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Host specificity testing of Diorhabda carinulata (Coleoptera: Chrysomelidae) as a biological control agent of Tamarix spp. (Tamaricaceae)

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Host specificity testing of *Diorhabda carinulata* (Coleoptera: Chrysomelidae) as a biological control agent of *Tamarix* spp. (Tamaricaceae)

Etienne Smit

A Dissertation submitted to the Faculty of Science, University of the Witwatersrand, in partial fulfilment of the requirements for the degree of Master of Science,

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Declaration

I declare that this Dissertation is my own work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted by me before for any other degree, diploma or examination at any other University or tertiary institution.



Etienne Rauch Smit

29-09-2017

Supervisors:

Dr Danica Marlin (University of the Witwatersrand)

Prof Marcus J. Byrne (University of the Witwatersrand)

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Abstract

Tamarix (Tamaricaceae) is an old world genus of riparian trees, which has been introduced into the USA and South Africa, where it has become invasive. In South Africa, two exotic *Tamarix* species have become invasive: *T. ramosissima* Ledeb. and *T. chinensis* Lour., as well as hybrids of these two species with one another and with the native *T. usneoides* E Mey. ex Bunge. The initiation of a biological control (biocontrol) programme against *Tamarix* in South Africa is being considered. To support this effort, the successful biological control programme initiated against *Tamarix* in the USA was used as a resource. The USA biocontrol programme uses leaf-feeding beetles of the genus *Diorhabda* (Coleoptera: Chrysomelidae), which defoliate *Tamarix* trees, leading to the eventual death of the trees after several seasonal defoliation cycles. *Diorhabda* spp. have therefore been considered for release in South Africa, against the invasive *T. ramosissima* and *T. chinensis*. The host specificity of *Diorhabda carinulata* was tested in open-field multichoice tests as well as laboratory-based choice and no-choice tests using invasive target *Tamarix* species as well as the indigenous non-target *T. usneoides*.

Open-field host choice tests and outdoor, caged multi-choice tests were conducted in Western Colorado, USA using native and invasive *Tamarix* material imported from South Africa, identified using molecular genotyping. Sixteen potted *Tamarix* plants were set out in a Latin Square array at each site, and presence of adults and egg clusters were recorded. Open-field multi-choice trials produced few data, but showed some evidence that *D. carinulata* preferred the target species. *Diorhabda carinulata* in outdoor, caged multi-choice trials initially showed preference for invasive target *Tamarix* species, but readily moved to the native *T. usneoides* upon removal of the target species from the array. Later outdoor-caged trials found that the beetles alighted and laid eggs on *T. usneoides*, indicating the possible inclusion of *T. usneoides* in the host range of the agent.

Laboratory paired-choice-trials for both alighting behaviour and oviposition found that adult beetles significantly preferred *T. usneoides* over *T. chinensis,* while the beetles did not significantly prefer *T. ramosissima* or *T. usneoides*. Laboratory no-choice tests using bouquets found that adults reared on *T. usneoides* did not significantly differ in weight from those reared on a *T. usneoides* x *T. ramosissima* hybrid. No-choice rearing tests found that the egg-to-adult survival rate was significantly higher for *D. carinulata* individuals reared on *T. usneoides* than for individuals reared on *T. ramosissima*. Additionally, the fecundity of females reared from egg to adult on *T. usneoides* was not significantly different from those reared on *T. ramosissima*, laying an average of 57.66 ± 13.71 eggs per female, and 32.48 ± 22.35 eggs per female respectively, thus reproductive potential was not reduced on *T. usneoides*.

A climatic suitability model was built using CLIMEX, and included day length induced diapause. The resulting suitability map indicated that no area of South Africa would be suitable for *Diorhabda* beetles, as the short day length would cause the beetles to remain in diapause for too long, causing their populations to eventually die out as they are unable to reproduce. The evolution of delayed diapause has been observed, therefore southern populations of *Diorhabda* from the USA may be considered for further study.

Diorhabda carinulata will not be a suitable biological control agent against invasive *Tamarix* in South Africa in light of its ability to readily develop and oviposit on the native *T. usneoides,* as evidenced by a 233% higher relative suitability for this species over the baseline *T. ramosissima*, as well as the unsuitability of the South African environment. An alternative biological agent with a higher host specificity and greater environmental suitability should be sought for further research.

Keywords: *Tamarix*, *Diorhabda*, biological control, host specificity, ecological host range, fundamental host range, climatic compatibility.

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Chapter 1 - Introduction

Literature Review

Invasive plants

Invasive success

Invasive plants are plants that are not native to a specific region, which spread beyond areas of introduction by passing through barriers to their dispersal (Richardson et al. 2000). Invasive plants also often have an environmental, economic and social impact, for example if the invasive species leads to the extirpation of native plants and the depletion of water sources. Several factors influence the ability of a plant to become invasive.

The intrinsic traits of an introduced plant, as well as features of the environment it is introduced into, can influence the invasiveness of the plant, either increasing or decreasing the ability of the plant to establish and become invasive. The characteristics of an ideal invader were initially and simplistically, outlined by Baker (1974) and have since been revised and reviewed by numerous authors (Mack et al. 2000; Sakai et al. 2001; Williamson & Fitter 1996). Pyšek *et al.* (2015) propose that the primary drivers of invasiveness are not purely biological traits but rather larger-scale processes, particularly the length of time for which the plant has inhabited the invaded region and the stage of the invasion. The number of habitats inhabited by the plant in its native region is also a good predictor of the invasive potential of a plant, as it indicates that the plant possesses biological traits which enable it to succeed in a diverse range of conditions (Pyšek et al. 2015). The propagule pressure applied by the plant, leads to large seed banks, and a high propagule load in the ecosystem, granting the invader greater reproductive success (Pyšek et al. 2015).

