RESEARCH ARTICLE

Love at first bite? Pre-release surveys reveal a novel association between a native weevil and the invasive *Nymphaea mexicana* Zuccarini (Nymphaeaceae) in South Africa

MK Reid[1]* [ID], MP Hill[1] [ID] and JA Coetzee[2] [ID]

[1]*Centre for Biological Control, Department of Zoology and Entomology, Rhodes University, Makhanda, South Africa*
[2]*Centre for Biological Control, Department of Botany, Rhodes University, Makhanda, South Africa*

Classical biological control aims to suppress alien invasive plant populations by introducing host-specific natural enemies from the native range. This relies on the assumption that invasive plant populations in the invaded range benefit from the release of natural enemies. Pre-release surveys in the invaded range are a useful way to determine if enemy release applies to a particular invasive alien plant, and to determine what other factors may contribute to the invasion. Similarly, pre-release surveys gather information that can be used to compare invaded sites before and after the release of biological control agents and may also identify whether natural enemies have been accidentally introduced into the country. Pre-release surveys were conducted in South Africa on the invasive *Nymphaea mexicana* Zuccarini (Nymphaeaceae) to gather such information about this species, for which a biological control programme is being developed. There was lower diversity and abundance of herbivores in the native range compared to South Africa, suggesting that *N. mexicana* does experience enemy release at most sites in South Africa. This support for the enemy release hypothesis justifies the investment in biological control for its management. However, a native weevil, *Bagous longulus* Gyllenhal (Coleoptera: Curculionidae), was found feeding and reproducing on *N. mexicana* at three sites, resulting in damage to the leaves and suggesting that a novel association has formed between these species. *Bagous longulus* may have potential to be distributed to sites of *N. mexicana* where it is not present, though further investigation is necessary to confirm if its host range is suitable for this to be a safe endeavour. With the exception of sites where *B. longulus* was present, leaf sizes were large and damage was low, and there is no evidence that any natural enemies have been accidentally introduced from the native range. Findings such as these emphasise the importance of conducting thorough surveys during the development of biological control programmes.

INTRODUCTION

To manage invasive alien plants, classical biological control makes use of host-specific insect herbivores from the native range of the alien plant to suppress populations in the invaded range (Müller-Schärer & Schaffner 2008). Biological control has been successful in controlling many weed species (McFadyen 2000; van Klinken et al. 2003; Herrick & Kok 2010; Coetzee et al. 2011, 2021) and is cost effective and environmentally friendly (de Lange & van Wilgen 2010; van Wilgen et al. 2020). Biological control for weed management relies mostly on invasion hypotheses such as the Enemy Release Hypothesis (ERH), which states that alien plants may become invasive due to reduced herbivory as a result of the lack of natural enemies in the invaded range (Keane and Crawley 2002), and the Evolution of Increased Competitive Ability (EICA) hypothesis. The EICA hypothesis states that invasive alien plants shift resource allocation from defence to growth and reproduction when they experience reduced herbivory (Blossey and Notzold 1995). While 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 and Vrieling 2005), understanding the drivers for invasions are important to determine the best means of managing problematic populations.

To initiate a biological control programme, a series of research steps should be taken to gather sufficient information about the target plant and maximise the chances that released agents will be effective at suppressing invasive populations (Jacob and Briese 2003; Sheppard et al. 2003; van Klinken and Raghu 2006). One such step is the completion of pre-release surveys, which involves identifying insect fauna associated with an invasive alien plant in its invaded range, before biological control agents are introduced. These surveys are useful to ascertain whether enemy release contributes to the plant's invasiveness (Keane and Crawley 2002; Canavan et al. 2014), and to determine whether potential biological control agents are already established in the invaded range (Dudley et al. 2006). This information is important in making 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 California, U.S.A., 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 (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.

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 and Hill 2009).

