromrivier (KR), and the other consisting of samples from Tsitsikamma (TK), Neil Ellis (NE) and Haga Haga (HAG). A fourth group clustered *N. tetragona* and the remaining samples from Durban (D), as well as samples from Kluitjieskraal (KK) and Maynardville (MAYN). There were high bootstrap values for the major separations. Samples from two sites (Durban (D) and Bellevue (Bell)) grouped in two different hybrid groups (*N. odorata* group and *N. tetragona* group, and group 1 and 2 respectively).

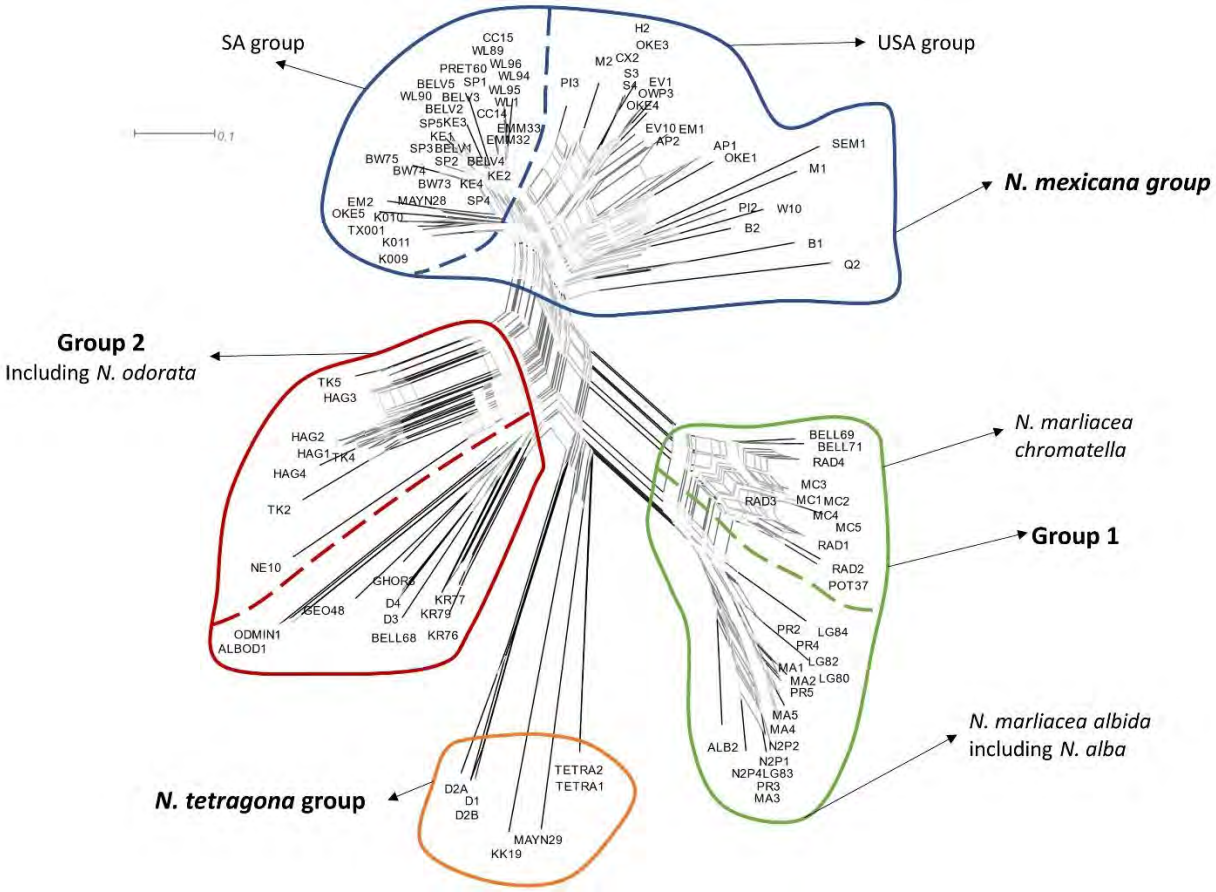

Figure 3.1: NeighbourNet tree constructed in SplitsTree using Jaccard's distance index to group genetically similar individuals based on ISSR binary data. Line shading indicates bootstrap (1000 repetitions) confidence values: the lighter the shading, the lower the support values. Generally black shading indicates support values of 80 and above. Species names beginning with 'N.' are for *Nymphaea*. SA = South African origin, USA = United States of America origin. Outlined groups 1 and 2 correspond with those in ITS analyses. Dashed lines indicate possible subgroupings within major groups.

Outputs from StructureSelector suggested an optimal K value of 8 determined by the Puechmaille Method (Puechmaille 2016) and K = 2 using the Evanno Method (Evanno et al. 2005). However, when selecting optimal K values, it is important to consider the biology of the organism under study (Gilbert et al. 2012). Considering that there are 5 to 7 possible populations included in the study depending on how populations are classified (*N. mexicana* as one group, or USA and South African populations considered as two groups, other putative parents including *N. tetragona*, *N. alba,* and *N. odorata,* and 1 or 2 hybrid groups), and that K = 8 plots did not add much more informative groupings compared to K = 7, we presented K = 5, 6, and 7 below.

For K = 5, *N. mexicana* from South Africa and USA formed one major grouping, but there was considerably more admixture in the USA populations compared to those from South Africa. Most South African populations had no or little admixture, with one sample from West Lake (WL) showing some genetic mixture with the *N. odorata* and *N. tetragona* groups. All the USA populations showed introgression with an unknown source (shown as light blue columns in K=5 for Figure 3.2, though this could be due to scoring errors) and most had admixture with *N. odorata*, *N. tetragona,* and *N. alba.* Several South African samples (of those that did not group with *N. mexicana*) clustered with *N. alba* (shown as orange with some slight admixture in Figure 3.2): *N. marliacea chromatella* (MC), *N. marliacea albida* (MA), samples from Plettenburg Bay (N2P), Pledge Nature Reserve (PR), Potchefstroom (POT) and Radway (RAD), with little to no admixture within these groups. Samples from Louw Geldenhuys (LG) and Bellevue (BELL) (excluding one sample) showed admixture with *N. odorata* and *N. tetragona* respectively. *Nymphaea odorata* itself presented as predominantly one group (green in Figure 3.2), but with some introgression from *N. alba.* Two samples from Durban (D), one sample from Bellevue, and samples from George (GEO), Haga Haga (HAG), Kromrivier (KR), Neil Ellis (NE) and Tsitsikamma (TK) all grouped with *N. odorata*, with varying levels of introgression from *N. tetragona*, an unknown source (indicated by light blue in Figure 3.2), and minimal introgression from *N. mexicana* in three samples. The remaining samples from Durban indicate hybridisation between *N. tetragona* and another unknown genetic group, whereas one Maynardville (MAYN) sample grouped with *N. mexicana* without introgression, and the other showed admixture between *N. tetragona* (predominantly), as well as *N. odorata* and *N. alba.* The sample from Kluitjieskraal (KK) indicated hybridisation between all three putative parents (without *N. mexicana*), as well as genetic contribution from the unknown group.

Similar trends were observed for K = 6, except that Kromrivier (KR) samples appeared as an entirely separate genetic group (shown as light purple columns in Figure 3.2). The samples from George (GEO), Bellevue (BELL) and Durban (D) that were grouped with *N. odorata* in the K = 5 plot showed admixture with the Kromrivier (KR) group as well as some mixing from *N. tetragona.* The *N. odorata* samples also included genetic mixing with the Kromrivier group, as well as from *N. tetragona*. In addition, the Durban (D) samples that suggested hybridisation between the unknown group and *N. tetragona* in the K = 5 group, no longer showed mixing in the K = 6 plot and instead were predominantly comprised of the unknown (light blue) genetic group. *Nymphaea mexicana* samples from the USA also included genetic contribution from *N. tetragona* in this plot.

Similar structuring was observed for the K = 7 plots except for the following: distinct and separate genetic groups indicated for Durban (D) and Kromrivier (KR) with minimum genetic mixing from these groups in other samples; distinct structuring for *N. odorata* and *N. tetragona* with USA *N. mexicana* populations showing introgression from *N. odorata* and a seventh group (indicated by darker camo green in Figure 3.2) of unknown source; hybridisation in *N. marliacea chromatella* (MC) and Radway (RAD) samples between *N. alba* and the seventh unknown group, and hybridisation also between *N. odorata* and the unknown group in Haga Haga (HAG) and Tsitsikamma (TK) samples. Finally, there was minimal contribution from *N. tetragona* in the genetic structuring of the South African hybrid groups, and little to no contribution to all the *N. mexicana* samples.

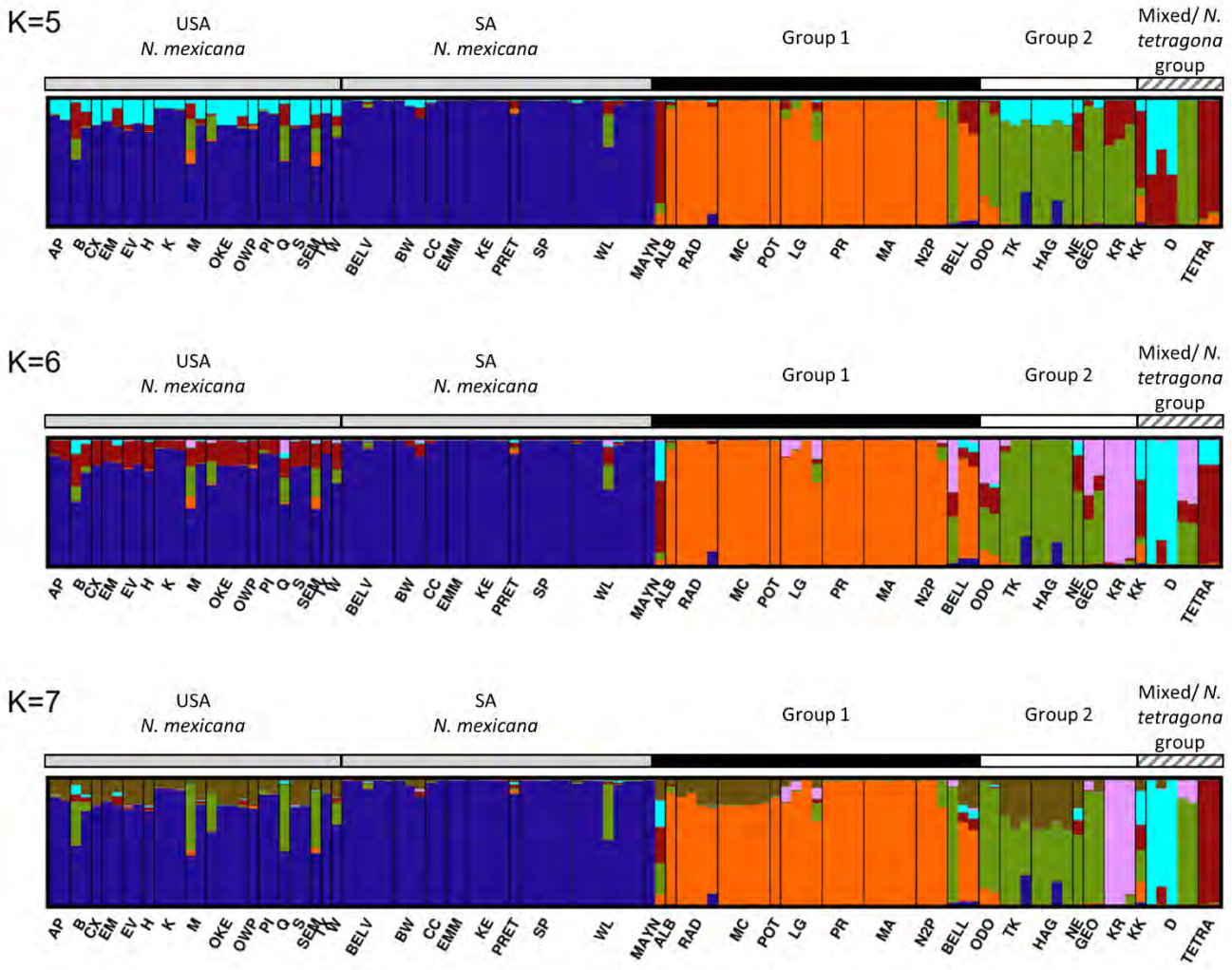

Figure 3.2: STRUCTURE analysis output for *Nymphaea* hybrids and their putative parents, showing outputs for K = 5, K = 6, and K = 7. Putative parents are *Nymphaea alba* (ALB), *Nymphaea odorata* (ODO), and *Nymphaea tetragona* (TETRA).

ITS Bayesian Inference and Maximum Likelihood phylogenetic trees

For only those samples sequenced for this analysis and excluding gaps, there was a mean of 787 base pairs. The total length of all the sequences used in the analyses, excluding outgroups but including gaps and missing base pairs, was 959 base pairs. Of these 959, 385 (40%) were variable, and 286 (30 %) were parsimoniously informative. There were 271.5 mean gaps inserted for the alignment, and mean nucleotide content was as follows: A = 174.9, T = 149.2, C = 194.8, and G = 168.5.

The maximum likelihood and Bayesian inference trees produced similar results (Figures 3.3 and 3.4). The first clade correlated with group 2 in the ISSR analyses, grouping the same samples (of those that were used for the ITS analyses) together. This was with the exception of ALB2, which was in group 2 for the ITS analysis, but in hybrid group 1 in the ISSR analysis. Of the additional samples included from Genbank accessions, *N. odorata* and *N. odorata tuberosa* were in group 2. Group 1 consisted of the same samples as for the ISSR analyses, as well as *N. marliacea, N. candida*, and *N. alba* var. *rubra* acquired from Genbank. *Nymphaea marliacea albida* samples (labelled MA in ISSR analyses) were included in the protocols but did not amplify successfully for use in the ITS analyses. As with the ISSR analyses, *N. tetragona* formed a separate group with *N. leibergii*, whereas *N. mexicana* was also placed in its own clade. Additional samples representing *Nymphaea* species native to South Africa formed a separate cluster. High support values were observed for the major branches in both the maximum likelihood and Bayesian inference trees, although there was slightly lower support in the maximum likelihood tree for the split between group 1 with *N. tetragona*, and group 2.

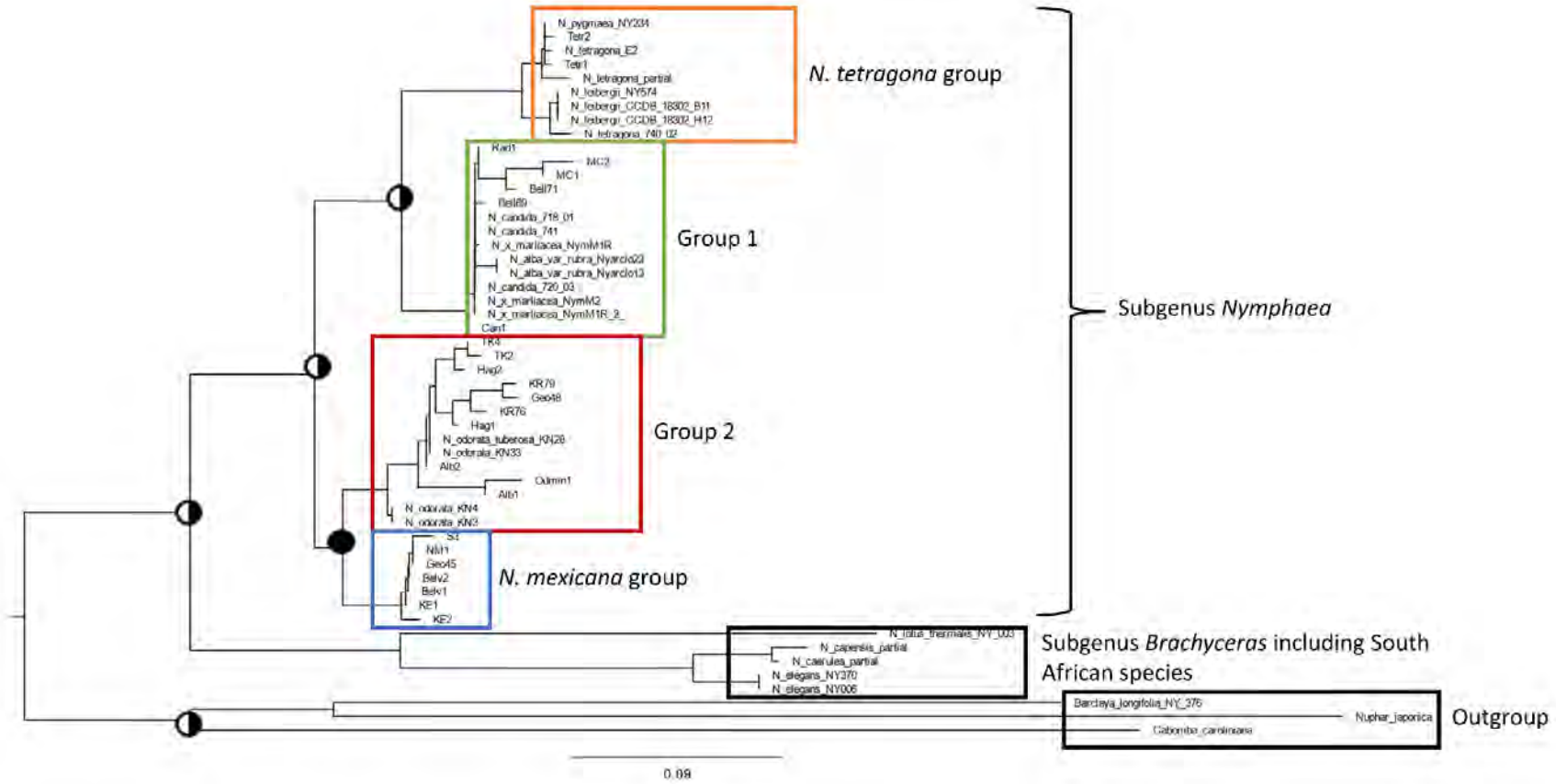

Figure 3.3: Phylogenetic reconstruction of *Nymphaea mexicana* and selected samples from suspected hybrid populations in South Africa, including putative parents and additional sequences from Genbank. Coloured circles at the nodes of major splits indicate values for 1000 bootstraps that are considered well supported for values > 95 for Maximum Likelihood (white), and > 0.90 for Bayesian Inference (black).

Morphology and summary

Overall, the samples were grouped similarly in all the genetic analyses (ISSR SplitsTree, STRUCTURE, and ITS) (Table 3.4). Although some admixture from *N. mexicana* was evident in some groups for K = 7 in the STRUCTURE analyses, generally the samples grouped more closely with the other putative parents (i.e., *N. alba, N. odorata*, or *N. tetragona*). The flower morphology correlated with conclusions drawn from the genetic data, especially for the STRUCTURE analyses (Figure 3.5). Samples, such as those from *N. marliacea albida* (MA), Potchefstroom (POT), and Plettenburg Bay (N2P), that grouped solely with *N. alba* in the analyses had white petals, with more rounded tips. Hybrids with pale yellow petals and more rounded tips (e.g., Radway Green (RAD) and *N. marliacea chromatella* (MC)) had admixture from both *N. alba* and *N. mexicana* in the STRUCTURE plots. The same was true for samples that had *N. odorata* as a putative parent (e.g., Tsitsikamma (TK), Haga Haga (HAG) and the yellow George specimens (GEO)), except that these hybrids had pale yellow flowers with bigger and more pointed petal tips. Although photographs of the flowers were not available for all the sites, the evidence for multiple species and hybrids at some sites was confirmed by the observation of multiple floral colours and types during subsequent surveys.

Table 3.4: Summary of genetic groupings of *Nymphaea mexicana* and suspected hybrids collected from sites around South Africa, according to ISSR analyses (SplitsTree and STRUCTURE) and ITS sequencing analyses (Maximum Likelihood (ML) and Bayesian Inference (BI)). Samples from the native range (USA) are not included in the table, and dashes (-) indicate that samples were not included in an analysis.

	Grouping result by analysis			
Population	**SplitsTree**	**STRUCTURE (K = 5 to K = 7)**	**ITS (ML and BI combined)**	**Closest grouping/conclusion**
ALB	1	*N. alba* group	2	*N. alba*
BELL	1	Multiple hybrids of *N. alba, N. mexicana*, *N. odorata,* and slight introgression from *N. tetragona* and unknown source.	1	*N. odorata* and hybrid of *N. alba* and *N. mexicana* as closest putative parents.
BELV	*N.mexicana*	*N.mexicana*	*N.mexicana*	*N.mexicana*
BW	*N.mexicana*	*N.mexicana*	-	*N.mexicana*
CC	*N.mexicana*	*N.mexicana*	-	*N.mexicana*
D	2 and *N. tetragona* group	Own genetic grouping, possible admixture with *N. tetragona,* some samples as *N. odorata.*	-	Multiple samples: *N. odorata* and unknown origin with admixture from *N. tetragona.*
EMMDAM	*N.mexicana*	*N.mexicana*	-	*N.mexicana*

GEO	2 and *N. mexicana*	*N. odorata* with possible admixture from unknown source.	2 and *N.mexicana*	Multiple species/hybrids at one site: *N. mexicana* and *N. odorata* (confirmed by morphological observations).
HAG	2	*N. odorata* with possible admixture from *N. mexicana*.	2	*N. odorata* and *N. mexicana* as putative parents.
KE	*N.mexicana*	*N.mexicana*	*N. mexicana*	*N.mexicana*
KK	*N. tetragona*	Hybrid including *N. tetragona, N. odorata, N. alba*, and unknown source.	-	Hybrid with admixture from multiple species/unconfirmed origin.
KR	2	Hybrid of *N. odorata* and *N. tetragona*, or separate genetic group.	-	Hybrid of unconfirmed origin.
LG	1	*N. alba* group with some admixture from *N. odorata* or unknown source.	-	*N. alba*, *N. odorata*, and unknown source as putative parents.
MA	1	*N. alba* group	Failed to amplify	*N. alba* as putative parent.
MAYN	*N. tetragona* and *N. mexicana* groups	*N. mexicana* and hybrid with admixture from *N. tetragona*, *N. odorata*, *N. alba* and possibly unknown source.	-	Mixed population including *N. mexicana* and hybrid with *N. tetragona*, *N. odorata*, *N. alba*, and unknown source as putative parents.

MC	1	*N. alba* group, admixture with *N. mexicana*.	1	*N. alba* and possibly *N. mexicana* as putative parent.
N2P	1	*N. alba* group with slight admixture with *N. odorata*	-	*N. alba* and *N. odorata* as putative parents.
NE	2	Hybrid with *N. odorata*, *N. tetragona*, and unknown sources as parents.	-	Hybrid with *N. odorata*, *N. tetragona*, and unknown sources as parents.
ODO	2	*N. odorata* group, with some admixture from *N. alba* (possibly also other sources if K = 6).	2	*N. odorata*
POT	1	*N. alba* group, slight admixture with *N. mexicana.*	-	*N. alba* as putative parent, with some contribution from *N. mexicana*.
PR	1	*N. alba* group	-	*N. alba* as putative parent.
PRET	*N. mexicana*	*N. mexicana*	-	*N. mexicana*
RAD	1	*N. alba* group, slight introgression with *N. mexicana*	1	*N. alba* and possibly *N. mexicana* as putative parents.
SP	*N. mexicana*	*N. mexicana*	*N. mexicana*	*N. mexicana*
TETRA	*N. tetragona*	*N. tetragona*, slight introgression with *N. alba* or unknown source.	*N. tetragona*	*N. tetragona*
TK	2	Hybrid of *N. odorata,* and *N. mexicana.*	2	*N. odorata* and *N. mexicana* as putative parents.