The most commonly invoked explanation for invasive success is known as ‘enemy release,’ which states that invasive plants are successful as a result of an absence of pressure from natural enemies, such as specialist herbivores (Keane & Crawley 2002). The resulting

lack of top-down stress allows the invasive weed to grow almost unrestricted. The enemy release hypothesis forms the basis for classical biological control, which seeks to re-introduce pressure from native enemies to the invasive plant.

Biological control

Classical biological control (biocontrol) involves the use of a specialist natural enemy of a target organism, in this case a weed, to reduce the population density of that weed by re-introducing the top-down stress which was lost when the weed was relocated (McFadyen 1998). Biocontrol is often considered preferable to other weed-control methods as it is more directed and less environmentally damaging than herbicide use, more economical and less labour-intensive than manual removal of the weed, and more permanent than either alternative treatment (McFadyen 1998).

In South Africa, many biocontrol programmes have been implemented, with varying degrees of success. The earliest biological control programme in South Africa was the release of the cochineal bug *Dactylopius ceylonicus* (Green) (Hemiptera: Dactylopiidae) in 1913, which was highly successful in controlling the target weed *Opuntia monacantha* Haw. (Cactaceae) (Klein 2011; Lounsbury 1915). As of the year 2015, 41% of all of the potential biocontrol agents that have undergone host specificity testing in South Africa were released, 19% were still under investigation, 17% were shelved for potential future consideration, and 24% of agents tested were outright rejected for release (Klein 2015 unpublished data). Many of the rejected agents were rejected on the basis of insufficient host specificity.

Reasons given for the failure of some agents to establish are varied but often attributed to climatic incompatibility. For example, the biological control programme against *Chromolaena odorata* (L.) R.M.King & H.Rob. (Asteraceae) has seen two congeneric agents fail to establish for different reasons. Initially *Pareuchaetes pseudoinsulata* Rego Barros

(Lepidoptera: Arctiidae) failed to establish as a result of egg predation by ants (Zachariades et al. 2011). *Pareuchaetes insulata* (Walker) (Lepidoptera: Arctiidae) was subsequently released as it was believed to have potentially evolved defences against egg predation, however failed to establish as a result of inability to survive the dry summer conditions in the release area (Zachariades et al. 2011). Two weevils, *Neochetina bruchi* Hustache (Coleoptera: Circulionidae) and *N. eichhorniae* Warner have been released against water hyacinth, *Eichhornia crassipes* (Martius) Solms-Laubach (Pontederiaceae), in South Africa to mixed success (Hill & Olckers 2000). In some areas the weevils achieved complete control over the weed, while in other areas the weevils failed to establish, and no impact was made on the weed population (Byrne et al. 2010). Reasons cited for the failure of the weevil agents against water hyacinth include climate incompatibility, predation, and the resilience afforded to water hyacinth by the high levels of eutrophication found in South African waters (Coetzee & Hill 2012; Coetzee et al. 2011).

To date no biological control agents released in South Africa have exhibited any considerable non target effects. A notable non-target effect which occurred in the United States of America (USA) was that of the Eurasian flower-head weevil, *Rhinocyllus conicus* (Frölich), which has shown non-target feeding on approximately 22 native thistle species including severe impacts on some native thistles (Louda 2000).

Leaf beetles in the genus *Diorhabda* have been successfully used as biocontrol agents in the USA to control populations of invasive tree species of the genus *Tamarix*, therefore the same agents may be useful in controlling invasive *Tamarix* in South Africa.

Tamarix

Biology

Tamarix (Tamaricaceae) is a genus of riparian trees and shrubs commonly known as saltcedars which have become invasive along river systems in several countries including South Africa and the USA. The name saltcedar arises from the ability of *Tamarix* trees to tolerate very high soil salinity (Brotherson & Field 1987). Salt taken up by the roots is excreted through specialised salt glands on the leaves of the plants (Bosabalidis & Thomson 1985). The presence of *Tamarix* increases salinity at the surface of the soil through foliar secretions and leaf litter to the exclusion of many riparian plant species (Shafroth et al. 1995).

Tamarix plants are facultative phreatophytes, and they have a very deep root system, sometimes reaching as much as 30m deep, allowing them to exploit the water table at a great depth (Brotherson & Field 1987). Physiological drought tolerance and the ability to reach a deep water table therefore gives *Tamarix* exceptional ability to survive periods of drought (Brotherson & Field 1987). *Tamarix* plants are also flood tolerant as they are able to survive for up to 70 days under complete inundation (Warren & Turner 1975).

Tamarix plants reproduce through both sexual and asexual means. Seeds are wind and water dispersed, and are produced in large numbers of up to 500 000 per plant per season (Brotherson & Field 1987). A dense stand of *Tamarix chinensis* Lour. can deposit 17 seeds per square centimetre of soil, a much higher seed load than that of *Baccharis glutinosa* Pers. or *Populus fremontii* S. Watson, riparian species native to the USA (Warren & Turner 1975). Seeds germinate within days of settling on suitable soil, and require very wet soil for several weeks after germination for seedlings to establish (DiTomaso 1998).

Tamarix plants are self-compatible, which means that seed production can continue even in the absence of cross-pollination (Brotherson & Field 1987). They can also reproduce vegetatively, through the sprouting of new plants from adventitious roots (Brotherson & Field

1987) although vegetative reproduction is not as important as germination. *Tamarix* is fire-adapted, and is able to resprout rapidly after a fire, as well as being able to survive and out-compete other species in the highly saline and alkaline post-fire soil environment (Busch & Smith 1993).