Nymphaea mexicana Zuccarini (Nymphaeaceae) is an invasive alien plant that originates in southern U.S.A. and Mexico that has become problematic in South Africa. Owing to the popularity of *Nymphaea* spp. in the horticultural trade, several hybrids of *Nymphaea mexicana* also exist in South Africa (Reid et al. 2021). The existence of hybrids can pose a challenge for biological control due to difficulties in morphological differentiation and because host-specific agents may not accept hybrids as host plants (Hoffman 2004; Urban et al. 2011; Williams et al. 2014). Research into potential biological control agents of *N. mexicana* is ongoing, but pre-introductory faunal surveys of *N. mexicana* have not previously been conducted in South Africa. Hence, in this study, 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. Although multiple landowners have indicated that the presence of *N. mexicana* has negative consequences, quantification of the impacts of the plant have not been carried out but are beyond the scope of this study. 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 conducted in the native range. This study will determine the role of enemy release in *N. mexicana* invasions and collect valuable information to assist in the development of a biological control programme for this species, as well as make data available for comparison after the release of future biological control agents to establish their effectiveness.

MATERIALS and METHODS

Site descriptions

Initial surveys were conducted in Gauteng province 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, while the Gauteng surveys were repeated in December 2020–January 2021. Thirteen sites comprising either *N. mexicana* (*n* = 10) or hybrids (*n* = 3) were sampled on two occasions each 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 two 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*. This weevil was identified by Riaan Stals at the South African National Collection of Insects. In light of this result, 12 additional sites across the country were included for once-off sampling to increase the likelihood of finding additional herbivores
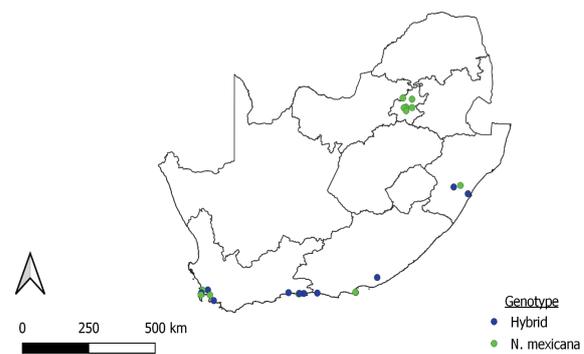
Figure 1. Sites invaded by *Nymphaea mexicana* and *Nymphaea* hybrids included in pre-release surveys. Genotype indicated as hybrid (blue) or *N. mexicana* (green)

including the weevil, and to incorporate more sites in which hybrids and native *Nymphaea* species were growing (Figure 1).

Descriptions of each site, including estimates of percent coverage by *N. mexicana*, notes on levels of damage (estimated from the area of leaf damage from mining, chlorosis, or chewing, in addition to petiole mining), and observations of leaf growth density (with dense leaf growth visualised when leaves were growing above water and crowding each other) 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 were used as metrics 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 selected based on their accuracy in other studies (González-Oreja et al. 2010; Martínez-Sanz et al. 2010). Species accumulation curves were generated for sampling occasions for all sites.

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 $\mu g\, l^{-1}$. 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 sulphanilic acid were added, followed by 1.5 g 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 µg 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.

Sites where nitrate and phosphate levels were tested were classified as oligotrophic (< 500 µg N l^{-1}, < 5 µg P l^{-1}), mesotrophic (500–2 500 µg N l^{-1}, 5–25 µg P l^{-1}), or eutrophic (2 500–10 000 µg N l^{-1}, 25–250 µg P l^{-1}) 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 2012). 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, *N. 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. These measurements were compared with similar measurements taken in U.S.A. and reported in Reid et al. (2020).

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: damage was rated as "high" if more than 50% of the leaf showed signs of chewing, mining, or chlorosis from sap sucking; "moderate" if between 25 and 50% of the leaves were damaged; and "low" if less than 25% of the leaf was damaged. Plants were monitored for insect feeding behaviour and mode of damage by wading through the water or accessing leaves from boats if necessary. 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.

Statistical analyses

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). 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).

Bagous longulus was present at seven sites, three with *N. mexicana* and four with hybrids. 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. Comparisons of leaf length, herbivore species richness, and observations of levels of damage were also compared with data collected from native range surveys recorded 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.