WL	*N. mexicana*	*N. mexicana,* possibly some slight introgression with *N. odorata.*	*N. mexicana*	*N. mexicana*

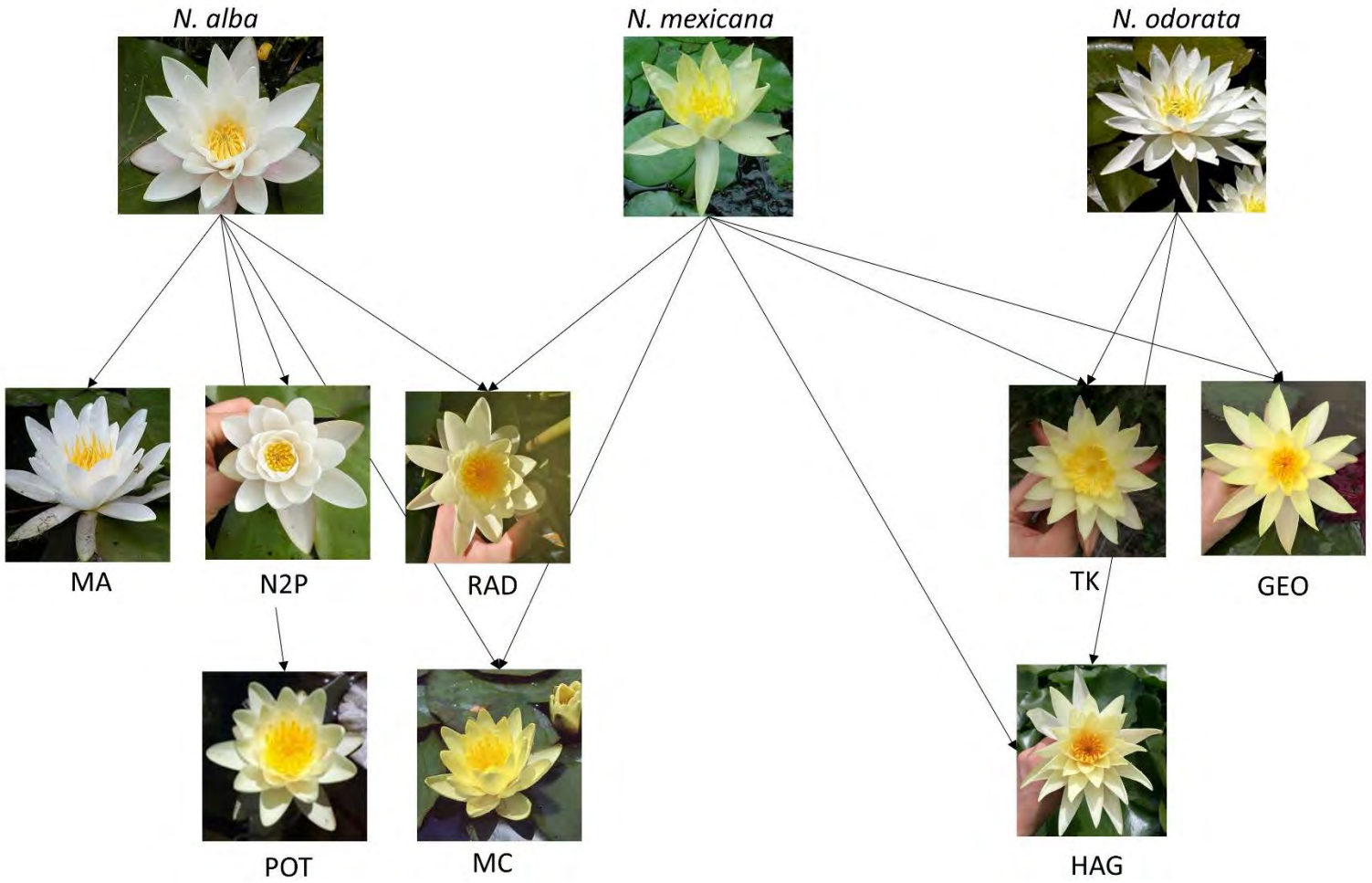

Figure 3.4: Flower morphologies of *Nymphaea* hybrids and their putative parents, *Nymphaea alba, Nymphaea mexicana,* and *Nymphaea odorata*. Abbreviations correlate to site names given in Table 3.1. Photographs obtained from field collections (Reid et al. 2021) and from Waterlily Galleries Index (victoria-adventure.org) (Kathryn Perry, Kit Knotts, Werner Wallner, Perry Slocum).

3.4 DISCUSSION

The samples included in this study, aside from a few inconsistencies, were separated into four different groups in all the analyses: a group containing "pure" *N. mexicana,* a group containing *N. tetragona*, and two hybrid groups. The hybrids *N. marliacea chromatella* and *N. marliacea albida* were both in the first group (group 1), which included *N. alba* in the ISSR analyses, and *N. alba* var. *rubra* along with *N. candida* in the ITS analyses. These results suggest that *N. marliacea chromatella* and *N. marliacea albida* are closely related, and that they may have the same or similar putative parents, namely *N. alba* or *N. alba* var. *rubra*, as well as *N. candida*. Other studies have suggested similar parentage for *N. marliacea chromatella*: Dkhar et al. (2012) and Slocum (2005) reported that *Nymphaea marliacea chromatella* was the result of a cross between *N. alba* var. *rubra* and *N. mexicana,* as the first hybrid produced by J.B. Latour Marliac (Latour-Marliac 1899), whereas the parentage of *N.* 'Marliacea Albida' Marliac is reportedly unknown (Slocum 2005). Similarly, genetic analyses conducted by Dkhar et al. (2011) supported the placement of *N. marliacea* as a close relative of *N. alba.* Although the SplitsTree and ITS analyses did not demonstrate *N. mexicana* as a putative parent for the group 1 samples, STRUCTURE analyses did suggest genetic contribution from USA *N. mexicana* populations for some of the members of the group, but only when 7 groups were considered. This genetic contribution is reflected in the morphotype of such specimens through the yellow colouration of the flower petals: those samples that had genetic evidence for admixture with *N. mexicana* had light yellow flowers, while the samples that did not have such evidence had solely white petals. Nevertheless, many of the samples in group 1 may be more closely related to *N. alba* compared to *N. mexicana*, and if these are to be targeted for biological control, natural enemies adapted to *N. alba* may be more likely to be successful agents.

The second group (labelled group 2) consisted of multiple hybrids and *N. odorata,* suggesting that *N. odorata* could be a putative parent of these hybrids. The ITS analyses resulted in a similar grouping, including both *N. odorata* and *N. odorata tuberosa*. *Nymphaea odorata* and *N. mexicana* overlap in their native distributions and are known to hybridise naturally, so hybrids between these species are not surprising. Indeed, two hybrids, namely *Nymphaea* 'Moon Dance' (named by Florida Aquatic Nursery) and *Nymphaea* 'Osceola' Connelly, are listed as natural hybrids collected from Lake Okeechobee in Florida, USA (Slocum 2005). Other hybrids have been created by deliberate crosses of these species, but the presence of naturally occurring hybrids provides the opportunity for the discovery of

potential biological control agents that may be better suited to managing these hybrids. Indeed, admixture between *N. mexicana* and *N. odorata* was seen in the samples collected from the USA in the STRUCTURE analyses. Surveying natural populations of *N. odorata* as well as populations in the US that have admixture from both species will broaden the range of insects that could be used to manage hybrids with *N. mexicana* and *N. odorata* as putative parents.

Interestingly, both ISSR and ITS analyses provided evidence that the second group may be more closely related to *N. mexicana* than group 1. This observation may suggest that biological control agents that are adapted to feeding on *N. mexicana* might be more likely to impact plants that cluster in group 2 compared to group 1. However, the STRUCTURE analyses showed minimal genetic contribution of *N. mexicana* to specimens within this group. Nevertheless, it is evident that the genetic makeup of *Nymphaea* hybrids is complex, as cultivars may be produced from plants that are hybrids themselves or differing varieties of a particular species. An illustration of this is provided by the list of numerous *Nymphaea* hybrids that have *N. mexicana* reported as a parent according to Slocum (2005). These include, but are not limited to: *Nymphaea* 'Andreana' Marliac, *N.* 'Arethusa' Marliac, and *N.* 'Aurora' Marliac (all with parents listed as *N. alba* var. *rubra* X *N. mexicana*); *Nymphaea* Barbara Dobbins Strawn (*N. odorata* X *N. mexicana*); *N.* 'Berit Strawn' Strawn (*N.* 'Rembrandt' X *N. mexicana*); *N.* 'Helvola' Marliac (probably *N. tetragona* X *N. mexicana*), *N.* 'Marliacea Chromatella' Marliac and *N.* 'Moorei' Adelaide Botanic Gardens (both with parents listed as *N. alba* X *N. mexicana*); among others. A variant of *N. mexicana* is also listed as *Nymphaea mexicana* f. *canaveralensis* Frase (*N. mexicana* 'Cape Canaveral'), which has a larger flower and is also parent to hybrids such as *N.* 'Yellow Sensation' and probably *N.* 'Sunrise'.

The complexity of such genetic mixing within *Nymphaea* is also evident when considering the samples that displayed genetic contribution from multiple putative parents as well as unknown sources in the STRUCTURE analyses. For example, there was genetic contribution from an unknown source in the *N. mexicana* originating from USA, and also in some of the hybrid groups. It is important to note here that this could be a by-product of the algorithms used in the STRUCTURE analyses, or errors in scoring, especially considering the small sample sizes of the putative parents in the analysis. The small sample sizes are a caveat to this study, so future investigations could elicit improved results if more putative parent samples are used. Alternatively, owing to the long horticultural history of *Nymphaea* hybrids,

it is possible that these hybrids were formed from crosses of multiple species and other hybrids and are more likely to be challenging to manage using biological control. Similarly, there was evidence for the presence of multiple species or hybrid individuals within a single population for a few of the sites, which is not unusual considering the appeal of waterlilies for water garden design. Without flowers blooming, discerning between these hybrids may be challenging, but this emphasises the importance of prioritising those hybrids with aggressive and invasive growth and reproduction for biological control.

Owing to the high genetic variability within this group, including a greater number of samples from a more diverse geographic region may clarify the unknown genetic contribution in the USA group. Alternatively, it is also possible that the *N. mexicana* in South Africa do not share some of the genetic structure found in the USA samples due to genetic bottlenecks occurring during the introduction of *N. mexicana* to South Africa. This hypothesis is supported by the apparent tendency of *N. mexicana* to reproduce vegetatively in South Africa but is unlikely if the infestations of *N. mexicana* (excluding hybrids) resulted from multiple introductions from multiple sources (Müller-Schärer & Steinger 2004).

Introgression between *Nymphaea* within the same subgenus is not uncommon. Although the placement of *N. candida* and *N. alba* in the same clade in the ITS analyses was unexpected, *N. candida* has been reported as an allopolyploid originating as a hybrid with *N. alba* as the maternal parent, and *N. tetragona* as the paternal parent (Volkova et al. 2010). This could explain the closer grouping in the SplitsTree analysis of group 1 and *N. tetragona*, as *N. candida* was not included in the ISSR analyses due to failed amplification. The reported parentage of *N. candida* might also explain the grouping obtained in the ITS Maximum Likelihood and Bayesian Inference analyses, which grouped *N. candida* and *N. alba* var. *rubra* closely with *N. marliacea* and some of the hybrid samples, with a separate group including *N. tetragona* and *N. leibergii*. Similarly, Dkhar et al. (2011) demonstrated that material that had been identified as *N. alba* var. *rubra,* was actually a hybrid with *N. alba* as the paternal parent, and *N. odorata* as the maternal parent. Cultivars and varieties may originate as hybrids and later develop a unique genotype compared to the parents' genotypes. Nevertheless, studies such as these are useful to ascertain the species with the closest genetic structures, and hence focus surveying efforts. For this to be possible, it is important to include a broad range of samples from multiple populations to match groups.

Hybridisation has been reported to either increase or decrease palatability to insect herbivores (Fritz et al. 1998, 1999, Campbell et al. 2006, Roley & Newman 2006, Williams et al. 2014), which may also depend on which traits are selected for during breeding programmes. It is unclear how differing genetic structure might impact palatability (through the production of defensive chemicals, or lack thereof) in *Nymphaea,* but host specificity and multi-generational feeding trials using plants within and between the hybrid groups highlighted in this study would reveal more information in this regard. Nevertheless, the improved understanding of hybrid genetic structure achieved in this study allows us to prioritise agents based on their compatibility with particular genotypes. In addition, further investigating and comparing the competitive and invasive traits of each of the hybrid groups will allow us to prioritise those that are more of a threat to invaded ecosystems. For example, *Lantana camara* has been problematic to control for many years, owing to the difficulty in managing the numerous hybrids that exist. Recent advances in understanding the genetic structure of this plant using molecular studies, however, has been instrumental in improving management efforts (Neser & Cilliers 1989, Urban et al. 2011). Obtaining information such as this in the early stages of a biological control programme minimises financial and temporal outlay.

This study revealed useful information through the inclusion of putative parents/sources of genetic contribution as an addition to the data collected by Reid et al. (2021). However, a weakness of this study is the small sample size of those putative parent samples. Although efforts were made to obtain as many samples as possible, only 1 or 2 representatives of each species (barring *N. leibergii*) were used in the ISSR analyses, and one of these (*N. candida*) did not amplify sufficiently for use in these analyses. The ability to include multiple samples from Genbank sequences in the ITS analyses, however, strengthened the evidence to make the conclusions discussed here. Although some samples may be more closely related to other exotic *Nymphaea* (such as *N. alba*) with minimal contribution from *N. mexicana*, we now have a much better understanding of the genetic lineages of *Nymphaea* present in the country so that we can focus efforts at prioritising and investigating management options for each lineage.

This study also emphasised the importance of using both morphological and genetic data when attempting to identify plant populations. Similarly, using multiple analytical techniques for molecular data is important to account for limitations to the analyses and to provide stronger evidence for suspected relationships in different regions of genetic

sequences. Many suspected hybrid groups had mixed morphological traits to varying degrees and genetic information, using SplitsTree and STRUCTURE for ISSR, and Bayesian Inference and Maximum Likelihood analyses for ITS, was useful to better understand the origins of these groups. However, it is also important to note that detecting hybridisation through genetics alone can be tricky, as samples can group solely with one putative parent or another, leading one to deduce that they can be identified as that species alone, even when they are confirmed to have arisen from hybridisation and have intermediate morphological traits (as, for example, with *N. marliacea albida*, which grouped with *N. alba* and has white flowers, but is cultured as a hybrid with unconfirmed parentage, or *N. marliacea chromatella* which has pale yellow flowers and grouped with *N. alba* but is known to be a cross between *N. mexicana* and *N. alba* var. *rubra*). Other molecular markers and genetic analyses could be useful to better disentangle such issues, but for the purposes of this study, we can establish general groupings and thus survey similar naturally occurring plants for potential natural enemies.

In conclusion, to maximise biological efforts, naturally occurring populations of *N. marliacea chromatella/albida* and *N. albida* should be surveyed for natural enemies that would be most likely to be suitable for biological control of hybrids that fall within hybrid group 1. The hybrids within group 2 would have a higher probability of being managed successfully using natural enemies found from surveys on the parent plant, *N. odorata*. Finding agents that are specific to these groups without harming native South African *Nymphaea* may be possible if they are specific to members of subgenus *Nymphaea.* Remaining hybrid groups with high levels of genetic mixture are likely to be most challenging to manage using biological control and should be prioritised instead for manual/chemical control. Further research should aim to prioritise certain genetic groups and populations. This study furthermore shows that many sites are invaded by 'pure' *N. mexicana,* and because these group with *N. mexicana* from USA, this should be where potential biological control agents are sourced. These results are highly useful to focus efforts to manage the genetically diverse *N. mexicana* populations in South Africa.

Chapter 4: Distribution and host preference of the South African *Bagous longulus* on *Nymphaea* species

4.1 INTRODUCTION

Aquatic weevils make up a small percentage of the known weevil species in the world (Oberprieler et al. 2007), and many of these are in the genus *Bagous* Germar. This genus has a worldwide distribution with the exception of central and South America (Skuhrovec et al. 2011). *Bagous* is the single constituent of the tribe Bagoini, which does not currently belong to an existing subfamily, owing to their distinctness from other weevils and difficulty in discerning their closest relatives (Oberprieler 2014). The genus *Bagous* is a diverse group containing approximately 350 species (Caldara et al. 2017). Many *Bagous* species are important in that they can act as environmental indicators of water quality (Sprick 2001, Barman & Gupta 2015), and a number have been used or are being investigated for use in biological control programmes of invasive alien species. For example, *Bagous affinis* Hustache and *Bagous hydrillae* O'Brien, collected from India and Australia respectively, are used as biological control agents for *Hydrilla verticillata* (L.f.) Royle (Balciunas et al. 1996, Buckingham & Bennett 1998, Center et al. 2013). Similarly, *Bagous collignensis* Herbst, *Bagous longitarsis* Thomson, *Bagous geniculatodes* O'Brien, and *Bagous vicinus* Hustache have been considered as potential biological control agents for *Myriophyllum spicatum* (Cock et al. 2008), while *Bagous nodulosus* Gyllenhal and *Bagous validus* Rosenhauer are being studied as potential biological control agents of *Butomus umbellatus* L. (Häfliger et al. 2020). For African *Bagous* species, aside from being known to have a narrow host range on aquatic plants, there is a paucity of detailed information (Griffiths et al. 2015). Within southern Africa, *Bagous* is the only known genus of aquatic weevils, with seven recorded species, and there are likely to be multiple undescribed species present (Griffiths et al. 2015).

In Chapter 2, *Bagous longulus* was found feeding and reproducing on *N. mexicana* at three sites in South Africa. This species, originally known as *Pseudobagous longulus* (Gyllenhal), belongs to the subgenus *Hydronoplus*, and is commonly recorded in still or slow flowing water in South Africa, Mozambique, Zimbabwe, Botswana, and Namibia (Riaan Stals, pers. comm., museum records). Records of this species also exist as far north as Côte d'Ivoire (Kra et al. 2018, Seniva et al. 2020). The biology of this species has not yet been studied, and the larvae are undescribed (Riaan Stals, pers. comm.). Native *Nymphaea* are known to host *B. longulus*, but details about its host range are not well known. Indeed, the

taxonomy of *Nymphaea* in South Africa is somewhat debated. In addition to *Nymphaea lotus* L., Verdcourt (1989) listed five varieties of *Nymphaea nouchali* Burm. f. native to southern Africa: var. *petersiana* (also treated as a synonym of *Nymphaea capensis* by Conard (1905)), var. *ovalifolia* (Conard) Verdc., var. *caerulea* (Savigny) Verdc., var. *mutandaensis* Verdc., and var. *zanzibariensis* (Casp.) Verdc. More recent molecular evidence, however, suggests that these varieties should be elevated to species level (Borsch et al. 2007, Löhne et al. 2007). Borsch et al. (2007) argued that the molecular differences between these varieties are so great that *N. nouchali* var. *petersiana* should be moved from the subgenus *Brachyceras* (where *N. nouchali* is placed), to subgenus *Lotos*. If these placements based on molecular evidence are supported, four species of *Nymphaea* are native to South Africa: *Nymphaea caerulea* Savigny, *Nymphaea capensis* Thunb., *Nymphaea lotus* L., and *Nymphaea petersiana* Klotzsch (USDA Agricultural Research Service 2021). Including the invasive *N. mexicana*, these species represent three subgenera in South Africa: *N. mexicana* in subgenus *Nymphaea, N. capensis* and *N. caerulea* in subgenus *Brachyceras*, and *N. lotus* and *N. petersiana* in subgenus *Lotos* (Borsch et al. 2007). The relations of these species could be important to understand the potential host specificity of *B. longulus*.

Bagous longulus is being investigated as it has expanded its host range to include the exotic *N. mexicana*. The use of native insects for the management of an invasive plant is advantageous, as less money is required for overseas research and quarantine studies, there may be little impact on non-target native species that have coexisted with the natural enemy, and there is a lack of coevolved interspecific balances between the insect and the exotic plant (Hokkanen & Pimentel 1984, Creed & Sheldon 1993, Sheldon & Creed 1995, Roley & Newman 2006). Nevertheless, it is important to test the host specificity of native natural enemies to ensure that releasing them in large numbers will not negatively affect native flora to which it is already known to be adapted. Additionally, it is important to understand the distribution of the natural enemy: the climatic conditions of its natural distribution reflect physiological limits, and deliberately expanding the range of an organism could alter ecosystem dynamics and so should not be undertaken without caution. Notwithstanding, the risks of managing indigenous natural enemies (i.e., mass-rearing them) should be compared with the negative impacts of not taking action to manage invasive plant populations at all (Sheppard et al. 2003, de Clercq et al. 2011).

Ideally, biological control agents will be host specific, abundant, cause high levels of damage on the target plant, be easy to rear, and be adapted to the climate of the invaded

environment (Harris 1973, 1991, van Klinken & Raghu 2006) . Hence, in this study, I aim to accomplish the following:

1) Determine the distribution (and hence climatic suitability/abundance) of *B. longulus* in South Africa,
2) Determine the field host range of *B. longulus,*
3) Explore methods for culture rearing,
4) Determine host plant preferences.

Answering these questions will enable us to determine if *B. longulus* might control *N. mexicana* in South Africa, while also improving knowledge of fauna associated with native *Nymphaea* in the country. The potential for the use of augmentative biological control, using a native agent that has expanded its host range, is a promising means of conserving time and money in the development of this biological control programme.

4.2 METHODS

Surveys and field host range of *Bagous longulus*

Native *Nymphaea* sites were selected from records on the Global Biodiversity Information Facility (GBIF.org) and iNaturalist (inaturalist.org). Sites were selected to represent a range of climates and habitats across the country to develop an understanding of the distribution of *B. longulus* and its ability to survive in different environments. Sites that experienced less disturbance were preferred, as they were considered more likely to host *Bagous* species unaffected by human activity. Surveying took place in the Eastern Cape, Western Cape, North West, Mpumalanga, and Limpopo provinces from February to May and September to November in 2021. KwaZulu-Natal sites were surveyed in March 2022. No records of naturally occurring native *Nymphaea* were found in Gauteng, Free State, and Northern Cape.

At each site, total visible plant cover was estimated by two to three independent observers and the mean of these estimations taken as the value for each species at each site. Measurements of leaf length (cm) were taken from five random plants using a tape measure as a measure of plant size, and flowers, stems and leaves were photographed. Five randomly selected damaged leaves were photographed from the top and processed using ImageJ (Schneider et al. 2012) to determine percentage damage estimates for each site (calculated as damaged leaf area/total leaf area x 100 %). During the surveys, plants were searched for *B.*

longulus adults by hand for approximately 30 minutes, by turning over the leaves. The stems were pulled apart by hand to search for larvae and pupae. Where other emergent vegetation or other *Nymphaea* species were present, these were also surveyed to determine the likely field host specificity of the *Bagous* species encountered.

Five to ten *Bagous* adults were preserved in 70% ethanol and sent to Riaan Stals at the South African National Collection (SANC-ARC) for identification. An additional five *Bagous* adults were preserved in 95% ethanol for DNA preservation for future studies. Three to five replicated readings of pH, temperature (°C), total dissolved solids (ppm), electrical conductivity (µS), and salinity (ppt) were taken using a Sanxin PC5 pH/conductivity multimeter (Shanghai San-Xin Instrumentation Inc.) at each site.

The distribution of *B. longulus* was mapped in QGIS (QGIS Geographic Information System 2021) using records obtained from this study; records of specimens in the South African National Collection of Insects and Ditsong National Museum of Natural History in Pretoria; Iziko South African Museum in Cape Town via the SAMC database through GBIF (https://www.gbif.org/); and also iNaturalist records (https://www.inaturalist.org) and selected publications (Marshall 1907, Bird et al. 2017).

Survey data analyses

All analyses were carried out in R version 4.1.0 (R Core Team 2021). Model diagnostics and analyses were carried out using lme4 (Bates et al. 2015), glmmTMB (Brooks et al. 2017), and DHARMa (Hartig 2021) packages and plots were produced using ggplot2 (Wickham 2016). ANOVA tests using the car package (Fox & Weisberg 2019) were used to determine the significance of factors in the models, and post hoc tests for the models were conducted using the package emmeans (Lenth 2022).

Photographs of leaf damage were processed using ImageJ (Schneider et al. 2012) by cropping the images, adjusting the colour thresholds to target whole leaf areas, converting the images to binary format, removing outliers, and measuring the desired area. This was repeated to target and measure the damaged leaf area, and the percentage of total leaf area damaged was calculated from these measurements for five leaves from each site.

Firstly, to determine if there were statistically significant differences in percentage leaf area damaged for each species, a mixed effects model was run after carrying out model diagnostics. Leaf area percentage damage was set as the response variable, plant species as a

fixed effect, and site as a random effect, modelled using the tweedie distribution. A second model was run to determine whether there were significant effects of percentage leaf area damage and plant species (fixed effects) on leaf length (response variable) with site as a random effect and using the Gaussian distribution. Finally, general linear models were run to determine whether leaf percentage damage had an effect on plant cover for each species.

Rearing

Bagous longulus cultures were started by adding over 100 individuals (collected and added in a staggered format after use in experiments as detailed above) to ~2000 L pools lined with sediment, planted with *N. mexicana* collected from Westlakes, Cape Town, and fertilised using Osmocote® (Dynatrade) slow release fertiliser at 10-15 mg N/L of water. The pools had 100 % cover of *N. mexicana* at the time that weevils were added. Weevils were collected from the Assegaai and Skate Park sites and kept in separate pools. Preliminary trials for culture establishment occurred in a similar way, with 5-20 weevils added to 20 L buckets placed in 600 L tanks filled with water.