Ecological impacts

One of the main concerns with the invasion of *Tamarix* plants is their high rate of water uptake. Stands of *Tamarix ramosissima* Ledeb. generally use more water than stands of native riparian vegetation as a result of their high leaf surface area, allowing more evapotranspiration, therefore requiring greater water uptake as compensation (Sala et al. 1996). The daily water use rate of *Tamarix* spp. has been reported from as little as 6.1*l*/day (Sala et al. 1996) to as high as 757.1*l*/day by un unknown original source (Owens & Moore 2007). Realistically the water usage rate of any riparian system will depend on the atmospheric conditions influencing transpiration rates, and Owens & Moore (2007) suggested a more conservative estimate of 122*l*/day.

Paired with the high water use of *Tamarix* is its effect on water flow, caused by altering the structure of river banks. Modifications caused by *Tamarix* include increased sedimentation, leading to larger sand banks, and narrower and deeper waterways (Graf 1978). *Tamarix* spread stabilises previously unstable sand banks causing the further accumulation of unstable sand, narrowing the water channel (Graf 1978). Waterways may also become more turbid, and are sometimes blocked by debris as a result of the presence of *Tamarix* (Graf 1978).

Tamarix plants grow more rapidly than many other riparian plants, with cut stems able to grow at approximately 75 cm per year (Goldsmith & Smart 1982). In extreme cases a seedling is potentially able to grow to 3 m in a single growing season (Friederici 1995). As a

result of the dense canopy cover of *Tamarix* thickets, other riparian plants are shaded. The shading effect as well as the other factors mentioned allow *Tamarix* to form monospecific stands, excluding native species from the area. *Tamarix* may invade disturbed or undisturbed areas, and once invasion has occurred, the area eventually becomes a monospecific *Tamarix* thicket (DeLoach et al. 2003).

Stands of *Tamarix* have also been shown to increase fire frequency, as their fallen leaves and above ground wood structure increase fuel loads and are highly flammable (Busch 1995; Busch & Smith 1993; Wiesenborn 1996). *Tamarix* is a fire-adapted genus and therefore is not killed by the fires it promotes, as it is able to recover much more quickly than most native plants (Busch & Smith 1993). The salinity tolerance of *Tamarix* also contributes to its impact, as it creates a snowball effect in which leaf excretions by *Tamarix* increase soil surface salinity, reducing survival of seedlings other than *Tamarix*, thus creating the opportunity for further spread of *Tamarix* (DeLoach et al. 2000). The invasion in the south-western USA was so severe that Howe and Knopf (1991) predicted that the native riparian woodland vegetation along the Rio Grande would become completely dominated by saltcedar and Russian olive (*Elaeagnus angustifolia* L.) by the years 2040 to 2090.

Not only do *Tamarix* thickets affect native plants, but wildlife as well. Reptiles (Jakle & Gatz 1985) and large and small mammals (Engel-Wilson & Ohmart 1978) have been found to occur in lower numbers within *Tamarix* stands than in native vegetation. In most cases, birds are less attracted to the canopy structure of *Tamarix* than that of native plants, and therefore experience a loss in habitat with increasing *Tamarix* density (Anderson et al. 1983). Additionally, birds which cannot find suitable food within *Tamarix* plots, such as frugivores and insectivores, were found either to be absent or in limited numbers in *Tamarix* infested areas (Cohan et al. 1978). *Tamarix* stands have also had some positive effects on wildlife, with some native arthropods having been found to preferentially use *Tamarix* stands in the

Rio Grande Valley (Ellis et al. 2000), and some *Tamarix* stands in the same region having been found to contain higher rodent diversity than stands of native vegetation (Ellis et al. 1997). The southwestern willow flycatcher, *Empidonax trailii extimus* (Passeriformes: Tyrannidae), has also benefitted from the presence of *Tamarix* stands as it is able to nest in the branches of *Tamarix* spp. which is especially beneficial in areas where nesting by the flycatcher is not successful (DeLoach et al. 1996). Some other riparian bird species are known to utilise *Tamarix* for nesting, but have been found to have larger populations on native plant communities (Shrader 1977).

More direct impacts of the *Tamarix* invasion on humans include recreational and agricultural effects. A large *Tamarix* infestation on a river can block access to the river for recreational purposes purely because of the density that *Tamarix* plots can achieve. Agriculture is affected in a similar way to natural vegetation. Specifically, farmers may have less access to water for irrigation as a result of high water use by *Tamarix* causing small streams to dry out, and increases in soil salinity and fire frequency caused by *Tamarix* reduce agricultural success (DeLoach et al. 2000).

In the South African context, research has been conducted into molecular genotyping and phylogeography of the native and invasive *Tamarix* species (Mayonde et al. 2016; Mayonde et al. 2015). However, more research is needed into the ecological role of the native *T. usneoides* E Mey. ex Bunge and the invasion biology of the invasive *Tamarix* spp.

Distribution and Taxonomy

Tamarix has its native distribution in the 'Old World': Asia, Europe and Africa. Former taxonomic analysis using molecular methods placed the Tamaricaceae in their own order, the Tamaricales (Spichiger & Savolainen 1997). However, more recently, the Tamaricaceae have been placed in the order Caryophyllales (APG 2009; Gaskin 2003). The

genus *Tamarix* contains 54 species (Baum 1978) spread across the Old World. The genus is believed to have originated during the Cretaceous Period in deserts in Asia (DeLoach et al. 2003), expanding across Asia, Europe and Africa (Baum 1978). No native *Tamarix* plants are found in the Americas or Australasia.