RESULTS

Site descriptions

Twenty-five *N. mexicana* sites were surveyed, of which 15 were invaded by *N. mexicana* and 10 were colonised by 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 and aphids or was due to 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 compared to a mean species richness of 5.4 ± 1.5 SD in the native range (Reid et al. 2020). 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. This contrasts with the high levels of damage at sites in the native range (Reid et al. 2020). Overall, eight natural enemy species were encountered in South Africa compared to 15 in the native range (Reid et al. 2020). Most of the species encountered in South Africa were generalists, such as Aphididae, snails, Erebidae, or crambid moths such as *Parapoynx* sp. Hübner (Lepidoptera: Crambidae) and most were incidental visitors. There was no evidence that any natural enemies from the native range were present in South Africa due to accidental introductions.

The mean estimated percentage coverage of *N. mexicana* and its hybrids in the water bodies sampled was 41.17% ± 32.36 SD. Based on the percentage coverage using the DAFOR classification, of the 25 sites surveyed, one was classified as rare, six were classified as occasional, six as frequent, five as abundant, and seven as dominant. Sites with lower coverage were either large water bodies where *N. mexicana* grew on the edges, sites where plants were removed through chemical or manual control, or sites where there were higher levels of damage by herbivores. A detailed description of the conditions at each site is given in Supplementary Table S1.

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 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 (by observation) and with smaller leaf sizes compared to all other surveyed sites where *B. longulus* was not present (Figure 2). At Fountainhill Estate in KwaZulu-Natal province, coverage by *N. mexicana* was only 13% compared with 56% by *Nymphaea nouchali* Burman (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.). *Bagous longulus* was also found at four sites where native *Nymphaea* spp. were growing with *N. mexicana* hybrids, but lower levels of feeding damage were observed on the hybrids compared to the native *Nymphaea* and *N. mexicana*, and only early larval instars were found in the hybrid leaves.

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 18 additional species could be present (Figure 3).

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 7.21 (± 0.77), with a broad range of electrical conductivity (509.4 ± 517.6 µS), total dissolved solids (361.4 ± 369.3 ppm), and salinity measurements (241.11 ± 271.15 ppt). 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⁻¹), followed by Westlakes in Cape Town (34°04′52.8″S 18°27′19.4″E) (2090 ± 2220 µg l⁻¹) and Florida Lake in Gauteng (2000 ± 222 µg l⁻¹), while Florida Lake also had the highest phosphate levels (79.1 ± 6.26 µg l⁻¹), followed by Yellowwood Dam in Somerset West (21.5 ± 18.4 µg l⁻¹), and Intaka Island (12.7 ± 9.45 µg l⁻¹).
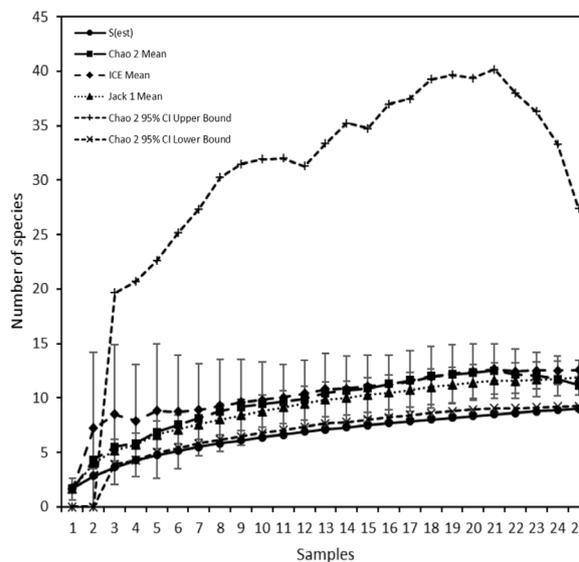
**Figure 3.** Species accumulation curves for *Nymphaea mexicana* sites sampled across South Africa

Leaf size

The mean leaf length of *N. mexicana* in the sites surveyed across the country was 18.9 cm ± 4.19 SD. Most of the sites had similar leaf sizes, but two sites, namely King's Beach Skate Park (9.78 cm ± 2.43) and Knysna Estate (9.14 cm ± 1.21), 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) (19.2 ± 3.05 cm) compared to *N. mexicana* sites where *B. longulus* was present (13.8 ± 6.58 cm) (Figure 4). This did not apply at the ten hybrid sites, where mean leaf length was similar whether *B. longulus* was absent (19.7 ± 2.83 cm) or present (21.4 ± 3.40 cm). 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 ($\chi^2 = 4.5538$, DF = 1, $p < 0.05$). Hence, *B. longulus* and genotype had a statistically significant interaction effect on *N. mexicana* plant length ($\chi^2 = 4.1957$, DF = 1, $p < 0.05$).