Host specificity experimental setup

Whole plant no-choice and multi choice experiments

To test host specificity and the ability of *B. longulus* to survive on other *Nymphaea* species, 24 plants each of *N. mexicana* collected from Westlakes in Cape Town (34°04'53.1"S 18°27'20.1"E) (*B. longulus* not present), *N. mexicana* from King's Beach Skate Park in Gqeberha/Port Elizabeth (33°58'26.2"S 25°38'41.0"E) (*B. longulus* present), *N. nouchali* collected from Kariega river near the Assegaai trails in the Eastern Cape (33°32'34"S 26°36'14"E) (*B. longulus* present), and *N. marliacea chromatella* supplied by AquaFlora nursery in North West Province (grown at Waainek Research Facility) (*B. longulus* not present), were planted in separate 20 L buckets held in flow-through tanks of 125 cm length, 108 cm width, and 174 cm height with approximately 540 L of water at Waainek Research Facility at Rhodes University in Makhanda/Grahamstown, South Africa. The *N. marliacea chromatella* represents group 1 of the genetic grouping determined in Chapter 3, grouping more closely with *N. alba,* and recorded as a hybrid of *N. mexicana* and *N. alba* var. *rubra.* All plants were fertilised with Osmocote® slow release fertiliser at concentrations of 10 mg N/L water. Although attempts were made to keep pest numbers low, aphids and snails were present on all the plants. All plants were completely free of *B. longulus* adults and damage prior to the start of the experiment. The buckets of each plant

species were placed randomly across 10 flow-through tanks and eight replicates were assigned to each of the following treatments: addition of two *B. longulus* females collected from Kariega River near Assegaai (hereafter referred to as "Assegaai"), addition of two female *B. longulus* collected from King's Beach Skate Park (hereafter referred to as "Skate Park"), and no *B. longulus* added (hereafter referred to as "Controls"). This setup was chosen to ensure that the water levels were high enough for the leaves for each species to lie flat on the water, which was deemed ideal for *B. longulus* oviposition to occur based on observations during establishment of *B. longulus* cultures at Waainek Research Facility. Before the experiment commenced, photographs were taken for use in analyses in ImageJ to calculate the percentage change in damaged leaf area for each species.

Owing to difficulties in sexing *B. longulus* reliably based on external morphological features, *B. longulus* individuals were collected and separated in petri dishes holding small leaf portions collected from the host plant populations moistened with a few drops of distilled water. These individuals were left overnight in a controlled environment (CE) room at 25º C and approximately 39% humidity, and the leaf portions checked the following morning for oviposition. Oviposition served as proof that an individual was female and mated, and these individuals were used in the experiments. Due to the nature of this sex determination method, the experimental treatments were initiated in a staggered setup within a few days of one another.

Each of the buckets was covered with white mesh netting and fastened with string or elastic to prevent escape of *B. longulus* and colonisation by greenhouse pests. Two HOBO temperature and light intensity meters (Onset® Computer Corp Firmware version 140.59) were placed at the first and sixth tank to monitor temperature changes during the experiment, and data were extracted and graphs generated using the HOBOmobile® Onset® app for android version 2.0. The buckets were left for two weeks to allow feeding, oviposition, and mine development from *B. longulus.* Originally, it was planned to leave the buckets for four to five weeks to allow for full lifecycle development (determined by observations during establishment of *B. longulus* cultures at Waainek Research Facility), but high levels of damage during the experiment deemed it necessary to collect the final data before the plants completely disintegrated. At the end of the two week period, the number of leaves in each bucket was recorded, and photographs were taken for ImageJ analyses. The number of larval mines and eggs per bucket were counted, and where the larval mines reached the petiole, the stems were dissected to count the number of larvae.

A subsequent procedural control experiment was carried out to quantify the effect that the nets may have had on the previous environmental setup. One plant of each species was added to each tank, so that there were four plants in each of 10 tanks. Photographs were taken of the leaves, the plants were left for two weeks without the addition of *B. longulus*, and photographs were taken again at the end of this period for comparisons of leaf area damage.

A similar experimental set up was utilised to conduct multi-choice experiments. The same buckets containing one plant of each species (*N. mexicana* from Westlakes, *N. mexicana* from King's Beach Skate Park, *N. nouchali* from Kariega River near Assegaai trails, and *N. marliacea chromatella* grown from nursery-bought plants) were placed in the tanks, so that there were four buckets per tank. The plants were placed such that the leaves were in as close proximity as possible, and the plants were not covered with netting. There were no controls for this experiment, as the goal was to determine if there was a preference in feeding and oviposition on different species. Two females of *B. longulus* from each population were collected as described above and added to each tank, such that five tanks were populated by weevils from Kariega River near Assegaai, and five tanks with weevils collected from King's Beach Skate Park in Gqeberha/Port Elizabeth. Photographs were taken for ImageJ analyses as before, and the tanks were left for two weeks before photographs were again taken, and mines, eggs and larvae counted.

Leaf square no-choice and multichoice experiments

Leaf squares (2.5 x 2.5 cm) were cut from freshly collected leaves of each of the species under investigation and placed in petri dishes moistened with distilled water and kept in a controlled environment (CE) room at 25^{o} C and 39 % humidity. One female *B. longulus* collected from each population (Skate Park and Assegaai) was placed in each petri dish and left for approximately 24 hours to feed and oviposit. After this time, the number of eggs on each leaf segment were counted, and the leaves were photographed to calculate leaf area percentage damage in ImageJ, before the segments were replaced with fresh leaf cuttings and the experiment repeated with the same weevils. This was repeated for five days. For the no-choice tests, only one leaf section was placed in each petri dish, with five replicates per plant species per weevil treatment. For the multichoice tests, one leaf section of each plant treatment was placed in a petri dish altogether so that there were four leaf squares in each dish, with five replicates per weevil treatment.

Segments from five leaves of each of the plants under investigation in this study were cut and the widths measured under a dissecting microscope using an eyepiece graticule. Segments from various parts of the leaf (at varying distances from the petiole) were measured from leaves of different sizes to calculate the average leaf width of each of the plants. These widths were multiplied by the leaf area damage determined by ImageJ analyses to calculate the volume (in mm^3) of each plant species consumed by *B. longulus* in each of the treatments.

Data analyses

Whole plant no-choice and multichoice experiments

Like the survey data, ImageJ was used to calculate the percentage damage incurred to the leaves of each species. To investigate differences in the percentage change in damaged leaf area between plant species and weevil treatments for the no-choice tests, a mixed effects model was run using the percentage change in damaged leaf area as the response variable, plant species/population and weevil as fixed effects, and tank as a random effect to account for the lack of independence between treatments. The MuMIn::r.squaredGLMM (Barton 2020) function in R was used to calculate the total variation in this model accounted for by the tank effect. Percentage changes in damaged leaf area from the procedural control experiment were compared with the controls from the no-choice netted experiments using a mixed effects model with treatment (either as procedural control or control) and plant as fixed effects, and tank as the random effect.

To investigate the same parameters in the whole plant multichoice experiment, a general linear model was run using the percentage change in damaged leaf area as the response variable, and plant species/population and weevil treatment (including their interaction) as the predictor variables.

Differences in number of mines and eggs between treatments for both the no-choice and multichoice whole plant experiments were modelled using the glmmTMB function, with the number of mines or eggs as the response variable, weevil population and plant species/population as the explanatory variables, using a negative binomial distribution. The model for the no-choice experiments included tank as a random variable.

Leaf square no-choice and multichoice experiments

A mixed effects model was used to determine the effect of plant species and weevil treatment on the volume of leaf consumed by *B. longulus*. For the no-choice tests, volume

was set as the response variable, plant species and weevil population as the fixed effects, and day measured as a random effect to account for the lack of independence in taking measurements of the same weevils on different days. For the multichoice experiments, owing to high occurrences of zeros in the dataset where *B. longulus* elected not to feed on leaf segments of a particular plant species on each day, a mixed effects model using a tweedie distribution was used, including a dispersion model to allow heteroskedasticity between plant species, with the same response, fixed, and random variables as described for the no-choice tests. The same tests were repeated for the leaf percentage damage for both experiments.

To investigate effects of plant and weevil population on egg counts for both the no-choice and multichoice plant segment petri dish experiments, a mixed effects model was run using the number of eggs as the response variable, plant species/population and weevil population as fixed effects, and count day as a random effect, using a negative binomial distribution. A zero-inflation model was added to the no-choice data to improve model fit.

4.3 RESULTS

Surveys, field host range, and rearing

Bagous longulus was found in a range of environments in all provinces of South Africa except for Northern Cape, Free State and Gauteng, where no records of naturally occurring native *Nymphaea* were found (Figure 4.1). The sites included natural and artificial water bodies with varying water parameters and abundances of *B. longulus* (Table 4.1). There was considerable variation in electrical conductivity, total dissolved solids, and salinity within the sites, with a mean pH of 7.3 (+/- 0.5). The plants found at the sites included *N. caerulea, N. capensis, N. lotus, N. mexicana,* suspected *N. petersiana*, *Nymphaea* hybrids, *Nymphoides thunbergiana* (Griseb.) Kuntze, *Aponogeton* L.f. spp., as well as various reed species (Poaceae). Of these species, *Bagous* feeding damage, larvae, and adults were only found on *Nymphaea* species. All specimens were confirmed as *B. longulus* (identified by Riaan Stals, South African National Collection (SANC)). Owing to the presence of *B. longulus* on all *Nymphaea*, and the difficulty in obtaining comprehensive descriptions of all variations of *N. nouchali* (i.e., *N. caerulea, N. capensis*, and *N. petersiana*), these were all regarded as *N. nouchali* in the further analyses.

At some sites where both *N. nouchali* and *N. lotus* were present, *B. longulus* was found in greater abundance on *N. nouchali.* The same was true where *N. nouchali* occurred with *Nymphaea* hybrids, except that only low numbers of early instar larvae were found on

the hybrids, whereas later instar larvae and teneral adults were found on *N. lotus* as well as *N. nouchali.* Larval abundances were not always directly linked with adult abundance, probably owing to cyclical and seasonal differences between sites (Table 4.1).

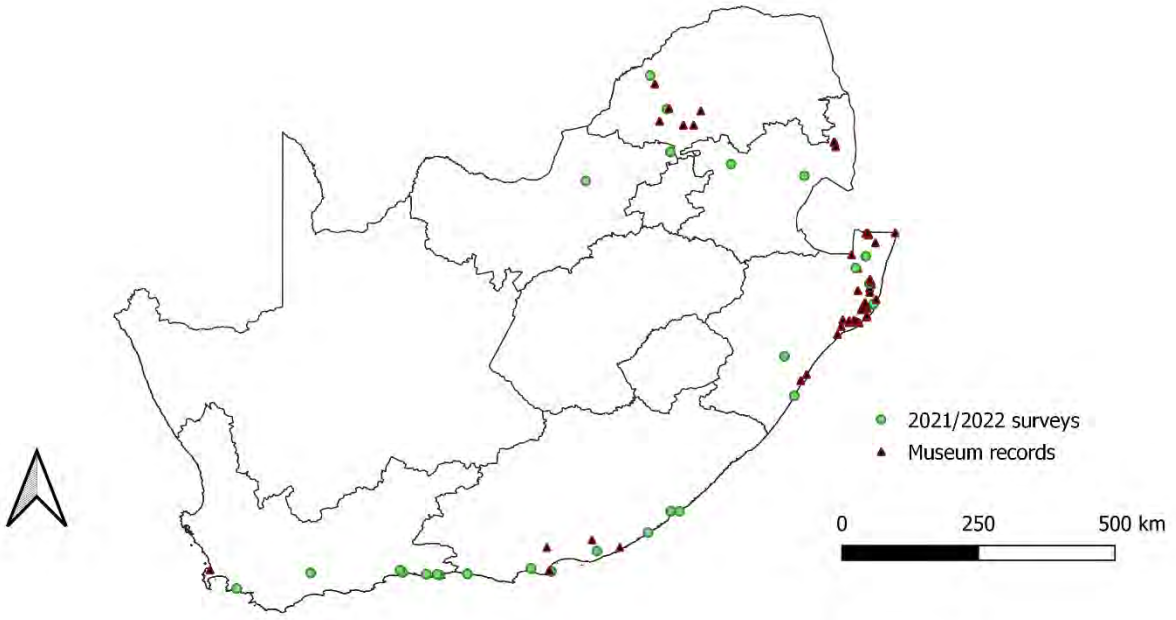

Figure 4.1: Distribution of *Bagous longulus* in South Africa based on records compiled by Riaan Stals from South African National Collection of Insects and Ditsong National Museum of Natural History in Pretoria, Iziko South African Museum in Cape Town via the SAMC database through GBIF (https://www.gbif.org/), iNaturalist records (https://www.inaturalist.org) and selected publications (Marshall 1907, Bird et al. 2017). These records are shown as maroon triangle icons. Records contributed from the surveys described in this study shown as green circle icons. Map created using QGIS (QGIS Geographic Information System 2021).

Table 4.1: Mean values for water parameters and estimates of *Bagous longulus* abundance (as number of larvae per 5-10 dissected stems, or an estimate of adult abundance based on the ease of collection) measured at surveyed sites across South Africa. Water samples for sites marked with an asterisk may not be accurate due to the discovery that the meter used was uncalibrated.

Site	**Plant**	**pH**	**Temperature (°C)**	**Electrical conductivity (µS)**	**Total dissolved salts (ppm)**	**Salinity (ppt)**	***Bagous longulus* abundance (n larvae per stem)**	***Bagous longulus* abundance (adult/feeding estimate)**
Eastern Cape								
Van Stadens Nature Reserve	*N. nouchali*	7.5	27.5	283.0	200.8	0.14	0.6	Moderate
Kariega River near Assegaai	*N. nouchali*	7.8	25.7	2822.0	1990.0	1.40	N/A	Moderate
Morgan Bay Beach Road	*N. nouchali*	7.4	27.0	1180.6	835.2	0.57	0.2	Moderate
Haga Haga farm dam	*N. nouchali* Hybrid	7.0	30.7	219.6	154.6	0.10	0.2 0	Moderate Low

Kidds beach, East London	*N. nouchali*	7.2	31	332.0	235.0	0.15	1.4	Low
Kings Beach Skate Park, Port Elizabeth/Gqeberha	*N. mexicana*	8.3	26.2	1538.0	1092.0	0.77	N/A	Moderate
Western Cape								
Tsitsikamma National Park	*N. nouchali*	6.8	25.3	93.3	66.5	0.05	0.1	Moderate
Farm near Plettenberg Bay	*N. nouchali*	7.2	28.5	236.2	168.2	0.11	1.5	Moderate
	White hybrid						0.2	Low
Knysna Lifestyle Estate		7.4	23.0	325.6	230.2	0.16	0.1	Moderate
George horse farm	*N. nouchali*	7.6	25.0	311.4	222.8	0.16	0.2	Low
	Hybrid						0.3	Low
George Botanical Gardens	*N. nouchali*	7.5	24.5	169.8	120.0	0.08	0	Low
Somerset Gift Farm, Swellendam	*N. nouchali*	7.0	24.0	243.8	174.0	0.12	1.8	Moderate - high

Kleinmond Golf Club	*N. nouchali*	6.4	25.7	260.8	185.8	0.13	1.0	Moderate
	Hybrid						0.4	Absent
Dam Park, Somerset West	*N. nouchali*	6.9	19.3	152.1	108.2	0.08	0	Absent
Green Point Park, Cape Town	*N. nouchali*	7.5	26.2	172.1	122.6	0.09	0	Absent
Masescha Lodge, Plettenberg Bay	*N. nouchali*	8.5	16.1	342.8	242.2	0.17	2.2	Low
	Hybrid						0	Low/absent
North West								
Marico Oog*	*N. nouchali*	9.8	21.0	26.2	186.2	0.13	0.6	Low
Dube Game Reserve*	*N. lotus*	11.3	24.1	323.8	230.2	0.16	0	Absent
Limpopo								
Zaagkuilsdrift*	*N. nouchali*	10.7	27.7	2213.0	1563.0	1.11	0	Low
	N. lotus						0	Absent
Verloren Estate*	*N. nouchali*	8.3	24.8	36.3	26.0	0.02	0.5	Low

Joubert Pro Hunting Farm*	*N. nouchali*	9.5	25.9	24.1	17.1	0.01	1.3	Low
Ephemeral pan, Lephalale*	*N. nouchali* *N. lotus*	8.5	21.0	396.6	281.8	0.20	3.4 0	High Absent
Mpumalanga								
Aylestone 8 Farm, Barberton	*N. nouchali*	8.0	20.8	34.5	24.8	0.02	0.8	Moderate
Kranspoort Golf Estate	*N. nouchali*	9.2	25.7	75.1	53.7	0.04	0.6	Low
KwaZulu-Natal								
Rennie's Beach, Port Edward	*N. nouchali*	6.4	25.9	198.6	140.7	0.10	1.6	Low
Scottburgh Golf Course	*N. nouchali* *N. lotus*	6.3	30.6	350.8	249.2	0.18	0.6 0	Moderate Moderate
Fountainhill Estate	*N. nouchali* *N. mexicana*	7.3	22.1	235.8	164.4	0.12	2.2 1.8	Moderate to high Moderate to high

St Lucia Estuary	*N. nouchali* *N. lotus*	7.4	28.2	415.8	295.2	0.21	1.2 1.6	Moderate Low
Farm near Hluhluwe	*N. nouchali*	7.8	29.7	844.8	610.2	0.42	1.2	Moderate to high
Ghost Mountain Inn, Mkuze	*N. lotus*	7.7	33.4	1339.0	981.6	0.73	0	Low
Stream near Jozini, Pongola	*N. lotus*	7.7	27.0	491.0	348.6	0.25	0.8	Low

The mixed effects model for leaf area percentage damage showed a significant effect of plant species (χ^2 = 36.0270, d.f. = 3, $P < 0.001$), with significant differences found between all plant species (*N. mexicana, N. lotus, N. nouchali*, and *Nymphaea* hybrids) except for between *N. lotus* and *N. nouchali*. Hence, *N. mexicana* had significantly higher percentage leaf area damaged compared to *N. lotus, N. nouchali*, and the hybrids, and the hybrids had significantly lower percentage leaf area damaged compared to the native species where *B. longulus* was present (Figure 4.2A). When investigating the effects of leaf area percentage damage and plant species on leaf length, there was no significant effect of leaf area percentage damage (χ^2 = 2.6693, d.f. = 1, $P = 0.1023$) but there was an effect of plant species (χ^2 = 122.4612, d.f. = 3, $P << 0.05$) with *N. mexicana* showing significantly smaller leaf lengths compared to the other three species (Figure 4.2B).

There was no significant effect of leaf percentage damaged (χ^2= 2.3955, d.f. = 1, P = 0.1217) nor of species (χ^2 = 6.1064, d.f. = 3, $P = 0.1065$) on overall plant cover but generally *N. lotus* had the lowest plant cover where present, followed by the hybrids, *N. mexicana* and *N. nouchali* (Figure 4.2C).

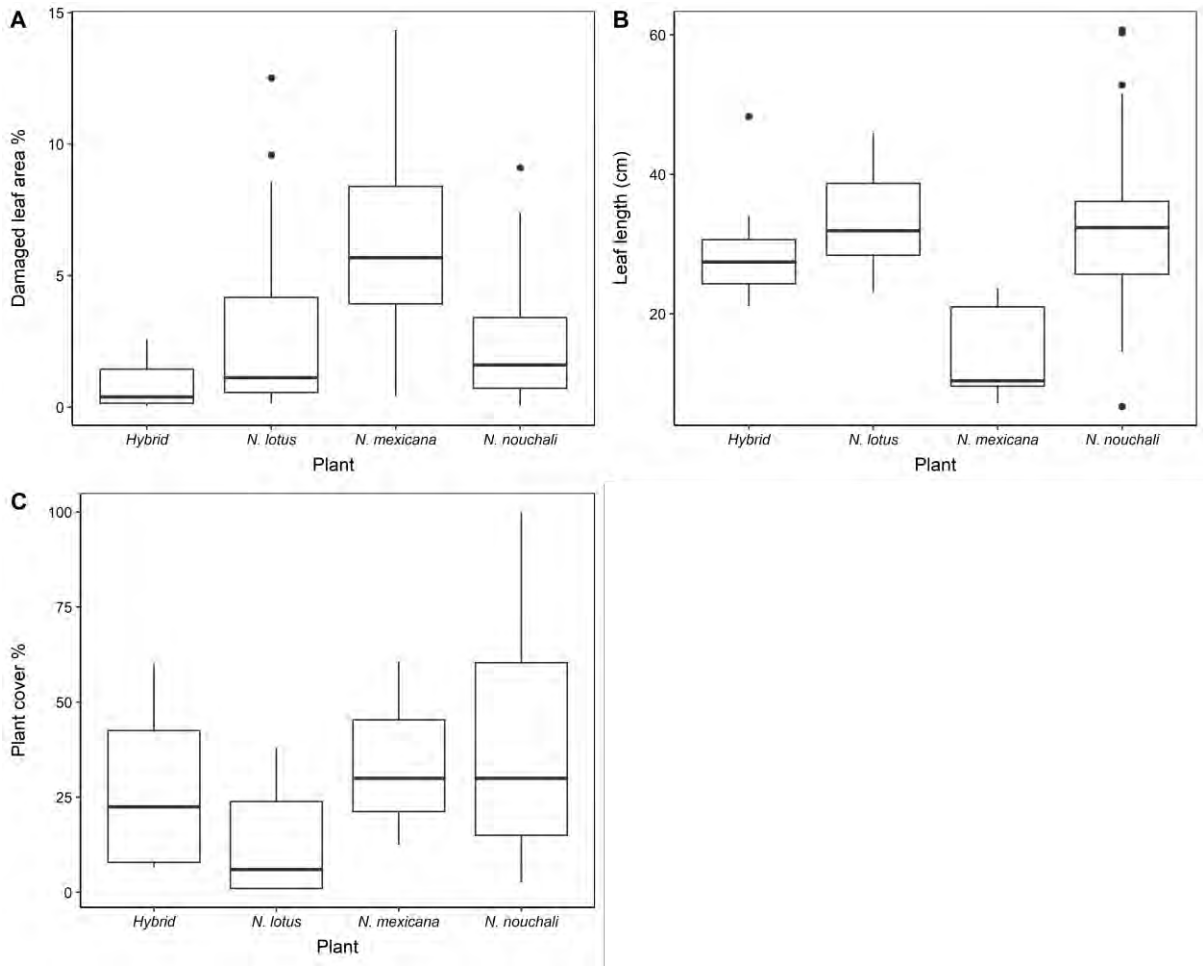

Figure 4.2: Percentage damaged leaf area (A), leaf lengths (B), and percentage plant cover (C) of each *Nymphaea* species sampled during surveys for *Bagous longulus* across South Africa. Boxes represent upper and lower quartiles and median values; whiskers represent highest and lowest values at 1.5 times the interquartile range. Outliers are marked as separate dots.

Bagous longulus cultures were reared successfully at the Waainek Research Facility tunnels. General observations suggested that leaf mining and successful reproduction was more likely when leaves were floating on the water surface to prevent dehydration of eggs and larvae. Reproducing populations were established in March 2021, and by May 2021, populations began to decline until reproduction (estimated by the number of mines on the leaves) decreased and ceased. By late September/early October, reproduction resumed without any further addition of adult weevils, indicating that *B. longulus* populations had successfully overwintered.

Host specificity experiments

The conditions in terms of air temperature and light intensity were similar for both the no-choice and multichoice whole plant experiments (Figure 4.3). For the no-choice tests, air temperature reached a maximum of 57.66 ºC, minimum of 16.08 ºC, with a mean of 29.03 ºC +/- 10.68. The maximum light intensity was recorded as 84 132 lux, with a mean of 14 007 lux +/- 21 095. For the multichoice tests, air temperature reached a maximum of 56.28 ºC, minimum of 9.61 ºC, with an overall average of 22.92 ºC +/- 10.98. Light intensity values reached 70 308 lux maximum, with an average of 10 769 lux +/- 16 861.