Tamarix ramosissima has a more western distribution in Asia, closer to Europe, whereas *T. chinensis* has a more eastern distribution, including Japan (Baum 1978). In South Africa, *T. ramosissima*, and *T. chinensis* have been introduced and become invasive, although the timeframe and reason for introduction are unknown (Henderson 2001; Mayonde et al. 2016; Mayonde et al. 2015). *Tamarix chinensis* and *T. ramosissima* are mostly distributed along the riparian zones near the south coast of South Africa, including the Western Cape and the Eastern Cape, as well as extending as far north as the Free State (Mayonde et al. 2016).

South Africa also has a native *Tamarix* species, *T. usneoides*. The native *Tamarix usneoides* is associated with an array of native insect species which are not found on the exotic *Tamarix* species (Buckham 2011). Research into the association of *T. usneoides* with other wildlife has not yet been conducted. *Tamarix usneoides* has the majority of its distribution in the Northern Cape province, and the North West province of South Africa, as well as Namibia. *Tamarix usneoides* is an economically valuable plant as it is used for phytoremediation of mine tailings (Weiersbye et al. 2006). Therefore it is imperative that any effort to biologically control the invasive *Tamarix* species must not harm *T. usneoides*.

Mayonde *et al.* (2016) found that *T. ramosissima and T. chinensis* in South Africa readily hybridise with one another, as well as with the native *T. usneoides*, and that hybrids are the dominant form of invasive *Tamarix* in South Africa, outnumbering their parental species. The case in the USA is similar, with hybrid *Tamarix* specimens comprising almost the entirety of the invaded range, whereas in the native range almost no hybridisation occurs (Gaskin & Schaal 2002). *T. ramosissima* and *T. chinensis* also hybridise more with one

another than they do with *T. usneoides* (Mayonde et al. 2016; Mayonde et al. 2015). *T. usneoides* has also been found to be less genetically similar to either of the two invasive *Tamarix* species than they are to one another (Mayonde et al. 2016; Mayonde et al. 2015). In phylogenetic terms, *T. usneoides* was found to be part of a separate clade from the two invasive *Tamarix* species found in South Africa, and *T. usneoides* was found to be more closely related to *T. aphylla* than to the other two species. *Tamarix aphylla* has also been found to hybridise with both *T. ramosissima* and *T. chinensis* (Gaskin & Schaal 2002).