Analyses of the effects of water parameters on leaf length using the GAM model revealed there was a significant effect of pH on leaf length ($F = 4.568$, DF = 1, $p < 0.05$), with leaf length decreasing with increasing pH, but the remaining water

Figure 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 (now Gqeberha) where *B. longulus* was present
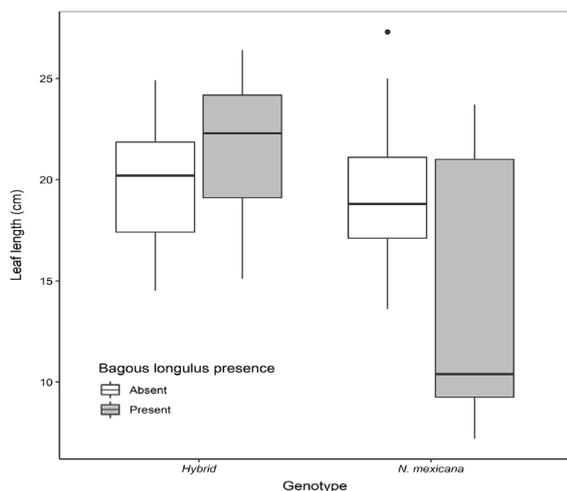
Figure 4. Leaf lengths of different *Nymphaea mexicana* genotypes (*N. mexicana* (*n* = 15) vs. hybrids (*n* = 10)) at invaded sites across South Africa where *Bagous longulus* was either absent (coloured white) or present (coloured grey). At each site for each genotype, the lengths of five leaves were measured. Hence, the number of replicates for each treatment were lumped together as follows: hybrids without *B. longulus* = 5 leaf measurements × 7 sites; hybrids with *B. longulus* = 5 × 4, *N. mexicana* without *B. longulus* = 5 × 11, *N. mexicana* with *B. longulus* = 5 × 3. The boxes represent upper and lower quartiles and median values; whiskers represent the highest and lowest values at 1.5 times the interquartile range. Outliers are marked as separate dots.

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 (χ^2 = 0.0183, *p* = 0.8923) and phosphate (χ^2 = 1.4698, *p* = 0.2254) concentrations between sites.

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) (18.9 cm ± 4.19) were significantly greater (χ^2 = 36.061, DF = 1, *p* < 0.001) than those in the native range (12.1 cm ± 3.17). This reflected a difference in length of approximately 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 5). 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 5).

At sites where *B. longulus* was present and surviving on *N. mexicana,* the greatest leaf length was recorded at Fountainhill Estate (22.48 ± 1.58 cm) and the highest percentage cover by *N. mexicana* at Knysna Estate (60.67%), 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.

DISCUSSION

Most of the sites invaded by *N. mexicana* in South Africa had dense growth, large leaves, and low levels of damage by herbivores. Twelve of the 25 surveyed sites had high (above 50%) percentage cover of the invaded water bodies. 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, species richness, and reduced growth density and leaf sizes of
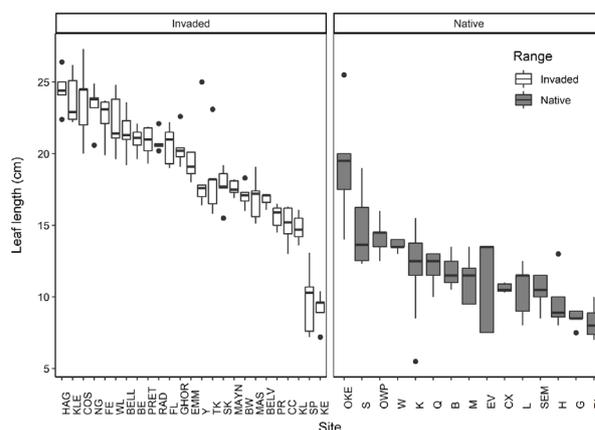
Figure 5. Ordered leaf lengths of *Nymphaea mexicana* in its invaded (South Africa) and native ranges (southern U.S.A.). Boxes represent upper and lower quartiles and median values; whiskers represent highest and lowest values at 1.5 times the interquartile range. Outliers are marked as separate dots. Name codes for each site are given in Reid et al. (2021). For invaded range sites which are not given in Reid et al. (2021), Y = Yellowwood Dam, Western Cape; FL = Florida Lake, Gauteng; KL = Klipriviersberg Nature Reserve, 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.