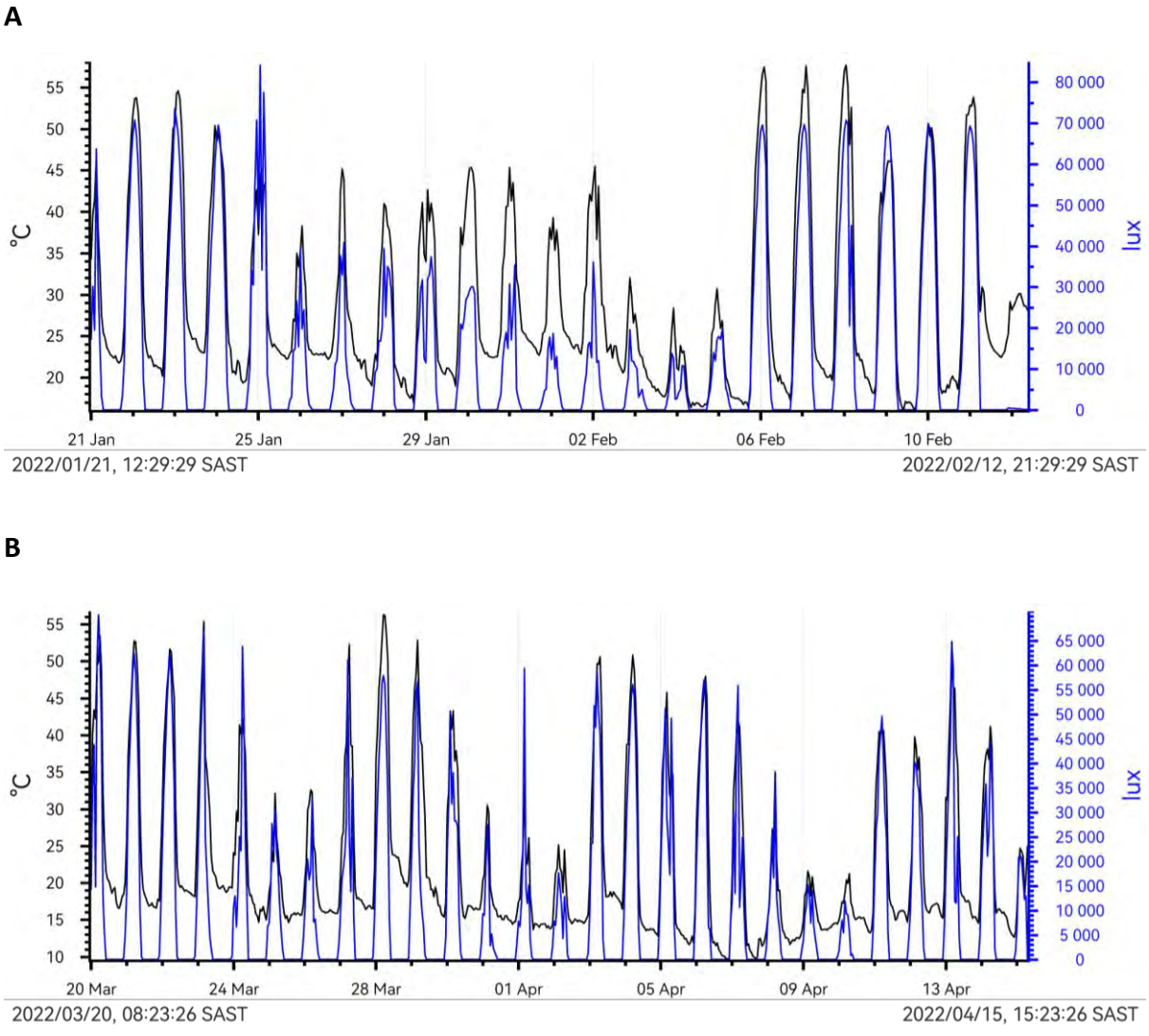

Figure 4.3: Air temperature (ºC) and light intensity (lux) readings for tank experimental setup during no-choice (A) and multichoice (B) host tests with *Bagous longulus* at the Waainek Research Facility tunnels in Makhanda. Blue lines represent light intensity measurements,

and black lines represent temperature measurements. Graphs generated from HOBOmobile® Onset® app for android version 2.0.

Whole plant no-choice experiments

Plant species did not affect the percentage change in damaged leaf area between treatments ($\chi^2 = 4.8594$ d.f. = 3, $P = 0.1823$), but the addition of weevils significantly increased damaged leaf area, in comparison to the control treatments (without weevils added) ($\chi^2 = 11.5860$, d.f.=2, $P < 0.05$) (Figure 4.4). There were no significant differences in damage between the weevil populations. Of the total variation in the mixed effects model, 2.34 % was accounted for by the tank effect. Use of the nets in the experiment did significantly impact the experiments, wherein the procedural controls had significantly lower reductions in undamaged leaf area over the course of the experiment compared to the netted control treatments in the no-choice experiments ($\chi^2 = 18.4870$, d.f. = 1, $P < 0.05$).

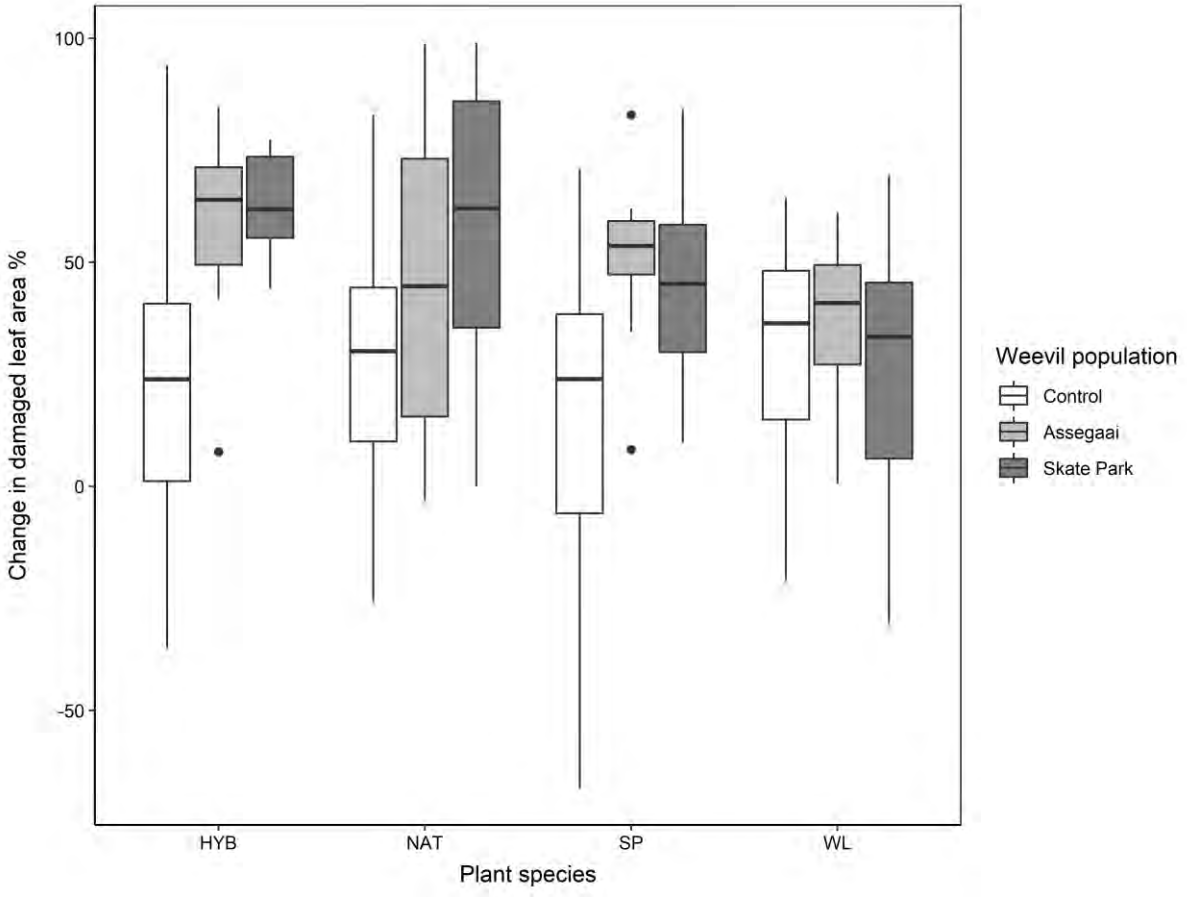

Figure 4.4: Change in damaged leaf area percentages for four different plant species/populations to which *Bagous longulus* from two different populations (Assegaai and Skate Park) were added in a no-choice experimental setup compared to insect-free controls. Boxes represent upper and lower quartiles and median values; whiskers represent highest and

lowest values at 1.5 times the interquartile range. Outliers are marked as separate dots. HYB = *Nymphaea marliacea chromatella* hybrid, NAT = native *Nymphaea nouchali*, SP = *Nymphaea mexicana* collected from King's beach Skate Park in Gqeberha/Port Elizabeth, WL = *Nymphaea mexicana* collected from Westlakes, Cape Town.

There was no significant effect of plant species/population (χ^2 = 7.5810, d.f.= 3, *P* = 0.0555) nor of weevil treatment (χ^2 = 1.0818, d.f. = 2. *P* = 0.5822) for the number of mines on the leaves of each species. The same was true for the number of eggs per treatment (χ^2 = 1.9711, d.f. = 3, *P* = 0.5784 for effect of plant species; χ^2 = 1.3148, d.f. = 2, P = 0.5182 for effect of weevil population). However, the number of mines on the plants were greatest in *N. nouchali* (5.41 +/- 9.25), followed by *N. marliacea chromatella* hybrid, and then *N. mexicana* from the Skate Park in Gqeberha/Port Elizabeth, and Westlakes. A similar trend was seen for the number of eggs, with the highest counted on *N. nouchali* (7.0 +/- 22.6) followed by *N. mexicana* from Westlakes and Skate Park, and lastly *N. marliacea chromatella* hybrid.

Whole plant multichoice experiments

Plant species/population did affect the percentage change in damaged leaf area between treatments (F = 3.8772, d.f. = 3, *P* < 0.05) with the hybrid (*N. marliacea chromatella*) plants showing significantly less reduction in damaged plant area compared to *N. nouchali* and *N. mexicana* (Figure 4.5). There was no effect of weevil population (F = 0.0940, d.f. = 1, *P* = 0.7612).

As with the no-choice whole plant experiments, the number of mines and eggs were unaffected by plant species (mines: χ^2 = 4.0253, d.f. = 2, *P* = 0.2587; eggs: χ^2 = 1.4276, d.f. = 3, *P* = 0.6991) and weevil population (mines: χ^2 = 0.4711, d.f. = 1, *P* = 0.4925; eggs: χ^2 = 0.1380, d.f. = 1, *P* = 0.7103). However, the greatest mean number of mines was found on the Westlakes plants (13.2 +/- 8.7), followed by the hybrids, *N. nouchali* and *N. mexicana* from Skate Park. The mean greatest number of eggs was found on *N. nouchali* (2.4 +/- 3.4), followed by *N. mexicana* from Skate Park, the hybrids, and then *N. mexicana* from Westlakes.

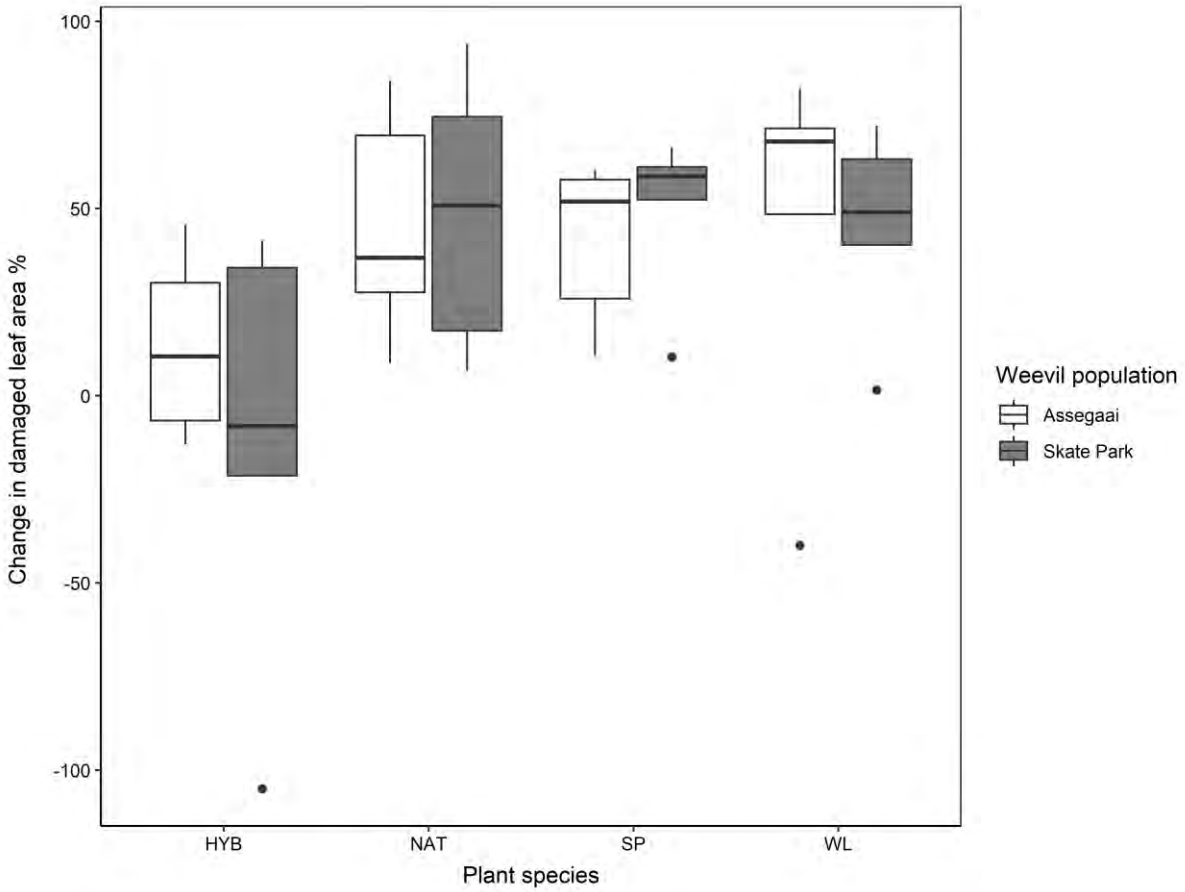

Figure 4.5: Changes in damaged leaf area percentages for four plants to which *Bagous longulus* from two different population were added and allowed to choose between plants. Boxes represent upper and lower quartiles and median values; whiskers represent highest and lowest values at 1.5 times the interquartile range. Outliers are marked as separate dots. HYB = *Nymphaea marliacea chromatella* hybrid, NAT = native *Nymphaea nouchali*, SP = *Nymphaea mexicana* collected from King's beach Skate Park in Gqeberha/Port Elizabeth, WL = *Nymphaea mexicana* collected from Westlakes, Cape Town.

Leaf square no-choice tests

There was a significant effect of both plant species/population (χ^2 = 25.9220, d.f. = 3, P <<0.05) and weevil treatment (χ^2 = 10.1090, d.f. = 1, P < 0.05) on the leaf volume consumed, with *B. longulus* consuming a significantly greater volume of *N. nouchali* compared to the other plant species, and weevils from Kariega River, Assegaai, consuming a significantly greater volume overall compared to the weevils from Skate Park (Figure 4.6A). In terms of leaf area damaged, however, there was no significant difference in percentage damage between *N. nouchali* and *N. mexicana* from both populations, but *N. marliacea chromatella* had significantly less damage compared to these species (χ^2 = 11.3317, d.f. = 3,

$P < 0.05$) (Figure 4.6B). Weevils collected from Assegaai inflicted a significantly greater leaf area percentage damage overall ($\chi^2 = 7.3312$, d.f. = 1, $P < 0.05$).

The number of eggs counted on each leaf segment did not differ significantly between plant species ($\chi^2 = 1.4609$, d.f. = 3, $P = 0.6913$) nor weevil population ($\chi^2 = 2.9084$, d.f. = 1, $P = 0.0881$).

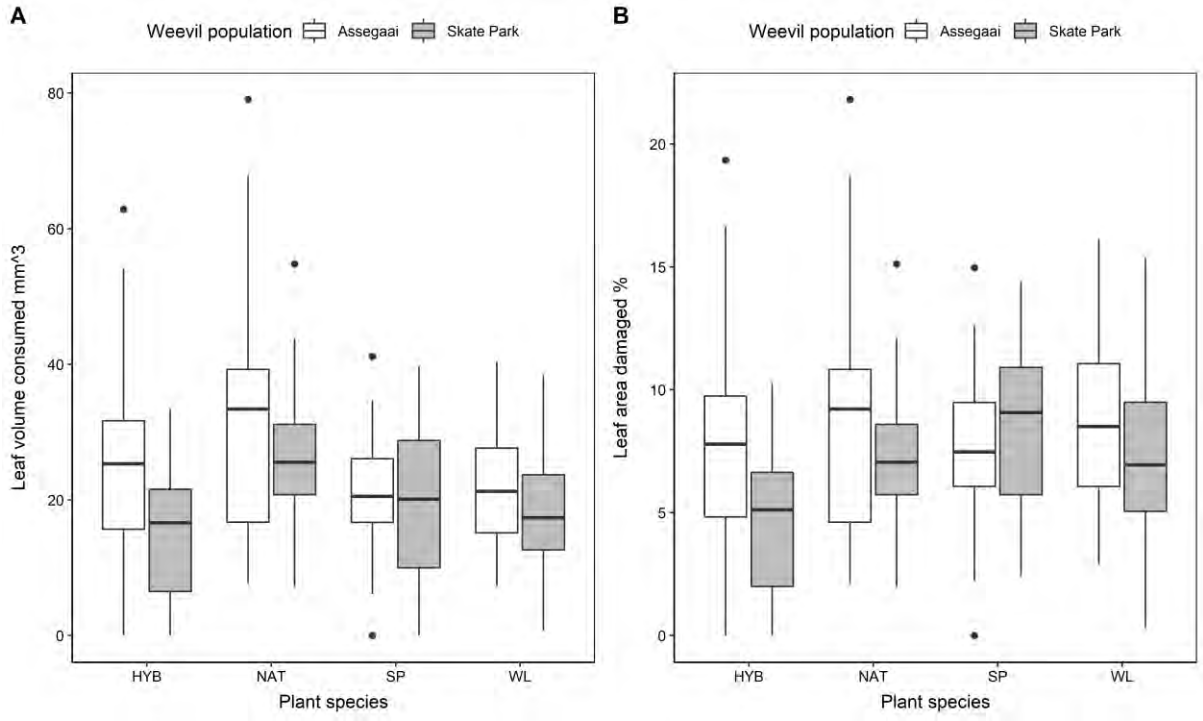

Figure 4.6: Leaf volume (mm^3) and leaf area (%) damaged in petri dish no-choice experiments completed using *Bagous longulus* from two different populations. Boxes represent upper and lower quartiles and median values; whiskers represent highest and lowest values at 1.5 times the interquartile range. Outliers are marked as separate dots. HYB = *Nymphaea marliacea chromatella* hybrid, NAT = native *Nymphaea nouchali*, SP = *Nymphaea mexicana* collected from King's beach Skate Park in Gqeberha/Port Elizabeth, WL = *Nymphaea mexicana* collected from Westlakes, Cape Town.

Leaf square multichoice tests

Plant species significantly impacted the volume of leaf consumed by *B. longulus* ($\chi^2 = 15.5772$, d.f. = 3, $P << 0.05$), but weevil population did not ($\chi^2 = 0.5185$, d.f. = 1, $P = 0.4715$). Within the different plant species, significantly lower volumes of *N. nouchali* were consumed compared to the other species, while significantly lower volumes of *N. mexicana* from Westlakes was consumed only when compared to *N. mexicana* from Skate Park (Figure

4.7A). The results for percentage damage values were similar, where plant effect was significant ($\chi^2 = 48.6402$, d.f. = 3, $P << 0.05$), with lower percentage damage values for *N. nouchali* compared to the other plants, but weevil population effect was not significant ($\chi^2 = 1.3141$, d.f. = 1, $P = 0.2516$) (Figure 4.7B).

Both plant species ($\chi^2 = 17.0799$, d.f. = 3, $P < 0.05$) and weevil population ($\chi^2 = 22.0794$, d.f. = 1, $P <<0.05$) had a significant effect on the number of eggs counted on each leaf segment, whereby there were significantly fewer eggs on *N. nouchali* segments compared to *N. mexicana* from Westlakes and King's Beach Skate Park, but not significantly so compared to *N. marliacea chromatella.* For the weevils, *B. longulus* collected from Assegaai oviposited significantly fewer eggs overall compared to *B. longulus* from Skate Park

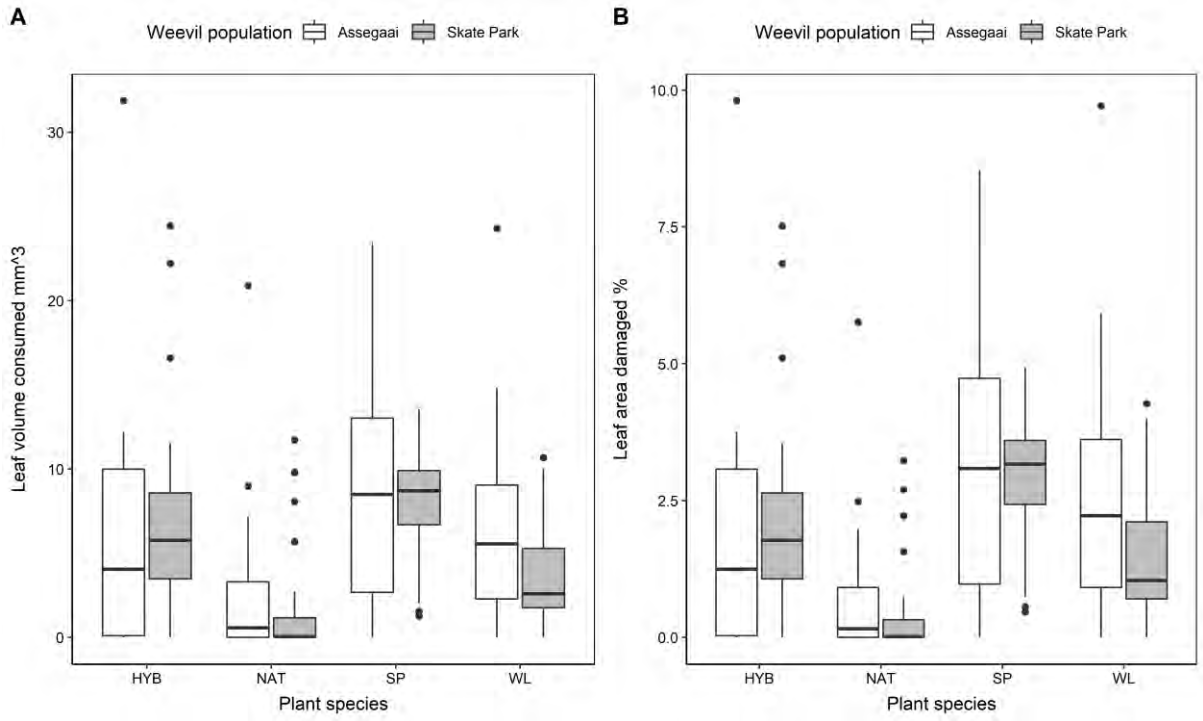

Figure 4.7: Leaf volume (mm^3) and leaf area (%) damaged in petri dish multichoice experiments completed using *Bagous longulus* from two different populations. Boxes represent upper and lower quartiles and median values; whiskers represent highest and lowest values at 1.5 times the interquartile range. Outliers are marked as separate dots. HYB = *Nymphaea marliacea chromatella* hybrid, NAT = native *Nymphaea nouchali*, SP = *Nymphaea mexicana* collected from King's beach Skate Park in Gqeberha/Port Elizabeth, WL = *Nymphaea mexicana* collected from Westlakes, Cape Town.

4.4 DISCUSSION

Bagous longulus was found throughout the country where native *Nymphaea* were found naturally, that is, in six of the provinces but not in Gauteng, Northern Cape, and Free State, where natural records of *N. nouchali* were not found. Hence, it is evident that *B. longulus* can survive in a range of climatic environments, and that it is already widely distributed in South Africa. Museum records also indicate that this species occurs further north into Africa (Riaan Stals, pers. comm.). The absence of *B. longulus* from Gauteng, Northern Cape, and Free State may not be due to climatic incompatibility, but perhaps rather to the lack of natural waterbodies (and thus native water lilies) in these provinces (Colvin et al. 2013). It is also possible that there are sites in these provinces where naturally occurring native *Nymphaea* and *B. longulus* do occur, but were not surveyed during this study, as I was reliant on iNaturalist and GBIF records for site selection. Nonetheless, the wide distribution of *B. longulus* suggests that augmentative introductions of this species to *N. mexicana* infestations will likely not pose issues of climatic unsuitability in at least six of the nine provinces. Release trials in the other three provinces, but most notably in Gauteng (Highveld) where there are several sites invaded by *N. mexicana* (see Chapter 2), and where the success of other biological control programmes has been limited due to difficulties with foreign agents surviving frosts and cold winters (Coetzee et al. 2011b, May & Coetzee 2013, Hill & Coetzee 2017), would confirm the ability of *B. longulus* to survive in these conditions. Indeed, temperature records from experiments conducted at Waainek Research Facility indicate the ability of the weevil to survive at high air temperatures (over 50 ºC), but less information is available about its ability to survive lower temperatures. Although it is not confirmed where *B. longulus* overwinters, its proximity to water may promote buffering of extreme low temperatures (Danks 2008). Alternatively, repeated exposure to extreme cold (as opposed to isolated exposure events) may be more influential to *B. longulus* mortality. For example, overwintering mortality of *Cyrtobagous salviniae* Calder & Sands (Coleoptera: Curculionidae) increased with the number of repeated cold exposure cycles (Obeysekara et al. 2015). Studies investigating the cold and heat tolerance of *B. longulus* could be useful to indicate its ability to survive different climates.