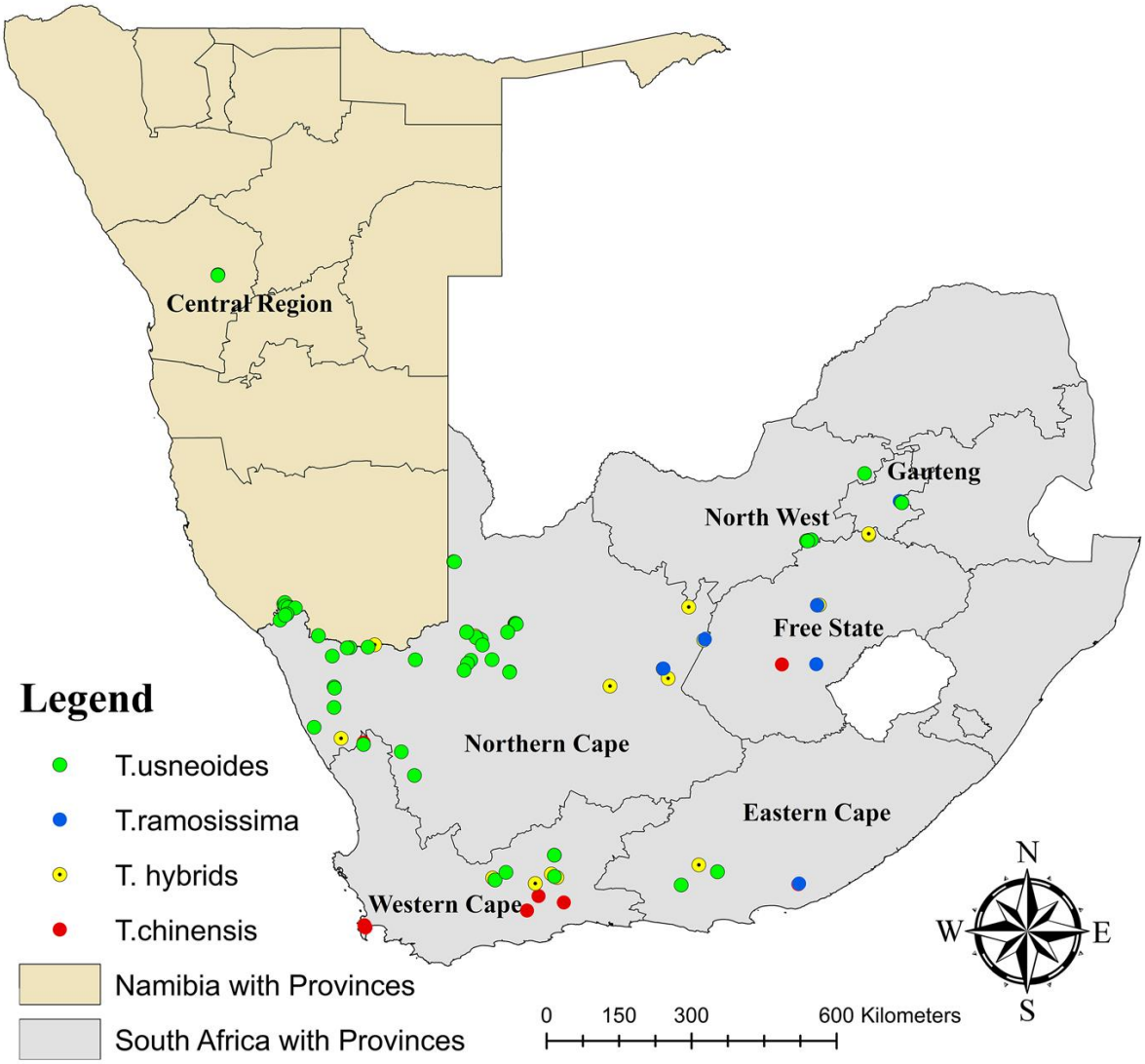


Figure 1.1: Distribution patterns of the *Tamarix* genotypes in South Africa as recorded by GPS and identified using molecular genotyping. Source: Mayonde et al. (2016)

The first introduction of *Tamarix* into the USA is believed to have occurred in 1823, the date of the earliest record, from a nursery in New York, in which the plant is listed as "French tamarisk" (Horton 1964). *Tamarix* was cultivated as a windbreak, soil retention plant, and stream bank stabiliser by the United States Department of Agriculture until it became naturalised in the south-western USA during the 1870s (Brotherson & Field 1987; Horton 1964). It spread rapidly along riparian zones, facilitated by human impacts on waterways through dam construction and water channel diversions (Busch & Smith 1995), moving into ever colder areas until the late 1990s when a biocontrol programme was initiated to combat the weed (DeLoach et al. 2003).

The biocontrol programme in the USA has been used successfully to reduce the *Tamarix* invasion using leaf feeding beetles in the genus *Diorhabda* (Coleoptera: Chrysomelidae) (DeLoach et al. 2004). The genus *Diorhabda* is an appealing source of a potential biocontrol agent for the South African *Tamarix* invasion, because the efficacy of the beetles has already been demonstrated in the USA, and because the biology of *Diorhabda* beetles is well established in the literature (Herrera et al. 2005; Lewis et al. 2003; Milbrath et al. 2007). It is predicted that the beetles may find *T. usneoides* to be a poor host given its genetic distance from their preferred hosts (Mayonde et al. 2016). The beetles released have been shown to have a slightly reduced intrinsic rate of population increase on *Tamarix aphylla* [L.] Karsten as compared to their preferred hosts *T. ramosissima* and *T. chinensis* (Milbrath & DeLoach 2006a). That is, the population increase of the beetles per individual was slower on *T. aphylla* than it was on a preferred host, and it is anticipated that *T. usneoides* will have a similar effect on *D. carinulata*. Moran et al. (2009) conducted open-field post-release evaluations of the suitability of *T. aphylla* for *Diorhabda* spp. and found that it would be unlikely that *Diorhabda* spp. would establish or have long term impacts on *T. aphylla*. Furthermore, *T. usneoides* has hypothetically been geographically separated from all

species of *Diorhabda* beetles for centuries as the beetles are largely restricted to the Palearctic realm with some species extending to the northern areas of the Afrotropical realm, (Tracy & Robbins 2009) whereas the distribution of *T. usneoides* falls within the southern area of the Afritropical realm. Therefore it is possible that the beetles will not even recognise *T. usneoides* as a host.

Diorhabda beetles

Biology and Taxonomy

When the *Diorhabda* beetles were initially introduced into the USA the beetles were classified as a subspecies: *D. elongata* Brullé *deserticola* Chen (DeLoach et al. 2003). Later releases of *Diorhabda* were treated as distinct taxa within the *D. e. deserticola* subspecies and each was subjected to host specificity testing. Tracy and Robbins (2009) performed a taxonomic revision of *Diorhabda* and showed conclusively that the beetles released in the USA were in fact four sister species. The first taxon released was elevated to a full species: *D. carinulata* Desbrochers. The remaining taxa were also elevated to *D. carinata* Faldermann, *D. sublineata* Lucas, and *D. elongata* Brullé. A fifth species, *D. meridionalis* Berti & Rapilly, was considered but not released along with the other four (Tracy & Robbins 2009). The identification of the five species in the *Diorhabda* complex did not warrant much concern, as the beetles had been treated as though they were separate taxa with different ecotypes since the early stages of the biocontrol programme (DeLoach et al. 2003). The ecotypes were referred to by the location from where they were originally collected, and were released in the USA at locations that corresponded with the latitude of their origin, although precise latitude matching was never fully realised in the USA (Table 1.1).

Table 1.1: *Diorhabda* beetle species released and considered for release in the USA as biological control agents. Species were initially classified as different sub-species of the monophyletic species *D. elongata*. The taxonomy was later revised, and resulted in the elevation of the sub-species to five different species. Adapted from Tracy and Robbins (2009).