N. mexicana in its native range (Reid et al. 2020). For example, five species exerted high levels of damage on *N. mexicana* in the native range compared to only one (*B. longulus*) in South Africa, which was only present at three sites (Reid et al. 2020). Species that exerted low levels of damage on *N. mexicana* in South Africa were generalists such as aphids or snails, in contrast to more specialist feeders in the native range. These data showing lower species richness and greater growth of *N. mexicana* in South Africa compared to the U.S. 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. Similarly, no natural enemies from the native range were found 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. For example, while pH seemed to have a slight effect on leaf size, no significant impacts were measured for nitrate and phosphate levels, and these measurements were not taken from all sites and have not been measured from the native range. 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, since leaf size and density was lower in the native range and was correlated with greater levels of herbivory.

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* spp. in South Africa. This weevil was found in abundance at these two 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. A similar scenario, where a native insect developed a new association with an invasive plant, occurred in northern U.S.A., where Eurasian watermilfoil *Myriophyllum spicatum* L. (Haloragaceae) is invasive. The native weevil, *Euhrychiopsis lecontei* Dietz (Coleoptera: Curculionidae), was found associated with declining populations of *M. spicatum* at some lakes in northern U.S.A., 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). Presently in the USA, *E. lecontei* is successfully being used as a biocontrol agent for *M. spicatum* (Smith 2010; Thorstenson 2011). Before *B. longulus* is considered for augmentative releases it is important that its host specificity is investigated to determine the risks of non-target impacts. If *B. longulus* is specific to *Nymphaea*, there would still be risk of spill over effects on the native *Nymphaea* spp., but most sites invaded by *N. mexicana* were not colonised by native *Nymphaea* spp., and if they were, *B. longulus* was already present.

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 five 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; 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 (Fountainhill Estate had slightly lower electrical conductivity, total dissolved solids, and salinity compared to the other two sites, with parameters at King's Beach Skate Park higher than the other two sites, but nitrate and phosphate measurements were not taken, and only pH had a slight effect on leaf sizes at other sites); 4) a much greater density of *N. nouchali* was present at this site compared to the other two sites where the weevil was present (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*. Hence, the combined effect of the weevils and clearing seems to have resulted in smaller leaf sizes. Nevertheless, further investigation is necessary to confirm this because 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 this is unlikely because *N. nouchali* was also present at other sites in much lower abundances, usually only along the edges.

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). *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 U.S.A. (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*, 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 to succeed using augmentative releases of *B. longulus*. The inability of *B. longulus* to survive on *N. mexicana* hybrids with varying parentage should, however, be confirmed with further experimentation.

Here we 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 study. We have also determined that it is likely that enemy release contributes to the invasion of *N. mexicana* in South Africa, and that mean *N. mexicana* plant size in South Africa is greater than in its native range in USA. 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 U.S.A. (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.

ORCID IDs

MK Reid – https://orcid.org/0000-0001-7995-5878
MP Hill – https://orcid.org/0000-0003-0579-5298
JA Coetzee – https://orcid.org/0000-0002-0364-3349

CONTRIBUTOR ROLES

MK Reid: Writing – original draft; writing – review and editing; data curation; formal analysis; methodology; investigation; conceptualisation; visualisation.

MP Hill: conceptualisation, funding acquisition, resources, supervision, writing – review and editing; methodology.

JA Coetzee: conceptualisation, funding acquisition, resources, supervision, writing – review and editing; methodology.

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