In the field, *B. longulus* demonstrated host specificity for members of the genus *Nymphaea*. The weevils were found reproducing on all native *Nymphaea* in South Africa, and no feeding or reproduction was recorded from other plants, even those with similar structures (i.e., floating leaves with submerged stems) such as *Nymphoides* and *Aponogeton* species

growing in the same vicinity as *Nymphaea* spp. It was noted, however, that although *B. longulus* can sustain isolated populations on the invasive *N. mexicana*, only sporadic evidence of feeding and larval mining was evident on *Nymphaea* hybrids, so it is unlikely that populations are sustained in the field. This was true for hybrids with *N. mexicana, N. odorata*, and *N. alba* as putative parents (see Chapter 3).

Noteworthy, is that at the three sites where *N. lotus* and *N. nouchali* were growing together in the same waterbody, higher levels of adult feeding and larval mining was observed in *N. nouchali*, suggesting either that *B. longulus* prefers this species, or that cryptic species, multiple races, or subspecies of *B. longulus* exist with slightly different host preferences. Cases of cryptic speciation are more common than initially thought, as is evidenced by the detection of cryptic speciation in *Eccritotarsus catarinensis* (Carvalho) (Hemiptera: Miridae) (Paterson et al. 2016), and this, along with the existence of multiple races and biotypes, can have important implications for biological control (Smith et al. 2018). The possibility that there are genetic differences between *B. longulus* populations around the country may be substantiated by the observation that *B. longulus* larvae in *N. lotus* were confined to the top of the petiole, whereas larvae in *N. nouchali* were often found several centimetres down the stem. Similar differences in feeding behaviour have been recorded in other cryptic species (Lu et al. 2021), but in this case, the contrasting mining behaviour may also be an effect of varying plant sizes. Molecular studies could provide evidence for or rule out the presence of cryptic species. Nevertheless, the narrow host range of *B. longulus* implies that the use of this species as a biological control agent for *N. mexicana* should not pose significant risks to existing native flora. Furthermore, experimental evolution experiments could be useful to select for *B. longulus* populations with preference for *N. mexicana* instead of native *Nymphaea* (Müller-Schärer et al. 2020).

One might argue that augmented releases of *B. longulus* may put pressure on native *Nymphaea* (Cofrancesco 2000), but only three of the known sites are inhabited both by *N. mexicana* (not *Nymphaea* hybrids) and *N. nouchali*, and *B. longulus* is already present at these. Furthermore, although augmentative use of a native insect species may indeed affect its native host, this may be preferable to using herbicides that would cause undesirable non-target effects (Jurado et al. 2011, Boutin et al. 2014). It is noteworthy that the dispersion ability of *B. longulus* is not well studied, so further research may be necessary in this regard to prevent widespread dispersal of *B. longulus* added in greater numbers to sites invaded by *N. mexicana*. The genus *Bagous* is reported to have low dispersal capability in general

(Sprick 2001, Skuhrovec et al. 2011, Caldara et al. 2017), so this may not be a major issue. On the other hand, contradictory reports of dispersal ability of other members of Curculionidae (Room & Thomas 1985, Herrick & Kok 2010) warrants further investigation into the dispersal ability of *B. longulus*.

For the whole host plant specificity experiments, there were no significant differences in plant area damaged between any of the four species/genetic lineages in the no-choice treatments, and no differences in the same parameter between weevil populations (aside from significantly lower damage in the control treatment). The same was true in the multichoice treatments, except that there was significantly lower damage on the hybrid plants. Although the use of the nets in the no-choice experiments did contribute to the plant damage levels (as evidenced by the procedural control experiments), these data may indicate that *B. longulus* is able to survive on *N. marliacea chromatella* in isolation, but that it prefers *N. mexicana* or *N. nouchali* when presented with a choice between these species. This is the opposite effect to that seen in *Euhrychiopsis lecontei* on *Myriophyllum* spp. in the USA, which also expanded its host range to include an invasive plant, but in fact demonstrated higher preference for the exotic plant and hybrids compared to the native species (Roley & Newman 2006). However, these hybrids were crosses of the original host and the invasive, unlike the hybrids in this study that were crosses of the invasive and other exotic species. Indeed, the experiments in this study only tested *N. marliacea chromatella,* which has *N. mexicana* and *N. alba* var. *rubra* as parents and is in group 1 as determined by genetic analyses (Chapter 3). Other hybrids with different parentage (i.e., group 2 and the *N. tetragona* group from Chapter 3) may thus demonstrate varying levels of herbivory from *B. longulus,* as hybrids may have novel, or varying combinations of parental secondary metabolites used in defence, and differences in backcrossing and mixing of genotypes in hybrids may result in different patterns of herbivore resistance (Krebs et al. 2011, Cheng et al. 2011, Torp et al. 2013). The rapid rate of plant degradation unfortunately did not allow me to determine varying ability of *B. longulus* to reproduce on each of the species, but the observation of the lack of full life cycles of *B. longulus* on *Nymphaea* hybrids in the field confirms the data obtained in these experiments.

In contrast to the whole plant experiments, there was significantly lower plant area damaged in the hybrid treatments for the no-choice petri dish studies. *Bagous longulus* consumed a significantly greater volume of *N. nouchali* in the no-choice experiments than leaf segments from the other plants. Additionally, for the multichoice petri dish experiments,

significantly lower volume and percentage area of *N. nouchali* was consumed compared to the other species, while there were also a significantly greater number of eggs oviposited on *N. mexicana* from Skate Park and Westlakes compared to the other two plants. It should be noted that in the no-choice experiments, the volume of leaf consumed and the area available for oviposition was restricted to one leaf disc, whereas more area was available in the multichoice experiments with four leaf discs. However, another possible explanation for the inconsistency between the whole plant and leaf segment experiments is that plant defence chemicals may be experienced differently by *B. longulus* when it is exposed to whole plants compared to cut segments. Plants may possess physical or chemical defences that act to repel insect herbivory, as well as stored constitutive defence compounds that are inactive and separated from their activating counterparts (Pentzold et al. 2014). These compounds, however, are mixed and the defence compounds are activated when tissues are damaged by herbivory to repel herbivores (Hopkins et al. 2009, Boeckler et al. 2011, Pankoke et al. 2012).. The act of cutting leaf segments from the plant may thus initiate an activation of such compounds, thereby potentially impacting the insects as soon as they are added to the petri dishes, as opposed to a delayed activation of these chemicals only as herbivory is occurring in the whole plant experiments. Similar inconsistencies were found in Altesor et al. (2014), where *Macrosiphum euphorbiae* Thomas (Hemiptera: Aphididae) showed no preferences for species tested in leaf disk experiments, but clear preferences were observed in field experiments. However, in the same study, host plant preference was consistent in both field and petri dish experiments for *Tequus* sp. Smith (Hymenoptera: Pergidae) and *Epitrix argentinensis* Bryant (Coleoptera: Chrysomelidae). Hence, it is important to consider how experimental setup may affect results, and how fundamental and realised host ranges may differ between tested biological control agents (Fowler et al. 2012).

The petri dish experiments also had inconsistent results in terms of differences between weevil populations compared to the whole plant experiments, wherein weevils from Assegaai consumed a greater volume and caused greater percentage area damage overall compared to the Skate Park weevils in the no-choice tests but exhibited overall lower oviposition in the multichoice tests. These trends may be explained by seasonal differences, because weevil collections for the petri dish experiments were staggered, while the whole plant experimental treatments were started within two days of each other. Assegaai weevils were collected in January for the no-choice petri dish tests and bordering on autumn at the end of March for the multichoice tests, but the Skate Park weevil no-choice and multichoice

tests were run simultaneously in late February/early March given the greater distance of this population from Rhodes University. Hence, it is likely that the Assegaai weevils were more active at the peak of summer when the no-choice experiments were first run and that the rate of feeding and reproduction decreased with lowering temperatures as winter approached when the multichoice experiments were completed. Indeed, it is noteworthy that during the frequent collection trips to this site, the sex ratio, number of mines, and abundance of adult weevils declined as the season progressed. For example, the sex ratio was approximately one female to two males in January, but this declined to less than one female to approximately three males in April. Similarly, during an hour of collecting, it was easy to collect over 100 weevils in January, but by April, the same amount of time would elicit only 40 individuals. These observations and discrepancies between the whole plant and petri dish experiments provide insight into the seasonality of *B. longulus*, but also highlight the importance of running experiments with conditions as similar to field conditions as possible.

Cultures of *B. longulus* were established successfully at Waainek Research Facility, which provides promising evidence for the use of this species as a biological control agent for *N. mexicana* in South Africa. Additionally, the differences in leaf size and coverage observed at *N. mexicana* sites where *B. longulus* is present compared to those where it is not (see Chapter 2), demonstrate its effectivity in reducing *N. mexicana* plant size. Furthermore, thus far, no parasitisation of *B. longulus* has been observed, though populations will be monitored for this.

The attributes of a desirable biological control agent include host specificity, abundance, high levels of damage, ease of rearing, and climatic suitability in the invaded environment. The results from this study have demonstrated that *B. longulus* possesses these attributes. Additionally, this species belongs to a group that is well known for use in successful biological control programmes. Clewley et al. (2012), in a meta-analysis to quantify the impact of biological control on target plants, found that Coleopterans in the families Chrysomelidae and Curculionidae caused greater reduction in target plant size compared to other insect groups, while Herrick & Kok (2010) showed that, at the time, Curculionidae was important in the suppression of over 50% of the targeted weed biological control programmes in the world. Owing to the great potential of *B. longulus* as a biological control agent, and its nativity to South Africa, I recommend that it is mass reared and released across more sites in South Africa, especially in the Highveld region, so that post-release monitoring can begin to gain further insight into its use for management for *N. mexicana* in

South Africa. In this scenario, it is believed that any possible risks of releasing *B. longulus* are lower than the risks of not taking action to manage *N. mexicana*.

Chapter 5: Host specificity trials of *Megamelus toddi* for management of *Nymphaea mexicana*

5.1 INTRODUCTION

Classical biological control is not without its risks, and some releases of weed biological control agents have resulted in direct negative effects on native non-target plant species (Zimmermann et al. 2000, Hinz et al. 2019). In a review by Hinz et al. (2019), 60 out of 457 (13.1%) agents intentionally released until 2008 were recorded utilising non-target plants in the field. Of all the non-target cases occurring on plant species, about 75% occurred on plant species in the same family as the target weed. However, half of the cases were predicted or predictable, and in 93.5% of the unpredicted cases, the affected non-target plant species had not been tested prior to the release of the biological control agent. In less than 1% of the cases, the impacted plant species had been tested before agent release and had been deemed to not be at risk. Hence, there are relatively few records of non-target impacts by biological control agents, and of these, many could have been predicted with the use of appropriate host specificity tests (Cory & Myers 2000, Hinz et al. 2019). Furthermore, unpredicted attack of non-target plant species has decreased over time as scientific advancements have improved (Hinz et al. 2019), and theoretical and empirical evidence suggest that evolutionary adaptation to the fundamental host range of an organism is unlikely (van Klinken & Edwards 2002). Despite this trend, it is becoming increasingly difficult to obtain release approval for new biological control agents (Briese 2005, Hinz et al. 2014), such that there is debate as to whether biological control researchers are being too cautious and rejecting agents unnecessarily (Sheppard et al. 2003). This emphasises the importance of developing effective and robust methods to determine host specificity of biological control agents and to predict host use in the field.

Part of improving host specificity testing comes with understanding that the types of tests used in trials have varying strengths, weaknesses, and behaviourally induced outcomes (Van Driesche & Murray 2004). For example, no-choice starvation and/or oviposition tests (in which potential agents are exposed only to one plant) are useful to determine the fundamental host range of an agent and provide valuable information on host suitability for various parameters of development, oviposition, and adult feeding (Sheppard et al. 2005). In some cases, no-choice tests may be the only test required if only the target is selected. On the other hand, choice tests (in which agents are presented with two or more host plants and are

allowed to choose a preferred host) are useful to rank hosts in terms of preference compared to the target plant (Singer 2004), and to assess how prior experience, motivation, and learning affect preferences of potential agents. However, laboratory tests may not always reflect field scenarios. Field tests that take place in the native range aim to allow opportunity for natural field behaviour and are useful for agents that are difficult to rear, highly mobile, and sensitive to artificial experimental conditions (Sheppard 1999). Despite the advantages of field tests, there are several political and logistic constraints, including but not limited to: high costs of foreign-based research, impracticalities associated with testing large numbers of non-target plants in the field, and difficulties in discerning between agent and other herbivore damage (Clement & Cristofaro 1995). Hence, this approach may not always be feasible, and estimates of host range must often be obtained in laboratory settings.

To remedy the difficulty in extrapolating results obtained in laboratory settings to field conditions (Sheppard et al. 2005), risk assessments can be used to improve field predictions of potential biological control agents (Paynter et al. 2015), through the calculation of risk scores from host specificity results of potential agents on non-target versus target plant species. These scores represent the risk that potential agents will feed and develop on non-target species under field conditions (Wan & Harris 1997), and provide a standardised, easy-to-interpret means of communicating laboratory results to regulatory authorities, stakeholders, and the public. Such risk scores can also be used to make comparisons of the environmental and economic risks of introducing a biological control agent versus not using biological control (Briese 2005). Furthermore, acceptable levels of risk may vary depending on different situations. For example, lower risk levels would be acceptable for valuable crops and native plants, but higher risk levels may be acceptable for exotic ornamental species for which alternatives exist (Pemberton 2002)

Megamelus species are found worldwide, with approximately 20 species found in the Americas north of Mexico (Beamer 1955). Most species in the genus are hosted by aquatic plants (Asche 1985 and O'Brien & Wilson 1985 cited in Tipping et al. 2011). For example, *Megamelus davisi* Van Duzee is known to be associated with a range of aquatic plants including *Potamogeton natans* L., *Nymphaea odorata* Aiton, and *Nuphar lutea* (L.) Sm. (Harms & Grodowitz 2009), whereas *Megamelus scutellaris* Berg originating from the Neotropical region is well known for its use as a biological control agent of *Pontederia crassipes* Mart. Solms (Grodowitz et al. 2014, Tipping et al. 2014, Hill & Coetzee 2017). During surveys on *Nymphaea* species in the southern United States for potential biological

control agents for *N. mexicana*, *Megamelus toddi* Beamer was found in abundance at 39% of the sites sampled in the states of Florida and Louisiana (Reid et al. 2020). At these sites, *M. toddi* was found only on *N. mexicana*, though at one site where another *Nymphaea* sp. (cf. *N. rubra*) was present, *Megamelus* sp. were collected but their identity could not be confirmed. Other information about *M. toddi*, such as association with other plant species and biology, is lacking. Nevertheless, owing to its abundance, lack of incidence on other species during the surveys, and the success of its congener *M. scutellaris* as a biological control agent, *M. toddi* was prioritised as a potential biological control agent for *N. mexicana*. As such, it was imported into quarantine facilities in South Africa for investigation.

Investigations of the host specificity of sap suckers may be more complex than for chewing insects, in the sense that damage is more difficult to observe and quantify, and whole plants instead of plant segments must be used. Some studies have used techniques such as the Electrical Penetration Graph (EPG) technique, wherein changes in resistance within plants related to stylet penetration is electrically recorded to generate data on the time spent by the insects under investigation at different locations on the plant (Tjallingii 1985, Sandanayaka et al. 2013). Methods such as this, however, while demonstrating feeding behaviour, do not show the ability of an insect to survive and develop on a non-target plant in the long term. Furthermore, the use of such equipment may be time consuming and expensive. Hence, the determination of host specificity can be determined by the survival of insects under investigation on whole plants. Indeed, trials such as these may be important to investigate the ability of an insect to survive on a plant over multiple generations (i.e., multi-generation continuation trials) (Coetzee et al. 2003, Day et al. 2016). Hence, continuation tests are a useful addition to choice and no-choice tests, and can be used to overcome some of the limitations in host specificity testing in laboratory conditions (Marohasy 1998). For example, in host specificity tests of *Hydrellia egeriae* Rodrigues (Diptera: Ephydridae), a potential agent for the submerged macrophyte, *Egeria densa* Planchon (Hydrocharitaceae) in South Africa, one non-target species was found to support larval development during paired choice tests (Smith et al. 2019). Results from multiple generation continuation tests showed that the non-target species could not sustain viable populations of *H. egeriae* for more than two generations, and population growth was negative for each successive generation. Hence, the agent was declared safe for release in South Africa. Similarly, although *M. scutellaris* oviposited and nymphs emerged on five non-target species, only a single female survived on one species (one replication of *Pontederia paniculata* Mart. [≡ *Eichhornia paniculata*

(Spreng.)Solms)]) (Tipping et al. 2011). This female survived for 11 days, and second-generation nymphs emerged after oviposition on the same plant, but both died after two days, while nymphs on the other plants did not reach adulthood. If feeding had been used as the only measure of evidence for host acceptance, both *H. egeriae* and *M. scutellaris* might have been rejected unnecessarily where both are now being used to manage problematic species without any known reports of non-target effects (Hill & Coetzee 2017, Coetzee et al. 2021).

In Chapter 4, I investigated the ability of the native *B. longulus* to feed and develop on *N. mexicana* from two different populations, as well as the native *N. nouchali*, and the exotic hybrid *N. marliacea chromatella*. Field and experimental evidence suggested that *B. longulus* could be effective at managing *N. mexicana* populations, but less likely to control *N. marliacea chromatella* and other exotic hybrids. Although it is desired that *M. toddi* will have the ability to manage *N. mexicana* and its exotic hybrids, if it has a host range that is too broad, such that it can survive and develop on *N. nouchali*, it will not be safe for release. On the other hand, if its host range is confined to the subgenus to which *N. mexicana* belongs (*Nymphaea*), it may be more effective at managing the exotic hybrids originating from exotic species of the same subgenus (Chapter 3). Hence, this study aims to investigate the host specificity of *M. toddi* by testing the same species investigated in Chapter 4 to allow a comparison between the South African *B. longulus* and North American *M. toddi*, providing a glimpse into the suitability of this species as a biological control agent for *N. mexicana* in South Africa.

5.2 METHODS

Insect and plant cultures

Megamelus toddi was imported under permit (P0107136) from the USDA-ARS Invasive Plant Research Laboratory in Florida, USA (courtesy of Dr. Melissa Smith and transported by Dr. Megan Mulcahy) to the quarantine facility at Waainek Research Facility, Rhodes University, Makhanda, RSA in December 2021. Cultures were maintained on *N. mexicana* plants planted in 20 L buckets and fertilized with slow release Osomocote® at approximately 10 mg N/L water and kept in mesh cages at approximately 25 ºC under UV lights with 12-hour light cycles.

Test plants were planted in 20 L buckets quarter filled with sediment, fertilized with slow release Osmocote® fertilizer at approximately 10 mg N/L water and filled with water. *Nymphaea mexicana* from Westlakes in Cape Town (34°04'53.1"S 18°27'20.1"E) was

collected from cultures at Waainek Research facility, as was *N. mexicana* from King's Beach Skate Park in Gqeberha/Port Elizabeth (33°58'26.2"S 25°38'41.0"E). *Nymphaea nouchali* was collected from Kariega river near the Assegaai trails in the Eastern Cape (33°32'34"S 26°36'14"E), and *N. marliacea chromatella* was supplied by AquaFlora nursery in North West Province (grown at Waainek Research Facility). These were kept in mesh cages in quarantine during the trials. Due to limitations in space and plant availability, the plants used in the host specificity trials had been used previously in experiments involving *B. longulus*. All *B. longulus* adults were removed from the plants as well as leaves and stems with signs of mining to ensure that the plants were insect-free before use. After the removal of *B. longulus*, the plants were left to grow first in the Waainek tunnels, and then allowed to acclimate in quarantine for at least three days before *M. toddi* was added.

Host specificity trials

Two female and one male adult *M. toddi* were added to five to seven replicates of each plant treatment (*N. mexicana* from Westlakes (WL), *N. mexicana* from King's beach Skate Park (SP), *N. nouchali* from Kariega river (NAT), and *N. marliacea chromatella* (HYB)) randomly arranged in mesh cages around the quarantine facility. The insects were left to feed and oviposit before being removed after seven days. All plants were kept pest free as much as possible by removing aphids and snails by hand every second day. Pesticides were not used to avoid risking possible negative impacts on *M. toddi*. During the seven days in which the *M. toddi* adults were present on the plants, disturbance was limited to prevent the insects from jumping out of the buckets. Once the adults were removed, the plants were monitored every second day for nymphal emergence, and nymphs were moved to fresh plants using an aspirator if plant quality declined such that they could no longer support the insects. If nymphs survived to adulthood, they were counted and these adults were allowed to feed and reproduce on the same host plants to determine whether multiple generations could be completed on the species in question (Tipping et al. 2011, 2018, Smith et al. 2019). The experiments were staggered but replicates of *N. mexicana* either from Westlakes or Skate Park were run simultaneously as controls with the non-target plant treatments, and some replicates were repeated due to lack of nymphal eclosion after the first adults were added, in order to confirm results. The multigeneration trials were run until the third generation or until the insects died out. If a single adult female eclosed on a replicate in the multigeneration trials, one male was added from the culture to allow mating. If only males emerged from a plant, the trial was terminated for that replicate.

Although plants were checked only every second or third day, the number of days from addition of the adults to first generation nymphal and adult eclosion were recorded to estimate the length of the *M. toddi* lifecycle.

Risk assessment

Results from both choice and no-choice tests as well as continuation trials may be utilised to calculate the risk of feeding damage and reproduction by a potential agent to non-target species (Wan & Harris 1997). In this study, choice tests and impact studies were not carried out, so risk assessments were only conducted for reproductive risk for the plant species under investigation. Relative values of survival from the no-choice tests were calculated by dividing the mean percentage survival of nymphs to adults of each test plant by the mean obtained for the original host plant, which in this case was taken to be *N. mexicana* from the Skate Park, because the greatest reproductive rates were recorded on this plant. Similarly, the relative numbers of surviving adults were calculated in proportion to that of *N. mexicana* from King's Beach Skate Park. The reproductive risk was then calculated by multiplying these two values, and this was calculated for the first and second generations.

Statistical analyses

All analyses were carried out in R version 4.1.0 (R Core Team 2021), with model diagnostics (including tests for zero inflation) performed as described in Chapter 4. General linear models were run using number of nymphs, number of adults, and percentage of nymphs reaching adulthood as response variables in separate models, with plant species/population as the factor. These were run for each generation separately. Negative binomial distributions were appropriate for most of the count models, and the Gaussian distribution for percent survival to adulthood. Models for F2 parameters were run as described above, in addition to mixed effects models that included the number of F1 adults or females as a random effect, to account for differences in the number of adults ovipositing in the first generation. Means and standard deviations for presentation in table format were calculated for all attempted replicates for three generations and were also calculated for each generation excluding replicates without any emerged nymphs.

5.3 RESULTS

There was a large amount of variation in the numbers of nymphs that hatched after the first addition of two females and one male for the seven-day period. The mean number of

nymphs that eclosed from eggs in the first generation was highest on *N. mexicana* plants from the Skate Park (mean ± standard deviation: 7.0 ± 5.7), followed next by the native *N. nouchali* (5.3 ± 10.6), *N. mexicana* from Westlakes (3.5 ± 2.6), and lastly the hybrid *N. marliacea chromatella* (2.2 ± 1.7) (Figure 5.1A). However, there were no statistically significant differences between plant treatments for the number of first-generation nymphs ($\chi 2 = 3.0061$, d.f. = 3, $P = 0.3907$). Similarly, there were no statistically significant differences between the number of adults counted for each species ($\chi 2 = 2.2944$, d.f. = 3, $P = 0.5136$) (Figure 5.1B), nor for the percentage survival to adulthood ($\chi 2 = 1.9862$, d.f. = 3, $P = 0.5753$). (Figure 5.1C).

Since there were replicates from which no nymphs eclosed for all plant treatments in the first generation (and for which experiments were subsequently repeated), a general linear model was run again excluding the replicates that did not have any emergence to determine if this would affect the results. Results were significantly different for the number of first-generation nymphs ($\chi 2 = 8.1729$, d.f. = 3, $P = 0.0426$), but did not change considerably for the other measure parameters.