Species scientific name	Countries of Origin	Release locations and years in the USA
Diorhabda carinulata	China, Kazakhstan, Uzbekistan	Colorado, Nevada, Utah, Wyoming (2001 – 2004)
Diorhabda carinata	Uzbekistan	Texas (2006)
Diorhabda elongata	Greece	California, Texas (2004)
Diorhabda sublineata	Tunisia	Texas (2005)
Diorhabda meridionalis	Iran	Not released in the USA

Diorhabda beetles complete much of their life cycle in the foliage of *Tamarix* plants, with pupation and diapause occurring in leaf litter or below the soil surface. Eggs of *Diorhabda* spp. are laid in clusters of between one and 25 eggs on the foliage of the host plant (Lewis et al. 2003), although *D. carinata* has been observed to occasionally oviposit up to 35% of total eggs on the stems or bark of the plant (Milbrath et al. 2007). When first laid, eggs appear yellow, but become tan-coloured after a short period (Lewis et al. 2003). Three larval instars are present, and distinguishable by size and colouration (Lewis et al. 2003). First instar larvae are completely black and grow to about 1 to 2 mm long. Second instar larvae have yellow spots around the spiracles and a yellowish stripe laterally, and grow to about 4 mm long. Third instar larvae have a more prominent lateral stripe (DeLoach et al. 2003) than the 2nd instars, and grow to about 9 mm in length. Larvae all feed on the foliage of the plant, including leaves and photosynthetic bark, and when fully grown they move to the soil surface by falling or crawling off of the plant and pupate within cells constructed from sand or sometimes litter found on the soil surface (Lewis et al. 2003). Pupae are yellowish

and approximately 6 to 7 mm in length. Adult *Diorhabda* beetles are largely yellow and brown, and also feed on the leaves of the host plant. Overwintering occurs in the adult form, within the leaf litter, and adults emerge to lay eggs the following spring (Cossé et al. 2005; Lewis et al. 2003; Milbrath et al. 2007).

Developmental times for the different *Diorhabda* species were measured by Milbrath *et al.* (2007), although each species was referred to by the geographic source of its population, as the taxonomy had not yet been revised (Table 1.1). The total development time of *D. elongata* under an average temperature of 28°C, was found to be 21 days, with 78% survival. *Diorhabda carinata* developed in 18.6 days under the same temperatures and showed 73% survival. *Diorhabda sublineata* was also found to have a development time of 18.6 days, however its percentage survival, 89%, was the highest of all five *Diorhabda* species in the study. *Diorhabda carinulata* collected in Fukang, China had a developmental time of 20.4 days and 74.5% survival, whereas *D. carinulata* from Turpan, China had a shorter developmental time of 18.5 days, and a higher percentage survival, 78.1%. *Diorhabda meridionalis* was not included in the study by Milbrath *et al.* (2007).

Diorhabda beetles will enter a state of reproductive diapause if the numbers of hours of daylight, or photoperiod, is below a certain temperature-dependent threshold, known as the critical day length (Bean et al. 2007; Lewis et al. 2003). For the *D. e. deserticola* complex, the threshold ranges between 14 hours 14 minutes, and 15 hours 8 minutes across temperatures from 34°C to 22°C (Bean et al. 2007), thus at warmer temperatures diapause induction occurs at a slightly shorter photoperiod, allowing delayed entry into overwintering. At latitudes in which the summer day length is below the threshold, the beetles enter diapause too early in summer, therefore depleting their metabolic reserves before the following season and dying during diapause. Populations are also unable to maintain synchrony with *Tamarix* availability. Therefore, south of 36°20’ N in the northern hemisphere, the beetles are only

able to complete a single generation in the summer and struggle to establish, whereas north of 36°20’ N, the beetles are able to partially complete a second generation and establish more successfully (Bean et al. 2007).

Diorhabda carinulata has been shown to have evolved a shorter critical day length within 10 generations (Bean et al. 2012). The critical day length is the number of hours in the day at which 50% of the population has entered physiological diapause. A decrease in the critical day length means that the population is active for more days, allowing them to reproduce more, and make better use of available *Tamarix* material by having more similarly-timed phenology (Bean et al. 2007), which is hypothesised to lead to higher efficacy of the agent (Bean et al. 2012). The populations of *D. carinulata* are also, consequently, able to establish and sustain themselves in areas where they previously failed to establish, leading to range expansion further south in the USA (Bean et al. 2012).

Male *D. carinulata* beetles emit a pheromone that acts as an attractant to other members of the species, and is therefore termed an aggregation pheromone (Cossé et al. 2005). The pheromone comprises a mixture of two very closely related compounds, an aldehyde and an alcohol. The alcohol on its own was found to be as attractive as a 1:1 mixture of the two compounds, and the pheromone attracted male and female beetles approximately equally (Cossé et al. 2005). The foliage of the host plant (*T. ramosissima*) emits its own compounds which induce beetle response, and was also found to emit both compounds without the presence of any male beetles. However the compounds are produced and emitted by the male beetles in much larger volumes than produced by the foliage. Additionally, different species of *Diorhabda* produce different ratios of the compounds than the ratios produced by other *Diorhabda* species or by the plant (Cossé et al. 2005; Tracy & Robbins 2009). The pheromone may be useful in traps to be used when surveying

populations of *Diorhabda* (Cossé et al. 2005) such as during post-release evaluation and monitoring of the agent.

The larvae of *D. carinulata* are small and they are only able to move a few metres between host plants (DeLoach et al. 2003; Lewis et al. 2003). Thus the larvae are not able to play a major part in host selection, and the primary host selection mechanism is expected to be driven by female oviposition (Bergman 2000).

Association of Diorhabda *with* Tamarix

Diorhabda beetles tend to feed on plants in the subclasses Caryophillidae, and Rosidae (Tracy & Robbins 2009). The family Tamaricaceae, of which *Tamarix* is a member, falls under the Caryophillidae, according to Spichiger *et al.* (2004) (Tracy & Robbins 2009). Of the five *Diorhabda* species that feed on *Tamarix*, *D. carinulata* is known to additionally feed on the genus *Myricaria* Desv. (Tamaricaceae) (Lewis et al. 2003). However, most of the *Tamarix*-feeding *Diorhabda* species are specific to the genus, as *Tamarix* spp. co-evolved with their herbivores in relative isolation (Kovalev 1995; Lewis et al. 2003).

DeLoach *et al.* (2003) reviewed the host range of *D. carinulata* (then referred to as *D. e. deserticola*) and concluded that it is likely to feed on all 20 *Tamarix* species that occur within its native distribution, and possibly on the majority of species in the genus. For example, *D. carinulata* has also been recorded utilising *T. parviflora* DC., although it usually avoids *T. parviflora* when not at high population densities (Dudley et al. 2012). The interaction between *D. carinulata* and *T. parviflora* represents a new association (Dudley et al. 2006), that is, *T. parviflora* does not occur within the native range of *D. carinulata*.

Invasive *Tamarix* may also be considered conflict of interest species, in both South Africa and the USA. In the USA, the *Diorhabda* beetles have had restrictive sanctions placed on them as a result of a secondary effect of their removal of stands (J. DeLoach 2014 pers.

comm). The southwestern willow flycatcher has been found to nest within the branches of invasive *Tamarix* trees, in areas where the bird's natural habitat tree has been extirpated by encroaching *Tamarix* (DeLoach et al. 1996). Thus there is a conflict between the benefits of biocontrol and the negative effects as perceived by those who wish to conserve the southwestern willow flycatcher.

Host specificity testing of the *D. elongata deserticola* species complex was conducted from 1999 to 2002 by DeLoach *et al.* (2003) in order to establish whether the complex was host specific to the invasive species: *T. ramosissima, T. chinensis, T. parviflora,* and *T. canariensis* Willd. Non-target plants included the genus *Frankenia*, as well as athel, or *T. aphylla*, which was a non-target due to its value as an ornamental and shade plant (DeLoach et al. 2003). It was concluded that the agent was sufficiently host specific for release in the USA, because *Frankenia* spp. were unsuitable hosts, as the weighted average of percentage survival on *Frankenia* spp. was 1.7%, in contrast with a weighted average percentage survival ranging between 53.2% to 65.8% on *Tamarix* spp. hosts (DeLoach et al. 2003). More recent laboratory research concluded that the *D. elongata* species complex may inflict damage on *F. salina,* especially in the absence of its preferred host and may also produce transient feeding when in the presence of its preferred host, *T. ramosissima* (Herr et al. 2009). However, even more recent field-based research has found that under field conditions, the rate of feeding and oviposition by *D. elongata* on *Frankenia* spp was lower than had been observed in prior laboratory tests and was not high enough to cause considerable damage to the native plant (Herr et al. 2014).

The agent was also concluded to be host specific as more feeding occurred on target *Tamarix* spp. than on the non-target *T. aphylla* (DeLoach et al. 2003). However, post-release evaluation by Milbrath and DeLoach (2006b; 2006a) revealed that *D. carinulata, D. elongata, D. sublineata* and *D. carinata* all completed their life cycles on *T. aphylla* in the

field, and the only negative effect on *Diorhabda* spp. larvae of utilising *T. aphylla* was a reduction in adult size of the beetles. More recent post-release studies of the impact of *Diorhabda* spp. on *T. usneoides* have found that *Diorhabda* spp. have a reduced preference for, and ability to establish on *T. aphylla*, and that *T. aphylla* is able to recover from defoliation by *Diorhabda* spp. much more effectively than saltcedar is (Moran 2010; Moran et al. 2009). Therefore, the genetic distance between the invasive *Tamarix* spp. in South Africa, and the native *T. usneoides* (Mayonde et al. 2016) may not necessarily mean that *Diorhabda* spp. will not attack *T. usneoides*. However, if the association of *Diorhabda* spp. with *T. usneoides* functions similarly to the association with *T. aphylla*, then *T. usneoides* may not be critically impacted by the agent.

Host specificity testing

Host specificity testing is the process whereby the host range of a potential biocontrol agent is evaluated in order to decide whether the agent is fit to be released against a target weed. The assessment of host range involves measuring the level to which the different life history stages of the agent utilise the target. Utilisation includes the processes of host plant finding, host plant acceptance and host plant suitability (Zwölfer & Harris 1971).

Host plant finding involves the insect locating a plant according to certain stimuli. For example, the aggregation pheromones used by *Diorhabda* may aid the beetles in locating a mate or a suitable plant (Cossé et al. 2005). Host plant acceptance involves the utilisation of the plant by the insect for activities such as oviposition and feeding, and is controlled by stimuli which can be detected at a close range, such as plant chemicals, or physical properties detected by touch or vision (Zwölfer & Harris 1971). Feeding and oviposition can both be measured quantitatively for the purposes of host specificity testing. Host plant suitability is the degree to which the agent is able to complete its life cycle on the host plant, which is

determined by whether or not the host meets the insect's nutritional requirements, and is not toxic or otherwise harmful to the insect (Zwölfer & Harris 1971). Suitability can be measured by comparing the survival rate of each life history stage of an insect on a plant species, as well as by the mass attained by the insects that reach the adult stage. By exposing the agent to different plants and measuring the feeding, oviposition and developmental response, it is possible to establish the host range of the agent.

Historically, the main motivation behind host specificity testing was to show a lack of damage caused by the agent to economically valuable plants, such as crops (McFadyen 1998; Wapshere 1989). However, in the last 50 years, the perception of what constitutes risk to native species has changed and methods have become more cautious and rigorous. More recently host specificity testing has followed a phylogenetic "centrifugal" method. This method involves testing the agent on plants closely related to the target weed, beginning with most closely related and proceeding with progressively less closely related plants (Wapshere 1974). After testing the taxonomically related plants, economically important plants may also be tested. (Wapshere 1974; Zwölfer & Harris 1971).