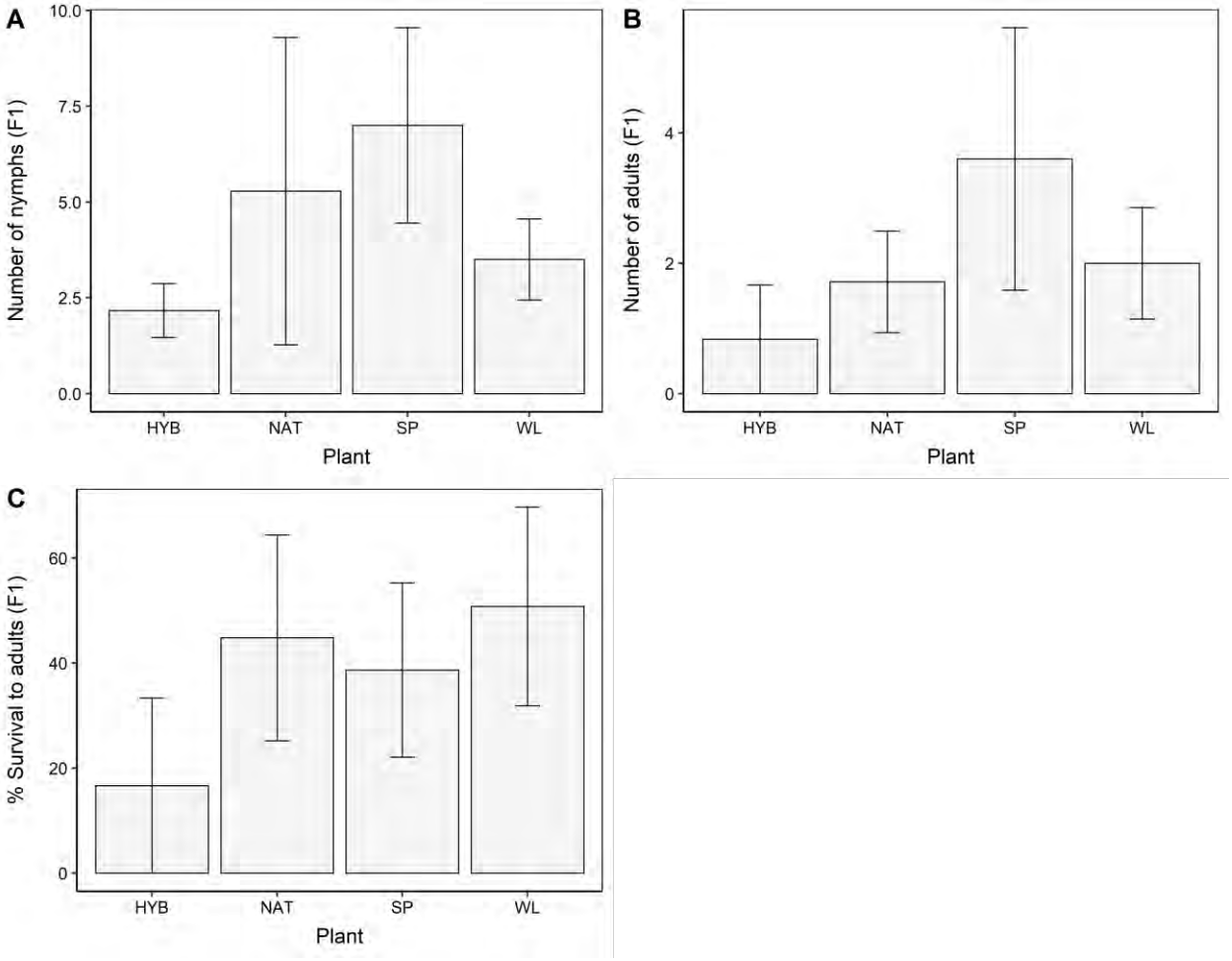

Figure 5.1: Parameters measured for first-generation host specificity trials of *Megamelus toddi* on *Nymphaea mexicana* from King's Beach Skate Park (SP) and Westlakes (WL), *Nymphaea nouchali* (NAT), and *Nymphaea marliacea chromatella* hybrid (HYB). Graphs show means ± standard error. A: Number of first generation (F1) nymphs to eclose from eggs; B: Number of F1 adults; C: Percentage survival from nymph to F1 adult.

Similar trends were seen for the second generation, with general patterns emphasised due to the carry-over of numbers from the first trials (Figure 5.2). Although there were still no significant differences between treatments for the second generation for mean number of F2 nymphs ($\chi2 = 3.3917$, d.f. = 3, $P = 0.2705$), numbers were again highest for *N. mexicana* from Skate Park (14.7 ± 22.0) followed by *N. nouchali* (11.3 ± 16.1), *N. mexicana* from Westlakes (5.3 ± 10.5), and lastly the hybrid (0.5 ± 1.0), with the same trend seen for the mean number of F2 adults ($\chi2 = 4.2274$, d.f. = 3, $P = 0.2379$) (Figure 5.2A and B). When mixed effects models were used to account for the number of F1 adults present, there was a slight statistically significant effect of plant species on the number of F2 adults ($\chi2 = 8.8607$, d.f. = 3, $P < 0.05$) but this effect was no longer significant when treating the number of F1

females as a random variable. The mixed effects models showed no significant effect of plant species for number of nymphs when treating the number of F1 adults or females as a random variable ($\chi 2 = 5.7902$, d.f. = 3, $P = 0.1223$ using F1 adults as random variable; $\chi^2 = 2.1673$, d.f. = 3, $P = 0.5384$ using F1 adult females as random effect). No statistically significant trends were found for F2 percentage survival ($\chi^2 = 2.8672$, d.f. = 3, $P = 0.4126$), although the Skate Park *N. mexicana* (37.5% ±33.1) and *N. nouchali* (16.0% ± 22.9) again had the highest survival, followed by the hybrid (12.5% ± 25.0) and *N. mexicana* from Westlakes (8.3% ± 16.7) (Figure 5.2C).

22.

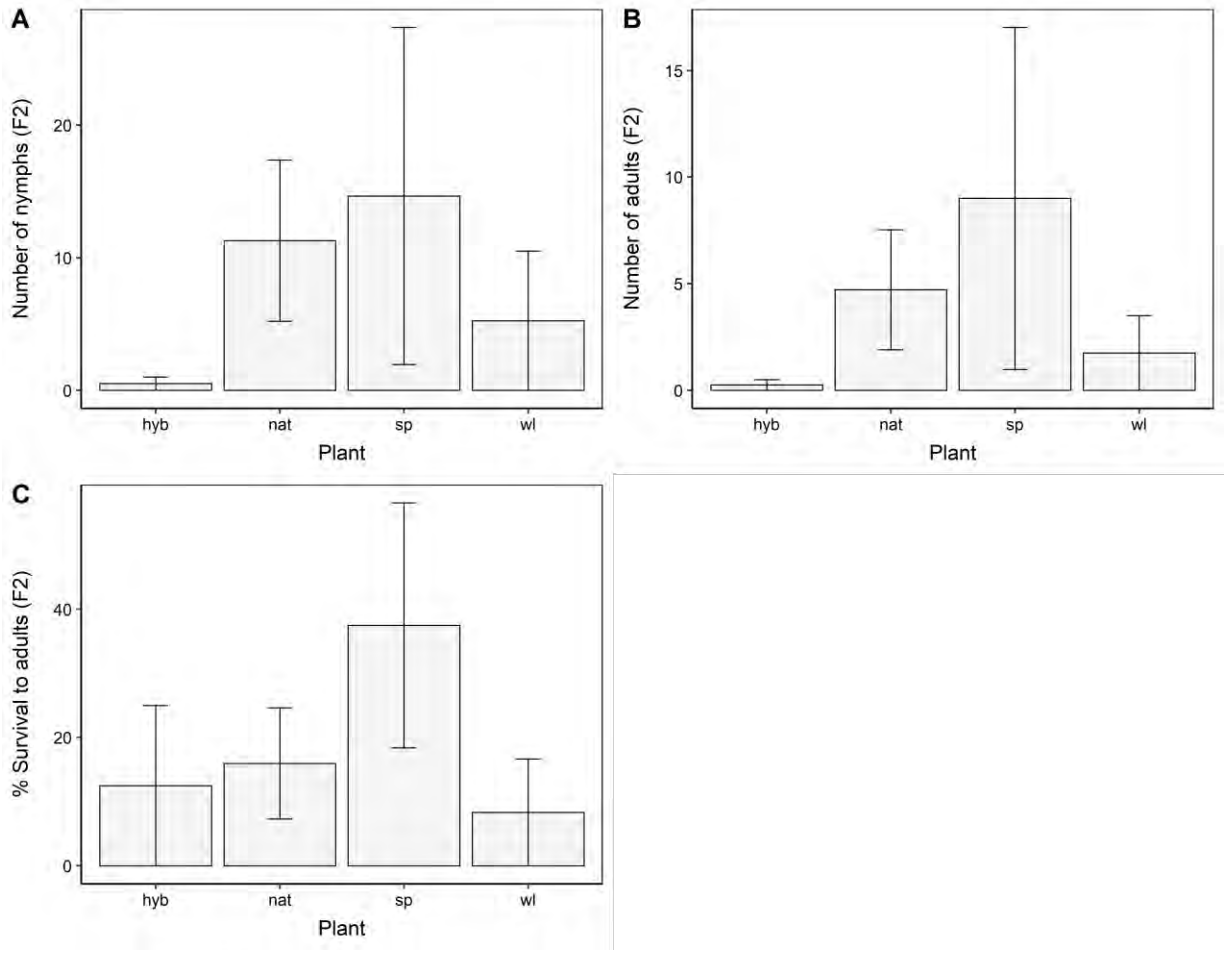

Figure 5.2: Parameters measured for second-generation host specificity trials of *Megamelus toddi* on *Nymphaea mexicana* from King's Beach Skate Park (SP) and Westlakes (WL), *Nymphaea nouchali* (NAT), and *Nymphaea marliacea chromatella* hybrid (HYB). Graphs show means ± standard error. A: Number of second generation (F2) nymphs to eclose from eggs; B: Number of F2 adults; C: Percentage survival from nymph to F2 adult.

As mentioned previously, the number of replicates differed for each treatment, and some replicates were repeated to ensure that *M. toddi* mortality was due to lack of host compatibility and not other factors, such as mortality due to transfer between replicates by aspirator. For the multigeneration trials, only 1 hybrid replicate showed emergence of F2 nymphs, as well as one Westlakes *N. mexicana* replicate (Table 5.1). However, an additional Westlakes plant and one additional Skate Park plant supported nymphal development to F1 adults, but multigeneration trials were not continued for these replicates as they were started late and would have required several more weeks for development to continue to generation 3. In another Westlakes replicate, four adult males eclosed and trials were terminated. Three of seven replicates of *N. nouchali* supported F2 adult development, compared with two out of five Skate Park plants (aside from the additional one replicate mentioned above). For the third generation, two *N. nouchali* plants supported F2 adults, but the plants died and could not be replaced due to difficulty in keeping this species healthy in quarantine conditions (and due to wild populations declining in the winter months).

The reproductive risk of *M. toddi* was relatively low in the first generation for the hybrid at 9.9%, and this decreased to 0.9% for the second generation. However, this increased to 55.7% in the first generation for *N. nouchali*, and 22.4% for the second generation. The reproductive risk on *N. mexicana* from Westlakes was similar to the baseline of *N. mexicana* from King's Beach Skate Park at 73.4% for the first generation, but this dropped to 4.2% for the second generation (Table 5.2).

Table 5.1: Mean (± S.D.) numbers of F1, F2, and F3 adults to survive on each tested species. The number of replicates for each treatment is shown and means for subsequent generations (F2 and F3) are calculated only for replicates that produced F2 and F3 nymphs. Lower replicate numbers for each generation reflect mortality in previous generation for other replicates, or in one case, eclosion of adult males only. Numbers marked with an asterisk indicate that one or more replicates for the previous generation were deliberately terminated due to time constraints, or that the other replicate plants died, and new plants were not available for transfer of nymphs.

Plant	**No. adults F1 (including reps with 0)**	**No. adults F1 (excluding reps with 0)**	**No. replicates with F1 adults/total replicates**	**No. adults F2**	**No. replicates with F2 adults**	**No. nymphs F3**	**No. replicates F3**
Hybrid (HYB)	0.8 ± 2.0	5.0 ± NA	1/6	1 ± NA	1	0	0
Nymphaea nouchali (NAT)	1.7 ± 2.1	3 ± 1.8	4/7	11 ± 7.94	3	43 ± NA	1*
Nymphaea mexicana (SP)	3.6 ± 4.5	6.0 ± 4.4	3/5	13.5 ±16.3	2*	61 ± 24.0	2
Nymphaea mexicana (WL)	2.0 ± 2.1	3.0 ± 1.8	4/6	7 ± NA	1*	101 ± NA	1*

Table 5.2: Relative nymphal survival and number of reproducing adults for each of the plants tested in host specificity trials for *Megamelus toddi.* Relative values were calculated as a proportion of values obtained in the *Nymphaea mexicana* SP (Skate Park) treatment, and reproductive risk was calculated by multiplying both values together and by 100% to obtain percentage values.

Plant	**Relative survival from nymph to adult (F1)**	**Relative reproducing adults (F1)**	**Reproductive risk (%) (F1)**	**Relative survival from nymph to adult (F2)**	**Relative reproducing adults (F2)**	**Reproductive risk (%) (F2)**
Hybrid (HYB)	0.43	0.23	9.89	0.33	0.028	0.92
Nymphaea nouchali (NAT)	1.16	0.48	55.68	0.43	0.52	22.36
Nymphaea mexicana (SP)	1.00	1.00	100	1.00	1.00	100
Nymphaea mexicana (WL)	1.31	0.56	73.36	0.22	0.19	4.18

The mean number of days from the addition of adults (removed after 7 days) to records of the first nymphs were similar for each of the plants, with a combined mean of 18.1 ± 3.5 days (Table 5.3). Similarly, the mean time from the addition of adults at the start of the experiment to the eclosion of the first F1 adults was similar across plant treatments, with a combined mean time of 42.5 ± 3.8 days (Table 5.3).

Table 5.3: Mean (± S.D.) number of days from the addition of two female and one male *Megamelus toddi* adults to each treatment (removed after seven days) until the eclosion of the first F1 nymphs and adults. Plants were checked every two to three days, so numbers shown are estimates, as eclosion could have occurred on days when plants were not checked. Data marked with asterisk indicates lack of standard deviation measure, as only one replicate supported development of F1 adults.

Plant	**Mean (± S.D.) days to first F1 nymphs**	**Mean (± S.D.) days to first F1 adults**
Hybrid (HYB)	19.0 ± 2.9	44.0 ± N/A*
Nymphaea nouchali (NAT)	17.8 ± 3.0	41.5 ± 1.0
Nymphaea mexicana (SP)	16.6 ± 4.2	41.0 ± 5.6
Nymphaea mexicana (WL)	18.6 ± 4.2	44.2 ± 4.9

5.4 DISCUSSION

Host specificity tests, by means of Wapshere's centrifugal phylogenetic testing method (Wapshere 1989) and despite some weaknesses, are a useful means of determining the safety of potential biological control agents (Briese 2003, Fowler et al. 2004, Grevstad et al. 2021). In the current study, *M.toddi* was able to fully develop for multiple generations on the South African *N. nouchali*, which is the closest relative of *N. mexicana* in the country. This was the case even for a low number of replicates in conditions where *N. nouchali* plant quality was not at its optimum, as this species was difficult to keep healthy in quarantine conditions, and wild stock populations were not available as the experiment was run during winter months. Although laboratory-based studies cannot be entirely reflective of field conditions, improved plant quality in field settings is likely to result in even greater reproductive ability of *M. toddi* if it were to reach *N. nouchali* in the wild. Two of the *N. nouchali* replicates died before F3 nymphs could emerge, but F2 adults survived for approximately two weeks prior to this, so it is likely that F3 nymphs would have emerged from these replicates as well. Furthermore, relative reproductive risks of approximately

55.36% for the first generation, and 22.36% for the second generation, are too high for safe release. Hence, *M. toddi* should be rejected as a potential biological control agent for *N. mexicana* in South Africa. This result, although disappointing, highlights the importance of vigorous host specificity trials, and the usefulness of the centrifugal phylogenetic testing method. It is unknown if *M. toddi* would be able to persist on more distantly related plant species, but time and financial resources would have been wasted if tests were first conducted on distantly related species, only to find that *N. nouchali* supported development.

The results of this study contrast the host specificity of the congener *M. scutellaris*. During host specificity trials in the USA, oviposition and nymphal emergence by *M. scutellaris* did occur on five nontarget plant species, but none of these plants supported development past the first generation, and the numbers of nymphs to emerge were much lower than on *P. crassipes* (Tipping et al. 2011). However, although *M. scutellaris* was sufficiently host specific for release in South Africa and North America, this was not the case in Australia, where feeding and development was recorded at levels equal to those recorded on *P. crassipes* on another species in the same family (Pontaderiaceae) (Heard et al. 2014). These opposing outcomes demonstrate the contrasting suitability of biological control agents in various regions of the world depending on the native flora present and emphasises the importance of conducting vigorous host specificity tests in each country. Varieties of *N. nouchali* and *N. lotus* were not tested in this study, nor were more distantly related plants in other genera/families, so the extent of *M. toddi* host specificity is not fully known. It is probable that this species could survive and reproduce at least on members of the *Nymphaea* and *Brachyceras* subgenera, based on the results of this study.

The hybrid, *N. marliacea chromatella,* was not suitable to host long-term survival and development of *M. toddi.* It is likely that the chemical and morphological cues/defences of this hybrid, due to inheritance of traits from its putative parents, *N. alba* and *N. mexicana*, has resulted in resistance to herbivory both from *B. longulus* (Chapter 4) and *M. toddi*. The popularity of this particular hybrid in the horticultural trade may suggest that artificial selection has occurred to favour resistance to herbivory. This is also evidenced by the relative lack of snail damage seen on this plant during culture growth compared to the other species used in this study. Finding a suitable agent to manage this species may thus be challenging, but investigating species known to attack its other parent, *N. alba*, may be helpful in this regard, especially because *N. alba* and *N. mexicana* do not generally intersect in their native ranges. Different results may be found for other hybrids, however. It is possible that *M. toddi*

would be able to survive and reproduce on hybrids with differing parentage, such as those parented by *N. mexicana* and *N. odorata*, both of which occur naturally in the native range of *M. toddi*. However, it is more advisable to test other agents collected from the native range of *N. mexicana* especially where this plant grows with other *Nymphaea* species, instead of focusing time and resources on the unsafe *M. toddi.*

There was reduced survival of *M. toddi* on *N. mexicana* from Westlakes compared to *N. mexicana* from the Skate Park. This is surprising, as both populations appear genetically similar according to ISSR analyses (Chapter 3). It is possible that there are morphological and/or genetic variations between these populations that were not identified in the genetic analyses, and that other molecular techniques utilising chloroplast or mitochondrial markers might reveal other information (Petit et al. 2005, Moriguchi et al. 2009). It is also possible that differences in environmental conditions between the populations could account for the variation in survival and reproduction of *M. toddi*. For example, performance (in terms of inflicted damage and reduction of plant biomass) of *Neochetina bruchi* Hustache (Coleoptera: Brachyceridae) was greater than that of *Neochetina eichhorniae* Warner on *P. crassipes* plants grown at high nutrient concentrations (Heard & Winterton 2000). Similarly, herbivory from *Cyrtobagous salviniae* Calder & Sands (Coleoptera: Curculionidae) was reduced in *S. molesta* plants grown in shaded conditions, possibly as a result of high insoluble nitrogen concentrations (Maseko et al. 2019). However, planting conditions were kept uniform across the treatments as much as possible, so this should have stabilised after plants were initially collected from their original populations. Furthermore, hundreds of *M. toddi* were maintained without issues on *N. mexicana* plants from Westlakes for the quarantine cultures, so even if differences were observed in this study, they are not enough to cause concern. The most likely explanation for the discrepancy in the number of nymphs and adults on *N. mexicana* from the Skate Park compared to Westlakes is that trends were masked in the low number of replicates used, or that growing conditions in some sections of quarantine were unsuitable for *M. toddi* survival. Indeed, some of the replicates for *N. mexicana* from both populations did not have nymphal emergence at all after the initial adults were added at the beginning of the experiment, so it is possible that the transfer of the adults from the cultures to the experimental plants resulted in mortality before oviposition could occur. This could also account for some of the high variation observed in the dataset, and the lack of significantly significant trends.

An additional consideration for the results of this study are the possible implications of source variation for host specificity. In Chapter 4, two populations of *B. longulus* were tested to account for possible variations in host specificity between populations, but the same was not carried out in this study. It is possible that *M. toddi* collected from different sources might exhibit varying levels of host specificity, or that cryptic species may exist (Jallow et al. 2004, Paterson et al. 2016, Smith et al. 2018, Szűcs et al. 2019, Lu et al. 2021). Although this is a possibility, I recommend that time and resources are spent investigating other species that could be used in biological control instead of spending time on a species that has a lower probability of being suitable. Nevertheless, this is an important consideration for this and future biological control programmes.

Although accurate measures of life history traits were not collected in this study, an idea of the length of *M. toddi* lifecycle was obtained, with first nymphs emerging after approximately 18 days after adults were added, and the full lifecycle being completed over about 42 days at 25 ºC. This would amount to a duration of about 24 days for the immature stage, which is just under 10 days longer than the immature stage duration of *M. scutellaris* (Sosa et al. 2005). Specific studies to investigate life history are necessary to confirm this.

The risks of introducing *M. toddi* would not outweigh the risks of not beginning biological control on *N. mexicana,* not only because of the potential damage on *N. nouchali*, but also because *M. toddi* could not complete development on the hybrid, *N. marliacea chromatella*. Although multi-choice tests were not carried out and we thus do not have an idea of preference between plants in the same environment, the fundamental host range of *M. toddi* is too broad for it to be safely released. Choice and impact tests could provide further insight in this regard, but I argue that time and resources should instead be focused on other potential agents (see also Chapter 6). Despite the unsuitability of this agent to manage *N. mexicana* in South Africa, this study has acted to prevent the release of an unsafe agent and thus increase the biosecurity of South African ecosystems, but valuable data has also been collected to improve knowledge about *M. toddi*, because little is known about the species in general.

Chapter 6: Discussion

6.1 INTRODUCTION

The primary aim of this thesis, as a continuation of previous work (Reid et al. 2020, 2021), was to carry out the next stages necessary to develop biological control for *N. mexicana*. Chapter 1 reviewed literature relating to biological control of invasive alien plants, how this is tied to the enemy release hypothesis, and a description of what has been achieved so far for the initiation of a biological control programme against *N. mexicana*. In Chapter 2, pre-release surveys of *N. mexicana* in South Africa provided a better understanding of the extent of invasion at various sites, including some environmental factors. Herein, the collected evidence supported the notion that *N. mexicana* and its hybrids have experienced enemy release, and that this has likely contributed significantly to its success in South Africa. However, water quality is known to be a major issue in South Africa, with eutrophication acting as an important factor for the invasion success of several aquatic macrophytes in South African waterbodies. Other factors, such as the mode of reproduction, may also contribute to the success of certain plant invaders. Understanding the relative importance of each of these factors is important to maximise control efforts, so a discussion of these, and the differences between top down (biotic predator) and bottom up (abiotic nutrient) processes, will be addressed in this chapter.

The genetic composition of invasive alien plants can crucially affect the ability of biological control agents to impact and survive on the target plant. In Chapter 3, several *Nymphaea* hybrids were highlighted, and their likely parentage ascertained. This information could have implications for understanding differences in host specificity of the tested agents and could provide useful strategies for the initiation of further field surveys to include hybrid parents and maximise the chances of obtaining agents with the required specificity. Other researchers have encountered similar challenges in attempting to manage species with complex genetic structures, including various hybrids and biotypes. Here I will draw on the available literature to determine the likelihood of successfully managing hybrid invasive alien plants by comparing the successes and failures in programmes for other species with this project.

Herbivorous insects may expand their host ranges to include species not previously encountered. Although this host range expansion is not desired in biological control

programmes where natural enemies are chosen for their host specificity, it can be useful in scenarios where exotic invaders are included as hosts. Chapter 2 provided evidence that the South African *B. longulus* accepted *N. mexicana* as an additional host, and Chapter 4 explored the host preferences of this species as a means to determine its suitability to impact *N. mexicana* and its hybrids. Similarly, Chapter 5 investigated the host preferences of the Floridian *M. toddi* in quarantine conditions. Here, the challenge is to find an agent that is specific enough not to cause damage to the native congeners in South Africa, but not so specific that it is not effective in managing *Nymphaea* hybrids. Hence, in the last section of this chapter, the complexity of host specificity and its relation to plant genotypes, as well as the relative importance of the origin of populations collected for rearing is addressed.

Finally, a general discussion of the limitations of the present study are presented, as well as a summary of recommendations for further research.

6.2 DRIVERS OF PLANT INVASIONS

In Chapter 1, a brief review was provided of three major invasion processes, namely the propagule pressure hypothesis (Lockwood et al. 2005), the disturbance hypothesis (Elton 1958), and the enemy release hypothesis (Keane & Crawley 2002). In Chapter 2, focus was placed on determining whether enemy release played a major role in the dominance of *N. mexicana* in invaded ecosystems, seeing as biological control is closely linked with this hypothesis. As hypothesised, *N. mexicana* in the invaded range had a lower species richness of herbivores and greater leaf sizes compared to sites in the native range. This provides motivation for the use of biological control through the introduction of a suitable agent to suppress population growth, especially considering the contrast in leaf sizes and coverage densities between the sites with and without *B. longulus* present. However, other factors are also likely to contribute to invasion success, with varying importance at difference stages of the invasion process, and these factors are likely to interact and reinforce one another. Although most of these factors were beyond the scope of this thesis for investigation, discussion around their role in the invasion success of aquatic macrophytes, and *N. mexicana* in South Africa in particular, is warranted. Importantly, the relevance of certain factors may be more or less applicable in different habitats, and single factors are unlikely to work alone. Instead, various factors often work interactively to explain invasion success (Mitchell et al. 2006). This is particularly important when considering the idea that invaders can be classified as: “passengers”, in which they benefit from disturbance of ecosystems but are less likely to

drive the decline of native species (Macdougall & Turkington 2005); "drivers", in which the plants themselves cause declines in native species and alter ecosystem characteristics (Macdougall & Turkington 2005); or "back-seat drivers", in which invasive species benefit from ecosystem disturbances that have reduced native plant diversity, but also contribute to further declines and alterations of ecosystem properties (Bauer 2012). For the purposes of this section, some of these factors and their likely role in the invasion of *N. mexicana* are discussed in order to begin to develop a more encompassing view of this and other macrophyte invasions.

Propagule pressure, though difficult to quantify, is one of the most important factors linked to plant invasion success, has greatest importance in stages of the invasion process prior to establishment, and may be highly useful to predict species invasions (Reaser et al. 2008, Xie et al. 2013, Thomaz et al. 2015). Indeed, propagule pressure may be more important than resident plant diversity, physical disturbance, and abiotic conditions in predicting habitat invasibility (Lockwood et al. 2005, von Holle & Simberloff 2005). For many of the invasive macrophytes that are problematic in South Africa (and the world), horticulture, aquaculture, and pet stores are largely responsible for the introduction of propagules into new regions (Coetzee et al. 2011a, Martin & Coetzee 2011, van Kleunen et al. 2018). Boating activity also contributes to the spread of invasive plants (Tamayo & Olden 2014), and dispersal of seeds by animals (Richardson et al. 2000a, Bass et al. 2006) and vehicles (von der Lippe & Kowarik 2007) is also common. The mode of propagule production by invasive plants is also relevant. Many aquatic plant species commonly reproduce both vegetatively (growing from rhizomes or other plant segments) and generatively (from seed), whereby dispersal often occurs predominantly with water movement in river systems (Boedeltje et al. 2003, Leyer 2006). Although seeds may be dispersed over longer distances and are more resistant to desiccation, vegetative propagules are more likely to successfully colonise a new region owing to their ability to photosynthesise and consume nutrients (Grace 1993, Johansson & Nilsson 1993). In aquatic plants, generative propagules are negatively correlated with vegetative propagules, demonstrating a trade-off in resource allocation between these reproductive strategies (Boedeltje et al. 2008).

For *N. mexicana*, reproduction of the species and its hybrids seems to occur predominantly asexually via its rhizomes based on observations from the field and culture maintenance. Propagule dispersal may occur via boat propellors if floating "brood bodies" are transported, but this is likely to be to a much lesser extent compared to small floating

plants such as *Azolla filiculoides* or *Salvinia molesta,* as the tubers are usually rooted in the soil. The role of seed dispersal, however, is not confirmed. During the surveys carried out in this study, the production of fruit and seeds was not noted in the field, nor in cultures grown in greenhouses. At many of the sites surveyed, *N. mexicana* was planted deliberately, but at one or two sites, the landowners claimed that no deliberate introduction occurred, and that it was more likely that seeds were deposited in the waterbodies by waterfowl. A previous investigation into the breeding strategy of *N. mexicana* and its hybrid, *N. marliacea chromatella*, suggested that *N. mexicana* produces fruit after cross pollination but the hybrid does not (Naidu 2018). Further investigation in this regard is warranted to confirm these preliminary findings, as the sample size was low in this study. Nevertheless, from general observation, vegetative reproduction seems to be favoured by *N. mexicana* and its hybrids in South Africa. This ability to reproduce asexually is common in plants that have become successful invaders (Thiebaut 2007, Bianchini et al. 2010, Schultz & Dibble 2012), as exemplified by species such as *Pontederia crassipes, Salvinia* spp.*, Hydrilla verticillata,* and *Myriophyllum spicatum* L. (Haloragaceae). Hence, asexual propagation through transplantation (accidental or deliberate) by enthusiastic horticulturalists is likely one of the most important instigators of propagule pressure for *N. mexicana* and its hybrids.

Another widely regarded influential factor for invasion success is the role of competition in macrophyte communities (Fleming & Dibble 2015). This is closely related to the hypothesis of biotic resistance, in which it is suggested that increased diversity in the invaded area results in greater competition for resources, thereby limiting establishment of exotic species (Elton 1958, Levine 2000). The empty niche hypothesis is also related, whereby communities are deemed more susceptible to invasions if there are unused resources available for invaders to utilise (Elton 1958). These hypotheses are certainly relevant for aquatic plant invasions in South Africa, as there are a relatively low number of native macrophytes, especially in areas like Gauteng where there are few natural waterbodies but many artificial dams. One sees a similar trend when considering cacti invasions in South Africa, where, again, there are few/no native cacti, and empty niches are thus available for colonisation by exotic species. In South African macrophyte invasions, the importance of competition and biotic resistance is further evidenced with the emergence of newly invasive species as previously problematic species are being effectively managed using biological control (Coetzee et al. 2021). Other interventions may therefore be necessary to prevent future invasions through improving biotic resistance (Petruzzella et al. 2018, 2020b, 2020a).

The underlying mechanisms that allow one species to outcompete another may be complex. We have already established that the predominant mode of reproduction may allow an organism to establish in an area and begin to dominate over species with slower modes of reproduction. For example, Spencer & Rejmánek (1989) determined that *H. verticillata* plants were stronger competitors when they grew from tubers compared to when they grew from turions, indicating that the type of propagule can impact competitive ability. Some plants may also be more efficient at utilizing resources, such as light and nutrients. For example, *M. spicatum* abundance was linked to total phosphorous levels in the water column (Madsen 1998), suggesting that competitive advantage may be achieved through a heightened ability to consume certain nutrients. Where newly emerging invasives are becoming problematic with the reduction of floating invasive macrophytes, greater access to light might allow for increased growth (Strange et al. 2018, 2019). Similarly, Herb & Stefan (2006), using a series of statistical modelling techniques, predicted that suppression of native species growth is achieved through the formation of dense surface canopies of invasive species, thereby monopolising light resources. When effects of nitrogen and phosphorous are included as limiting factors for the competitive growth of aquatic plants, light competition has been suggested to play a much larger role (Best et al. 2004). However, both of these studies were based on simulation models, so real-time processes may differ. While experiments have not been conducted to determine competitive effects of *N. mexicana* specifically, research is available for its close relative, *N. odorata*. For example, Monacelli & Wilcox (2021) found that *N. odorata* is more competitive than *Trapa natans* L. Additionally, evidence for traits to maximise light utilisation was found in a study that showed that *N. odorata* responds to restriction of light as a result of dense leaf cover by producing aerial leaves that rise above the water surface to access light (Etnier et al. 2017). It is possible that *N. mexicana* possesses some trait that gives it a competitive advantage over South African tropical *Nymphaea.* At all but one of the sites surveyed in this study, where *N. mexicana* or its hybrids were found growing in sympatry with *N. nouchali*, *N. mexicana* dominated regardless of whether *B. longulus* was exerting damage or not. For the one site where this was an exception, *B. longulus* was abundant both on *N. mexicana* and *N. nouchali*, and *N. mexicana* leaf sizes were bigger than at the other two sites where *B. longulus* occurred, but *N. nouchali* dominated the system.

It is important to reiterate that competition is often facilitated by other mechanisms, so it is difficult to distinguish between factors affecting invasion success. For example, enemy

release has been linked to invasion success but supporting evidence has been mixed across studies (Colautti et al. 2004, Joshi & Vrieling 2005, Liu & Stiling 2006, Heger & Jeschke 2014). In some cases, native herbivores may prefer exotic plants over indigenous hosts for feeding (Parker & Hay 2005, Morrison & Hay 2011), but this will likely differ case-by-case. For example, although herbivorous consumption of *P. crassipes* was high in its native range, the population was not significantly impacted, thus suggesting that the species is not likely highly impacted by enemy release (Franceschini et al. 2010). Other studies have hypothesised that if herbivory intensity is not strong in the native range of an invasive plant, enemy release is less likely to limit spread in the introduced range (Ding & Blossey 2005). It is thus again evident that other factors surrounding herbivory are notable, not just the presence or absence of a natural enemy itself.

Nevertheless, for *N. mexicana*, there is evidence that enemy release occurs in South Africa (Chapter 2), with lower species richness and much lower damage in the invaded range compared to native range studies (Reid et al. 2020). Without natural enemies exerting selective pressures for defences, invasive populations may evolve to allocate resources away from defences and instead to growth, via the Evolution of Increased Competitive Ability (EICA) hypothesis (Blossey & Notzold 1995). Although this has been a popular hypothesis in invasion ecology, evidence has been mixed. One meta-analysis found that there is evidence for genetic changes in defences and competitive traits after a species is introduced to a new range, but these changes do not follow the mechanisms outlines by the EICA (Felker-Quinn et al. 2013). Another meta-analysis, which focused on studies that investigated variation in genetic structure and trade-offs between resistance traits and fitness, found partial support for the predictions of the EICA hypothesis (Rotter & Holeski 2018). In this same study, there was stronger support for the hypothesis when direct measurements of herbivore performance were considered, and weaker when resistance traits were assessed. This varying support for EICA highlights the complexity of relationships between herbivory and resistant traits in plants. Experiments comparing herbivore performance on *N. mexicana* in its introduced and native range would shed more light on the extent to which EICA applies in this case. Similarly, although there is considerable evidence to support that *N. mexicana* is experiencing enemy release, other studies such as reciprocal plant studies could be useful to confirm the extent of enemy release and better test the hypothesis itself.

Another means by which invasive plants may achieve competitive advantage is through allelopathy (Callaway & Aschehoug 2000, Ervin & Wetzel 2003), although dilution

may limit the effectivity of this phenomenon in aquatic plants (Gross 2003, Morris et al. 2009). There is no known evidence of allelopathy in *N. mexicana,* but other studies have shown highly inhibitory allelopathic effects of *N. odorata* on lettuce seedlings (Elakovich 1989, Elakovich & Woofen 1989, Quayyum et al. 1999), and *N. lotus* allelopathic chemicals inhibit rice seed germination and growth (Hegazy et al. 2001). It is possible that allelopathy may have a role in *N. mexicana*'s ability to outcompete other species, but exudates from other invasive macrophytes are undetectable in the water column, so dilution effects and degradation by bacteria may nullify allelopathic secretions (Glomski et al. 2002).

Environmental abiotic factors are also important for the successful invasion of plant species. Ecosystems may be invaded as a result of increased susceptibility due to fluctuations in resource availability (fluctuating resource hypothesis) (Davis et al. 2000) coinciding with invading propagules. The opportunity window hypothesis (Johnstone 1986) is similar in that it postulates that invasions occur under a specific temporary set of conditions. As an example, invasive *Elodea nuttallii* (Planch.) H.St.John, *Myriophyllum aquaticum* (Vell.) Verdc., and *Myriophyllum propinquum* A.Cunn. exhibited higher performance in nutrient-rich sites than natives, with increased rooting efficiency and growth, and improved asexual propagation (Xie et al. 2010). Furthermore, disturbed ecosystems, such as those that experience eutrophication, may be highly susceptible to invasions (Hobbs & Huenneke 1992, Alston & Richardson 2006). In the South African context, eutrophication in water systems has been a critical factor in macrophyte invasions. South African phosphate standards for water treatments are much higher than international standards, and this, coupled with pollution from degraded infrastructure and a growing population, has resulted in high eutrophication levels in freshwater systems (van Ginkel et al. 2000). This has favoured invasions from floating macrophytes such as *P. crassipes*, which can increase its biomass eightfold in high nutrient compared to low nutrient settings (Reddy et al. 1990). In this case, herbivory from biological control agents is less important than water nutrient status, showing that a reduction of water nutrients is necessary to manage this species (Coetzee & Hill 2012). However, enemy release is still relevant for water hyacinth in South Africa, because the addition of large numbers of *M. scutellaris* to complement already present biological control agents has assisted in reducing plant cover at some sites irrespective of eutrophication (Sutton et al. 2016, Coetzee et al. 2021).

The idea of "invasional meltdown" is also relevant, whereby an alien species will invade a site and alter the habitat characteristics such that it is more susceptible to

colonisation by other alien invasive species (Braga et al. 2018). The importance of addressing environmental factors is important here to prevent recurring invasions of new species where those with a longer invasion history are effectively managed. Regime shifts or alternate stable states are being observed in this way with the presence of emerging invasive species filling niches where the predominant floating invasives have been managed in South Africa (as described in section 1.3 of Chapter 1) (Strange et al. 2018, 2019). Evidence of such transformation of invaded habitat with *N. mexicana* was described in (Motitsoe (2020), where *N. mexicana* formed a secondary invasion 18 months after the removal of *S. molesta*. Discussion of such factors reiterates the importance of understanding the multiple factors impacting invasions, and this is likely to vary case-by-case.

Phenotypic plasticity has also been implicated in driving plant invasion success, in the sense that a species may have the ability to adjust to varying environmental conditions and improve competitive traits in favourable situations (Richards et al. 2006). For example, invasive ability was enhanced in *Elodea canadensis* Michx. (Hydrocharitaceae) as a result of its highly plastic responses to temperature (Riis et al. 2012), and results from a study on the same species as well as *Lagarosiphon major* Ridl. Moss ex Wager (Hydrocharitaceae) and *E. densa* suggest that adaptation to a wide range of habitats occurs as a result of phenotypic plasticity instead of local adaptation (Riis et al. 2010). *Alternanthera philoxeroides* (Mart.) Griseb (Amaranthaceae) similarly exhibited plasticity that enabled it to colonise different habitat types (Geng et al. 2007), and invasive populations of *Ceratophyllum demersum* L. (Ceratophyllaceae) showed higher phenotypic plasticity than native populations in a study by Hyldgaard & Brix (2012). Specific studies for phenotypic plasticity in *N. mexicana* are not known to have been completed, but high phenotypic plasticity in the genus is known (Les & Philbrick 1993, Dąbrowska et al. 2015), so it is probable that this might have relevance in *N. mexicana* and hybrid invasions.

Although phenotypic plasticity does not reflect genetic heterozygosity (Scheiner 1993), high genetic diversity and hybridisation can also increase the invasive ability of plants (Hovick & Whitney 2014, Buhk & Thielsch 2015). For example, weedy lantana, consisting of numerous varieties and high levels of hybridisation and polyploidy, have greater concentrations of allelochemicals (Hart et al. 1976), higher levels of growth and reproduction, and greater resistance to herbivores (Day et al. 2003b, Sharma et al. 2005, Urban et al. 2011), while also enabling adaptation to a wide range of habitats (Cilliers 1983). Alternatively, low genetic diversity resulting from population bottlenecks may be

characteristic of invasive populations (Carter & Sytsma 2001, Zhang et al. 2010). Nevertheless, hybridisation may result in unique genotypes with traits from both parents that promote invasiveness and can produce considerable problems in terms of management with biological control. This is discussed in more detail in the next section.

Overall, it is likely that multiple factors are at play in the invasion of *N. mexicana* in South Africa. Asexual reproduction, enemy release, and hybridisation certainly contribute, and disturbance in the form of eutrophication and habitat modification is also a likely factor. At *N. mexicana* sites where phosphate and nitrate levels were recorded, meso- or eutrophic conditions were observed at some but not all the sites. However, all of the infestations were in artificial standing water bodies and most of these did not host native *Nymphaea* spp. Investigations of competition effects between *N. mexicana* and the native waterlilies would be useful to determine the extent and mechanisms of competition effects, but overall, *N. mexicana* may be considered a "back-seat driver", benefitting from disturbance but likely playing a role in driving declines of native flora. Management of this species (and to prevent further invasions) will require biological control as well as addressal of eutrophication and habitat disturbance.

6.3 MANAGEMENT OF HYBRID INVADERS

Biological control of plants with high genetic diversity and hybrids is challenging but not impossible, as is demonstrated by case studies for a few problematic plant invaders. For example, *Prosopis* L. (Fabaceae) is an invasive group that has many hybrids (Kleinjan et al. 2021). Within the genus, high levels of phenotypic plasticity and hybridization result in considerable taxonomic confusion (Castillo 2019). Generally, four species are invasive worldwide, namely *Prosopis juliflora* (Sw.) DC., *Prosopis pallida* (Humb. & Bonpl. ex Willd.) Kunth*, Prosopis glandulosa* Torr., and *Prosopis velutina* Wooton, as well as their hybrids (Pasiecznik et al. 2001, Patnaik et al. 2017). Although *Prosopis* species may exist as separate entities when individual populations of each species grow in isolation, these species boundaries are no longer clearly defined when they grow in proximity in the native or invaded range, owing to hybridization (Castillo 2019). These high levels of hybridization may contribute to their invasiveness (Zimmermann 1991).

As a result of the taxonomic uncertainty and blurred species boundaries in this group, the 'mesquite clade' recognized by Catalano et al. (2008) and consisting of representatives of Section Algarobia, include members of the group that have become invasive (Pasiecznik et

al. 2001, for detailed review see Kleinjan et al. 2021). Hence, for *Prosopis* invasions in South Africa, it is necessary for potential biological control agents to have a fundamental host range restricted to *Algarobia* s.s. or *Algarobia* s.l. clades, or to attack American *Prosopis* spp. but not related South African taxa. This is similar to the requirement for potential biological control agents of *N. mexicana*, in which they should be specific to subgenus *Nymphaea,* thereby impacting *N. mexicana* and its hybrids without harming members of subgenera *Brachyceras* and *Lotos*. Obtaining this level of host specificity, in which agents are general enough to impact both the invasive species and its hybrids, but not so much that it will affect native species, can be challenging to achieve. Another challenge is that other members of the targeted group that are not problematically invasive, but that may have uses horticulturally or commercially, would also be targeted. In these cases, the risks associated with the release of such an agent should be compared to the risks associated with lack of control of the invasive plant in question.

Initial biocontrol efforts for *Prosopis* spp. in South Africa focused on seed-feeding species, with the release of three bruchid beetles. Two of these species are established throughout South Africa, but their impact has been limited owing to complications caused by livestock feeding on seed pods and making them unavailable to the beetles (Moran et al. 1993, Impson et al. 1999). Subsequent research has demonstrated that a seed feeder, *Coelocephalapion gandolfoi* Kissinger (Coleoptera: Brentidae: Apioninae), and a leaf feeding moth, *Evippe* sp. #1 undesc. (Lepidopitera: Gelechiidae), are suitably host specific to the *Algarobia* s.s. clade without posing significant risks to South African related taxa (van Klinken & Heard 2000, Mc Kay et al. 2012, Kleinjan et al. 2021). Additional studies identified that another potential agent, *Asphondylia prosopidis* Cockerell (Diptera: Cecidomyiidae), was more appropriately considered as a cryptic species complex (Zachariades et al. 2011a). Although this group was deprioritised, two cryptic species within the complex that were associated with the production of specific gall shapes are considered to have promise as potential biocontrol agents (Park et al. 2019).

Another alien invasive for which researchers have encountered numerous challenges in establishing effective biocontrol is *Lantana camara* L. (Vernenaceae) (common name ‘lantana’). Hundreds of man-made horticultural varieties of this plant exist (Stirton 1977, Simelane et al. 2021) and the weedy forms have spread rampantly in various ecosystems, where they cause numerous socio-economic and environmental problems (Day et al. 2003b). *Nymphaea* is similar to *Lantana* in this regard, owing to the wide range of hybrids produced

by enthusiastic horticulturalists since the 1800s. As a result of the uncertain parentage of the numerous hybrids and varieties of *Lantana* as well as polyploidization (Spies 1984), there is enormous taxonomic complexity and uncertainty (Day et al. 2003b, Czarnecki & Deng 2009). However, four distinct sections may be listed within *Lantana,* with three of these groups including species closely related to African species (Day et al. 2003b), and the last group, section *Camara*, consisting of weedy taxa with over 650 hybrid varieties around the world (Thomas & Ellison 2000). Weedy lantana taxa have thus been referred to by several name groupings, including 'complexes', 'hybrids', 'aggregates', and so on (Urban et al. 2011). Many of these given names are taxonomically incorrect, but *L. camara* L. (*sensu lato*) may be considered more appropriate, with 'varieties' used to refer to the component entities (Urban et al. 2011).

Although there are varying views of the genetic origins of *L. camara* (s.l.), understanding the true origins is necessary to target native range surveys for potential biological control agents. This ties in with the necessity to determine the putative parents of the *Nymphaea* hybrids present in South Africa, to decide which regions are most likely to produce suitable agents. Globally, biological control research for the management of lantana began over a century ago, with research in South Africa beginning in the early 1960s (Oosthuizen 1964, Urban et al. 2011). With over 41 agents released and the establishment of over three dozen agents around the world (Simelane et al. 2021), in most of the Old-World, lantana is still not adequately suppressed (Urban et al. 2011). To a great extent, climatic unsuitability and difficulties in matching agents collected from naturally occurring genotypes with the weedy genotypes have contributed to the difficulty in establishing effective biocontrol (Day et al. 2003a, Urban et al. 2011). Most agents display varietal preferences, and this is problematic where agents are introduced onto varieties with which they are not compatible. For example, only 30% of the lantana complexes invading South Africa comprised the variety targeted by *Aceria lantanae* (Cook) (Acari: Eriophyidae), a flower galling mite from Florida (Mukwevho et al. 2018). Hence, it has been suggested that future research should focus on potential agents that attack a broader range of lantana varieties, especially because most agents do have varietal preferences.

Despite these difficulties, recent studies suggest that continued research for biocontrol of the species is warranted (Mukwevho et al. 2017, Katembo et al. 2020) and that without current biocontrol agents, lantana would have spread more and reached higher densities (van Wilgen et al. 2004, Zalucki et al. 2007, Katembo et al. 2020). For example, the combined

effects of the 16 biological control agents established on lantana in South Africa has resulted in reduction of stem and seed production by 16% and 13% respectively, 44% of leaf damage in Mpumalanga (Katembo 2018, Katembo et al. 2020), and between 16 and 29% plant dry biomass reduction and 94% seed reduction in KwaZulu-Natal (Mukwevho et al. 2017), overall decreasing lantana fitness in drier continental and humid coastal regions in South Africa. Notably, three of the 16 agents are indigenous to South Africa, indicating the potential of South African species to contribute to the suppression of invasives.

The existence of different biotypes of potential biological control agents or plants could be important for the management of *Nymphaea* hybrids, and hybrids in general. In an entomological context, "biotype" may be defined as "an individual or population that is distinguished from the rest of its species by criteria other than morphology, for example, a difference in parasite ability" (Maxwell & Jennings 1980). For biological control agents, populations might be distinguished based on varying host specificity, or for plants, this might be characterised by a suite of morphological and ecological characteristics. For example, *Chromolaena odorata* (L.) R.M. King & H. Rob. (Asteraceae: Eupatorieae) has two distinct forms/biotypes in its introduced range, distinguished by leaf pubescence, odour, flower colour, as well as cold and fire tolerance (Kluge 1990, Kriticos et al. 2005, Zachariades et al. 2011b). Limited success of biological control of this species in South Africa is believed to be impacted by incompatibility of the agents with the biotypes present in South Africa (Zachariades et al. 2011b), because substantial control has been achieved in other regions where agents were collected from the matching biotype (Zachariades et al. 2009). Similarly, *Dactylopius opuntiae* (Cockerell) (Homoptera; Dactylopiidae), a biological control agent for *Opuntia* species, consists of two or more distinct biotypes. Each of these has a restricted host range, such that one is hosted by *Opuntia ficus-indica* (L.) Millar, and the other is hosted by *Opuntia stricta* (Haw.) Haw (Githure et al. 1999, Volchansky et al. 1999). Examples such as these demonstrate that although it can be complex, it is possible to manage unique genotypes by sampling from similar biotypes. Although many of the *Nymphaea* hybrids are not known to occur from the wild, crosses between species such as *N. odorata* and *N. mexicana* do occur where these species overlap. Hence, sampling from sites where this occurs could produce agents suitable for management of these particular hybrids. The examples discussed here suggest that population level differences in host specificity do occur, but that this might be restricted to species with a demonstrated narrow host range. The populations of *B. longulus* tested, despite differences in the plants from which they were collected, did not show

differences in terms of preference between *N. nouchali, N. mexicana,* and *N. marliacea chromatella.* For *M. toddi,* although population level differences were not tested, its demonstrated broad host range (at least within *Nymphaea*) suggests that it is unlikely that a different population would exhibit differential levels of host specificity.

The genetic results from Chapter 3 did not provide significant evidence for the existence of *N. mexicana* biotypes in South Africa. However, it is interesting to note that there was some admixture in the native range populations in the STRUCTURE analyses. This may mean that *N. mexicana* has evolved differently in the invaded range, or that a population bottleneck restricted gene transference from the original propagules. Owing to the ease with which hybridisation occurs in *Nymphaea* within the same subgenus, perhaps it is possible that the *N. mexicana* considered as the 'pure' species in South Africa may have inherited features from ancestral hybridisation. For example, *N. mexicana* in North America is not regarded as an aggressive species (although this could be due to lack of enemy release or similar factors as discussed in the previous section), but natural hybrids of *N. mexicana* X *N. odorata,* with an inherited stoloniferous habit, exhibit highly aggressive characteristics (John Wiersema pers. comm.). Identifications of *N. mexicana* in other regions of the world have also been met with difficulty, because the infestations do not match morphological descriptions of the pure *N. mexicana* species (Hofstra et al. 2013, Zafar Reshi pers. comm)

Examples such as these show that there is promise in finding suitable biological control agents for targets with blurred species boundaries and rampant hybridisation, but that vigorous research and separation of collected agent populations is necessary. Also demonstrated herein is the importance of targeted biocontrol that is specific to the needs of the programme and the biology of the target weed. The results from Chapter 3 of this thesis have improved the understanding of *Nymphaea* hybrid genetic origins and thus provide further options for sampling potential biological control agents. The *N. marliacea chromatella* hybrid utilised in Chapters 4 and 5 for host specificity trials was not sufficiently damaged by *B. longulus* and resulted in reduced survival and reproduction of *M. toddi.* This shows that even though both species are able to survive and reproduce both on *N. mexicana* and *N. nouchali* (thus demonstrating a broader host range), they are not suitable for the control of this particular hybrid. It is not known how they might fare on other hybrids, however. *Nymphaea marliacea chromatella* was shown in Chapter 3 to have likely been parented by *N. alba* and *N. mexicana.* Other hybrids parented by *N. odorata* for example, might be more prone to herbivory by *B. longulus* and *M. toddi*. Nevertheless, the other

parents of the hybrids should be surveyed for potential agents to improve the efforts to manage the *Nymphaea* hybrids discussed here. Further studies could also test the ability of *B. longulus* to survive on other hybrids and building a strong taxonomic understanding of *Nymphaea* hybrids is necessary to correctly separate different hybrids for such experiments. Prioritisation of more invasive/problematic hybrids may also be necessary to focus management efforts. For example, several haplotypes of *Arundo donax* exist, but phylogeographic studies determined that haplotype M1 is the only form present in South Africa, thereby streamlining biocontrol efforts (Canavan et al. 2017). One form of *N. mexicana* exists in South Africa according to ISSR and ITS analyses, but multiple hybrids exist. Of the hybrids, focus should be placed on the two most widespread hybrid groups, namely the *N. odorata* and *N. marliacea chromatella/albida* groups, whereby surveys on *N. odorata* and naturally occurring crosses between *N. odorata* and *N. mexicana* should be completed for the former, and surveys on *N. alba* for the latter.

6.4 HOST SPECIFICITY AND HOST RANGE EXPANSION

The plant defences that need to be overcome by herbivores include mechanical structures, such as cuticular waxes or thorns, and chemical defences which may include over 200 000 metabolites (Chen 2008, Mithöfer & Boland 2012). Chemical cues are important for insect herbivores to identify a plant as an appropriate host for feeding and oviposition (Heard 1999). Acceptance of a host occurs through a series of selection steps that are governed by visual, olfactory, gustatory, and tactile cues, in addition to humidity and light intensity (Bernays & Chapman 1994). Plants may possess unique compositions of these cues, which act to initiate behavioural and physiological reactions in host-specific taxa to initiate feeding and oviposition, while non-host plants may possess deterrents which prevent feeding and oviposition (Bernays & Chapman 1994). It follows that plants that are more closely related to each other are more likely to possess similar morphological and biochemical traits than distantly related taxa (Wapshere 1974, Bernays & Chapman 1994). Hence, insects are more likely to feed on plants that are closely related to its field host plant than distantly related species. This idea forms the basis of the centrifugal phylogenetic testing method developed by Wapshere (1974), in which closely related species are tested for host specificity first, followed by more distantly related species. In this way, if closely related plant species are rejected by the agent in question, it is highly unlikely that they will accept distantly related species, and host specificity testing is thus simplified. Nevertheless, it is important to exercise caution when using these methods, and plant species (including economically important and

rare species) that occupy similar habitats or that display morphological and ecological similarities to the target plant should be included in test plant lists (Sheppard et al. 2005). Similarly, testing of the olfactory and visual cues governing agent host specificity is useful to complement traditional host specificity test procedure, as plants with similar biochemical profiles to the target plant may be at risk of non-target attack (Park et al. 2018).

It was demonstrated in this thesis that the native *B. longulus* expanded its host range to include a plant species to which it had not previously been exposed. Other instances of host range expansion are easily found in scientific literature, with various factors impacting the acceptance (or rejection) of novel hosts. For example, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae) (commonly known as mountain pine beetle) was originally hosted by *Pinus contorta* Douglas (Pinaceae) (lodgepole pine) but expanded its host range to include *Pinus banksiana* Lamb. (Pinaceae) (Jack pine) in western Canada (Erbilgin et al. 2014). Three phytochemical mechanisms were identified by Erbilgin (2019) to facilitate this expansion. Firstly, the historical hosts of *D. ponderosae* shared similar chemistry to the newly attacked *P. banksiana,* deeming it compatible with *D. ponderosae* in terms of pheromone production, larval development, and maintenance of interactions between the weevil and its beneficial symbionts. Secondly, there is a lower concentration of toxic and repellent chemical defences and a larger concentration of chemicals that promote host colonisation in *P. banksiana* compared to the historical hosts. Thirdly, hybrid zones of the novel and historical hosts exist, and these hybrids have chemical characteristics of both parent hosts. *Dendroctonus ponderosae* invaded the hybrid zone before moving to *P. banksiana*, which may have improved its success on the new host (see also the hybrid bridge hypothesis by Floate & Whitham 1993). These factors may further be facilitated by climate change, in which regions that were previously unsuitable become habitable. Relating this host range expansion to the expansion of *B. longulus* to *N. mexicana* and the acceptance of *N. nouchali* by *M. toddi*, whereas hybrid zones have not likely contributed to the host range expansions (because hybridisation does not occur readily between subgenera), it is possible that both *N. nouchali* and *N. mexicana* share similar chemistry, despite their being classified in separate subgenera. In contrast, intermediate chemical and morphological properties of *N. marliacea chromatella* inherited from each of its parent species deemed the plant unsuitable for the feeding and reproduction of both species investigated in the study.

The reduced feeding and survival of *B. longulus* and *M. toddi* on *N. marliacea chromatella* may thus be explained by genetic contribution from *N. alba.* Evidence from

Chapter 3 suggests that this hybrid originated from a cross between *N. mexicana* and *N. alba,* so perhaps chemical defences inherited from *N. alba* prevented the acceptance of *N. marliacea chromatella* by *B. longulus* and *M. toddi*. Also notable, however, is that hybrids bred for horticulture are often selected for traits desirable to plant growers, such as resistance to herbivory. The development history of the numerous *Nymphaea* hybrids is complicated and vague, so it is likely that many cultivars were developed over time to increase such desirable traits. It is evident that both *B. longulus* and *M. toddi* distinguish between the hybrid investigated in this study and *N. mexicana* and *N. nouchali*, strengthening the argument for a unique suite of chemical (and morphological) traits in the hybrid. Other examples of differences in performance on hybrid plants demonstrate the complexity of the factors governing host preference (Fritz et al. 1998, 1999, Hoffmann 2004, Krebs et al. 2011, Borrowman et al. 2014, Williams et al. 2014).

Geographic variations of host plant use may exist for certain species as a result of evolutionary processes (Funk & Bernays 2001, Nylin et al. 2009, Kohyama et al. 2012). Such variations of host plant use may develop where particular populations are exposed to new plant species, with adjustments in life history traits, such as oviposition and larval feeding behaviours, resulting to adaptations to novel hosts (Feeny et al. 1985, Sylla et al. 2019). For example, a population of the tomato leaf miner *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) from Senegal showed higher oviposition acceptance on two nontarget crop species compared to a population from France (Sylla et al. 2019). A likely explanation for this geographic variation is that the nontarget species tested are abundant in Senegal when tomato crops are scarce, such that the Senegal population has undergone adaptations enabling it to feed and develop on species to which it is exposed in contrast to the France population (Sylla et al. 2019). It may be possible that certain populations of *M. toddi* in the U.S. where multiple related *Nymphaea* spp. exist for example, may have a greater tendency to accept novel plant species as hosts compared to other populations, but the ability to accept new hosts at all deems the species unsuitable for use in biological control. Population level differences in host preference were not observed for *B. longulus* for the two populations tested, suggesting that preferences have not yet evolved in this case, but perhaps this would change with time, as occurred with *T. absoluta* (Sylla et al. 2019). Adaptations for the acceptance of novel hosts may, however, be less common in insects that are more host specific, compared to generalist herbivores that may prefer exotic species over natives (Bertheau et al. 2010, Morrison & Hay 2011, Parker et al. 2012). Furthermore, similarity in terms of taxonomy,

chemical makeup, and morphology may facilitate greater likelihood of host range expansion for specialist insects, as suggested earlier in this section (Lawton & D. R. Strong 1981, Goßner et al. 2009). Evidence from a meta-analysis contends this hypothesis, however, suggesting that phylogenetic relatedness may not always account for host range expansion of herbivores from native to exotic species (Chun et al. 2010). Instead, the following factors may be explanatory: differences in abiotic and biotic conditions resulting in geographic variation in adaptation to herbivores (Thompson 1999, Atijegbe et al. 2020); lack of co-evolved defences in exotic plants against native herbivores (Parker et al. 2006, Orians & Ward 2010); and local adaptation resulting in equal susceptibility of native and exotic plants in the introduced range. The idea of ecological fitting is relevant in this case, whereby host range expansions occur when two newly interacting species simply "fit" together without evolutionary processes (Janzen 1985, Agosta 2006). For the acceptance of *B. longulus* on *N. mexicana*, the similarity in life histories of *B. americanus* in the native range of *N. mexicana* and *B. longulus* may have aided this "fitting".

It has been suggested that acceptance of introduced plants as hosts by native herbivorous insects through such ecological fitting may occur readily (Agosta 2006), as demonstrated by several examples (Wilson et al. 1990, Solarz & Newman 2001, Louda & O'Brien 2002, Strauss et al. 2006, Branco et al. 2015). While this may be true for many species, invasions of alien plants might not be as problematic if insect herbivores in invaded ranges were frequently and readily accepting the introduced species as hosts, such that they reduced population growth. This phenomenon is not common in invasion biology literature, suggesting that there is reduced importance of ecological fitting in these cases. Emphasis of other drivers of invasion (as discussed in section 6.2) may be relevant, especially where evidence against enemy release is collected for invasive species (Colautti et al. 2004, Liu & Stiling 2006, Heger & Jeschke 2014). Similarly, even where generalist herbivores may accept novel invasive species, their impact on populations may be negligible, but other factors may be at play. For example, colonisation of exotic plants by native herbivorous insects depends on the likelihood that compatible species capable of ecological fitting encounter one another, as well as the ability to recognise a novel plant as a host and digest the ingested material (Pearse et al. 2013). This likelihood and frequency of encounter is governed by the species richness and abundance of native insects found on ecologically similar plants (Shmida & Wilson 1985), the area covered by the introduced plant (Neuvonen & Niemelä 1981), the time since an exotic plant has been introduced (in which the longer the plant has been present

in the introduced range, the more likely it will encounter suitable insect colonisers) (White et al. 2006, Brändle et al. 2008), and of course, the level of host specificity of the colonising insects (Fraser & Lawton 1994). For this last point, phylogenetic similarity may again be more important due to higher likelihood of similar traits governing host plant selection and host resistance in more closely related plants (Ricciardi & Ward 2006, Pearse et al. 2013).

The concept of host range expansion may be desirable when considering native herbivores attacking invasive alien plants but is alarming for biological control practitioners. If host range expansions and ecological fitting are supposedly common, questions around the ethical implications of biological control use are raised. This concern has been addressed in other studies (McEvoy & Coombs 2000, Louda et al. 2003, Pearson & Callaway 2005, Sheppard et al. 2005, Grevstad et al. 2021) and extensive reviews suggest that host range expansions are uncommon for biological control agents that have undergone strict host specificity testing and are decreasing with improvements in research techniques (Fowler et al. 2004, Paynter et al. 2018, 2020). As is often the case, the importance of trade-offs between safety factors and driving forces will likely need to be assessed for each situation, such that the risks for taking action through biological control should be weighed against the risks of not utilising biological control (and instead making using of environmentally damaging herbicides).

Extending from the above ethical consideration of introducing exotic biological control agents is the question of whether it is safe and ethical to introduce high numbers of native but less host specific insects, in this case *B. longulus*, to manage problematic exotic plants. A useful study can be considered for comparison, by examining the case of *Parapoynx diminutalis* Snellen (Lepidoptera: Crambidae: Nymphulinae) on *Hydrilla verticillata.* *Parapoynx diminutalis* has a native range in Asia, South-East Asia, Australia, and disjunct populations in central Africa and Europe (Cook & Lüönd 1982 cited in Bownes 2010) but may be native to southern Africa according to one source (Speidel 1984) and based on southern African records in the last century even where *H. verticillata* is likely absent. It is a polyphagous species, with field host records including *Egeria densa* Planchon (Hydrocharitaceae), *C. demersum*, *M. spicatum* (Zhang 1994 cited in Bownes 2010), as well as *Vallisneria spiralis* L. (Hydrocharitaceae) and at least three species of *Potamogeton* L. (Potamogetonaceae) (Baloch & Sana-Ullah 1974). Host studies conducted under laboratory conditions showed that 14 plant species across 13 genera and nine families were susceptible to feeding and development by *P. diminutalis* (Buckingham & Bennett 1989). *Hydrilla*

verticillata was first identified in 2006 in KwaZulu-Natal in South Africa and seems to be confined to this region. In 2008, *P. diminutalis* was found feeding on *H. verticillata* (Bownes 2010), and a monitoring study revealed that *H. verticillata* abundance was likely suppressed by populations of *P. diminutalis* (Bownes 2018). In the study, a native plant, *Potamogeton schweinfurthii* A. Bennett (Potamogetonaceae) was monitored in the same system, and although *P. diminutalis* was also hosted by this species, *P. scheweinfurthii* was more abundant than *H. verticillata* for most of the duration of the study. Furthermore, densities of the moth population were reduced after *H. verticillata* was overexploited, even though *P. schweinfurthii* was available as an alternative host, but *P. diminutalis* populations were able to persist on the native plant and exert damage on *H. verticillata* as it regenerated. Although evidence suggests that this moth is contributing to the suppression of *H. verticillata* at this site, *P. diminutalis* is unlikely to be intentionally released if other hydrilla sites are found across South Africa owing to its broad host range (Bownes 2018). Although the nativity of *P. diminutalis* to southern Africa is contested, *B. longulus* is confirmed as a native in the region. Although host specificity trials did not include genera other than *Nymphaea,* the surveys conducted in Chapter 4 did include other emergent aquatic species, and no evidence of *B. longulus* feeding damage was collected on species outside of genus *Nymphaea.* Introducing *B. longulus* to sites invaded by *N. mexicana* in South Africa is unlikely to pose significant threats to native vegetation, owing to its already wide distribution and the fact that most sites invaded by *N. mexicana* are not inhabited by native *Nymphaea*, or if they are, *B. longulus* is already present. This is not to say that investigations of other potential agents from the native range of *N. mexicana* should be halted, but for now, it is possible that *N. mexicana* populations could be suppressed with introductions of *B. longulus.*

The success of *P. diminutalis* in suppressing hydrilla in this example may be the result of evolutionary adaptations between the plant and the moth as opposed to old association (Hokkanen & Pimentel 1984, 1989). Although *B. longulus* and *N. mexicana* have certainly been isolated over their evolutionary histories, the similarity of *B. americanus* lifecycles and feeding to *B. longulus* may mean that *N. mexicana* already has adaptations to deal with this form of herbivory. Hence, new association may not be as relevant in this case. In contrast, surveys on native *Nymphaea* in South Africa did not reveal the presence of sap suckers similar to *M. toddi* at any of the sampled sites, implying that lack of coevolved defences against this form of attack (barring sap sucking aphids) could result in high levels of damage imposed on native *Nymphaea* by *M. toddi*. Although impact studies in quarantine were not

conducted, this risk further strengthens the lack of suitability of *M. toddi* for release in South Africa.

In summary, the factors governing host specificity and host range expansion are complex but are related to phylogenetic similarity between novel and original hosts and are influenced by abiotic and biotic factors that alter the probability of herbivores encountering and 'ecologically fitting' with novel hosts. Native insects are being successfully used to manage exotic plant invaders in other case studies (Thorstenson 2011, Bownes 2018, Simelane et al. 2021), and developing an improved understanding of specific insect-plant interactions can improve prospects for biological control safety and management of hybrids.

6.5 LIMITATIONS AND RECOMMENDATIONS

The pre-release surveys in this study were important to build a database documenting the extent of *N. mexicana* invasion, and to collect additional samples for genetic analyses. Species accumulation curves were generated for 24 sites sampled once and rarefaction estimators predicted that about 16 additional species were expected. Although the Chao 2 upper 95% bound estimated a large number of additional species expected, the rest of the estimators were very similar, and the estimation curves reached their asymptotes. It was possible that some species were unsampled, but the low levels of damage (barring where *B. longulus* was present), even where sites were visited twice, suggest that it is unlikely that any other highly damaging specialist herbivores were missed.

The role of eutrophication in the invasion of *N. mexicana* has been discussed, and water samples from some sites were analysed for nutrients during the pre-release surveys. The water nutrient analyses provided a better understanding of differences in effects between sites, especially in comparing leaf sizes and percentage coverage between sites with and without *B. longulus.* Water samples were not taken from all sites, however, and doing this would allow the most problematic sites to be prioritised in terms of addressing disturbance. Financial, logistical, and temporal limitations create challenges for this to be carried out for every invaded site, however, especially for plants that are widespread.

Several recommendations were made in previous research to continue the development of a biological control programme for *N. mexicana* (Reid 2019). One recommendation was to determine the likely parent species for *Nymphaea* hybrids, and this was achieved for the hybrids sampled in this study using both ISSR and ITS techniques. Although there are limitations to the use of these techniques (as with any genetic technique)

(Baldwin 1992, Sarwat 2012), the use of two replicates for each sample for the ISSR analyses and conforming results of the ITS analyses provides stronger support for the conclusions obtained in this study. Unfortunately, only one primer provided sufficient amplification for the ISSR analysis, and only a subset of representative samples (with a limited sample size for putative parent species from only one locality) was used for the ITS analyses, so the use of additional primers and markers for a greater number of samples could further strengthen the results. In a study by Li et al. (2011), of four markers tested (rbcL, matK, ITS, and trnH-psbA), ITS markers showed the highest discriminatory power, but a combination of ITS and a plastid marker improved discriminatory power compared to the combination of rbcL and matK. If more detail is deemed to be required, the use of additional plastid markers could provide greater insight. Finally, some regions of the native range of *N. mexicana* were not sampled, including Mexico and some parts of the southern United States. Additional samples collected from these regions may provide further clarity on the genetic structure of *N. mexicana* and its hybrids.

The host specificity trials carried out for *B. longulus* made use of both whole plant and laboratory tests to substantiate observations in the field. The limitations of the study design, including the impact of using nets in the whole plant no-choice experiments and the inconsistencies between the laboratory and whole plant experiments, were addressed in Chapter 4. Despite these limitations, the evidence from all three aspects (field, laboratory, and whole plant greenhouse experiments) all point to lower preference for the tested hybrid. Valuable information could, however, be obtained by conducting similar experiments comparing *B. longulus* feeding and survival over multiple generations for multiple different hybrids, focussing on the higher priority hybrids. No-choice host specificity trials using ecologically similar aquatic plants, such as *N. thunbergiana* and *A. distachyos,* would confirm whether or not *B. longulus* can survive on these species, but lack of feeding damage in the field and asynchronisation in life histories (*B. longulus* is active in summer, and *A. distachyos* grows in the winter) suggests that these are not high priority studies. The ability of *B. longulus* to survive in Highveld regions would be worth investigating to support the use of the species to manage *N. mexicana* across South Africa. Furthermore, while population level differences were not observed between the *B. longulus* populations tested in this study, it is possible that differences might be observed between more geographically isolated populations, or that cryptic species might occur. Genetic studies and investigations into varying host specificity between geographically separated species could yield useful results.

As recommended (Reid 2019), *M. toddi* was imported into quarantine and utilised in host specificity trials. The use of multigeneration trials provides a strong case for the ability for this species to survive on nontarget species, as evidenced in similar studies (Tipping et al. 2011, Smith et al. 2019). A small number of replicates was used for these trials, however, and considerable variation was observed within the data. The fact that observable nontarget effects over multiple generations was evident even with a low number of replicates strengthens the argument that *M. toddi* is not suitably host specific for release in South Africa. If no nontarget effects were observed, it would be highly advisable to repeat the experiments to increase certainty. Similarly, although an effort was made to standardise nutrient values across the plant treatments, differing sources of the plant populations used, and variations in plant age could account for the high variability observed for *M. toddi* survival across treatments. In some cases, plants that were used in previous experiments involving *B. longulus* were used for the quarantine studies, and though these plants were kept insect free for weeks before conducting the *M. toddi* trials, this could have affected the results. Nevertheless, fluorometry readings at the beginning of the experiments confirmed that the plants were not significantly stressed before insects were added. It is notable also that the experiments were carried out in quarantine over wintertime, and some plants, especially *N. nouchali*, were difficult to keep healthy in quarantine conditions during a time in which their growth is usually reduced. To counter these effects, the insects in running trials were transferred to new plants if plant quality declined across all treatments. Again, the possibility of variable plant quality in quarantine conditions strengthens the results obtained in the study: if high survivability was observed on plants that were of lower quality than those found in the wild, survival would likely be even higher in natural field conditions. Finally, for trials involving both *B. longulus* and *M. toddi*, *N. nouchali* from only one source was used, and the multiple varieties of *N. nouchali* (or species according to genetic studies (Löhne et al. 2007, Borsch et al. 2008)) as well as *N. lotus* were not tested. Differences in host preference may therefore exist if these taxa are also considered. However, this is not likely to be of high importance, because *B. longulus* naturally occurs on these taxa across its range (unless cryptic species exist as described above), and *M. toddi* is not safely host specific.

To summarise, I suggest the following recommendations for further development of biological control for *N. mexicana*:

1) Conduct further investigations on *B. longulus* to determine its impact at sites where it is intentionally introduced, and further explore its ability to survive on and impact other *Nymphaea* hybrids.
2) Reject *M. toddi* as a potential biological control agent of *N. mexicana* owing to its broad host range, and instead explore other species discovered during native range surveys. *Bagous americanus,* although challenging to rear in quarantine, would be worth further investigation (especially for hybrid management) because it is hosted by *N. odorata* and *N. mexicana*. Unidentified but damaging chironomids were also reported in native range surveys of *N. mexicana* (Reid et al. 2020) and have been recorded feeding on macrophytes including *N. odorata* on other studies (McGaha 1952, Kondo & Hamashima 1985) and thus warrant investigation.
3) Conduct further field surveys for natural enemies on the parent plants of the predominant *Nymphaea* hybrids in South Africa, such as *N. odorata* and *N. alba*, as well as on naturally occurring hybrids of *N. odorata* X *N. mexicana*.
4) Although of lower priority, other studies that would improve efforts to manage *N. mexicana* and its hybrids include confirming the contribution of seed production to the spread of *N. mexicana* in South Africa, and the use of a broader range of samples of putative hybrid parents to gain further resolution for the genetic structuring of the group in South Africa. Additionally, competition effects of *N. mexicana* and *N. nouchali* and *N. lotus* with and without *B. longulus* feeding could provide useful insights for the prospects of restoration of invaded ecosystems with native species.

6.6 CONCLUSION

This study has contributed valuable information needed for the continuation of establishing biological control for *N. mexicana* in South Africa. In Chapter 1, the importance of following a series of steps to initiate biological control was discussed, and this has become evident with the results obtained in this thesis. Without pre-release surveys, *B. longulus* would not have been found to have expanded its host range. The use of genetic methods has allowed us to improve our understanding of the complex genetic structuring of *Nymphaea* hybrids, enabling us to make targeted decisions to improve the likelihood of finding suitable agents to help manage problematic populations, whereas comparisons with programmes for other similarly problematic species provide insights for the way forward. Although it is disappointing that *M. toddi* is not sufficiently host specific for use in classical biological control of *N. mexicana*, it highlights the importance of host specificity and multigenerational

trials to prevent the release of high-risk species. There is still a long way to go before effective biocontrol of *N. mexicana* is likely to be established, but great steps have been taken in the right direction, and the presence of *B. longulus* as a native agent will enable us to suppress problematic populations while further research is conducted. As is evident in biological control programmes for other invasive species, a multi-faceted approach is necessary to maximise the chances of success.

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