Host specificity testing is usually conducted in quarantine, under laboratory conditions, which may be problematic as it does not allow for the agent to use its normal host finding process (Zwölfer & Harris 1971). Caged choice and no-choice tests affect the behaviour of the agent by physically limiting their movement, and restricting them to one or more potential hosts, as chosen by the researcher. Therefore, laboratory-based host specificity testing is expected to produce false positive results for agent utilisation of plants, because the agent may damage a plant which it never would have selected under field conditions, thereby broadening their perceived host range (Wapshere 1989; Zwölfer & Harris 1971). This effect is known as artificial host range expansion (Briese 2005; Marohasy 1998).

Open-field host specificity offers a potential solution to the problems of laboratory-based host specificity testing. Open-field testing involves exposing target and non-target plants to the potential agent in an area where the agent already occurs. The area may be within the native range of the agent, or an area in which the agent has already been released for biological control (Clement & Cristofaro 1995; Zwölfer & Harris 1971). This form of testing is advantageous because it allows the agent to go through the full behavioural process of host finding and selection. Additionally, it allows for testing under true environmental conditions in the field, including weather, climate, predation, and competition, which may not be adequately simulated in a laboratory. Thus, open-field host specificity testing can be used to gain insight into the ecological host range of the potential agent (Briese 2005; Briese et al. 2002; Clement & Cristofaro 1995).

Some criticism has been raised against open-field host specificity testing, specifically that open-field testing in the native range of the weed and agent would inadequately simulate the level of intraspecific competition in a released population of insects (McFadyen 1998). The high densities attained by a released agent, combined with removal of the host by the agent may lead to high levels of intraspecific competition, and therefore a broadening of host range. Marohasy (1998) argued that host selection is a function of the relative availability of the target weed and non-target plants, and that selection of non-target plants by the agent may only occur in the absence or low incidence of the target weed.

To attend to the criticism against open-field host specificity testing, Briese *et al.* (2002) developed a two-phase testing method. The first phase involves a set-design, in which target and non-target plants are laid-out in a set block pattern or Latin-square pattern. The second phase involves the removal of the target weed by cutting those plants down, creating a no-choice situation for the agent. The cut target plants are left in position in order to give the agents an opportunity to migrate to the non-target plants. The agents will either select a new

host, showing a broadened host-range; or if the non-target hosts are unsuitable, the agent population will crash.

Key Questions

Does *Diorhabda carinulata* include *T. usneoides* within its host range?

Does *Diorhabda carinulata* include hybrids of *T. usneoides* in its host range?

Does *Diorhabda carinulata* include South African genotypes of *T. ramosissima* and *T. chinensis* in its host range?

Objectives

1. Obtain cuttings of known genetic composition from South African *T. usneoides*, *T. ramosissima*, *T. chinensis* and their hybrids.

- Collect samples of full species and hybrids from individuals of known genetic composition.

- Successfully establish cuttings at the University of the Witwatersrand, Johannesburg.

- Export cuttings from South African *Tamarix* plants to Fort Collins Colorado, USA.

- Successfully establish South African cuttings at Colorado State University and at the Palisade Insectary in Palisade, Colorado.

2. Obtain cuttings from *T. ramosissima* x *T. chinensis* specimens in the USA to act as a control group.

- Successfully establish USA cuttings at the University of Colorado and at the Palisade Insectary.

3. Grow cultures of *Tamarix* samples until sufficient foliage is available for experimentation.

4. Establish the level of utilisation of *Tamarix* species and hybrids by *Diorhabda carinulata* in order to test host specificity.

5. Predict range of *Diorhabda carinulata* in South Africa under the current climate.

- Produce climate models.

Chapter 2 - Open Field Host Specificity Testing

Introduction

Tamarix trees have become invasive in several countries worldwide, including South Africa, and the initiation of a biological control programme against *Tamarix* in South Africa is being considered. In the USA, a successful biological control (biocontrol) programme has been put into effect using beetles of the genus *Diorhabda* (Coleoptera: Chrysomelidae) (DeLoach et al. 2004; DeLoach et al. 2003). The proven efficacy and well understood biology of *D. carinulata* make it a potential candidate to be used against *Tamarix* in South Africa.

In the field of biocontrol, host specificity testing is used to determine the host range of any potential agent to be released against a weed (Wapshere 1974; Zwölfer & Harris 1971). Determining the host range involves measuring the level to which the different life history stages of the agent utilise the target, where utilisation includes the processes host finding, host selection and host acceptance (Bernays & Chapman 2007; Wapshere 1989). When agents choose to utilise a host in the field, they undergo all three processes, and the host is referred to as part of their ecological host range.

The ecological host range includes only hosts which an agent will behaviourally select to use when given an opportunity (Cullen 1988; Marohasy 1998), whereas the fundamental host range includes all host plants which the agent can utilise to complete its life cycle (Briese 2005; Marohasy 1998). It is important to test ecological host range under field conditions as well as in the laboratory in order to identify only suitable host plants, thus reducing the rejection rate of suitable agents as a result of artificial range expansion in the laboratory (Briese 2005; Briese et al. 2002; Clement & Cristofaro 1995; Marohasy 1998). Laboratory testing, especially no-choice testing, often restricts the behaviour of the agent and thus it is not able to complete all three host choice processes, which normally confine the

agent to its fundamental host range. As the ecological host range is a subset of the fundamental host range, the agent may be falsely rejected for release on the assumption that it has a broad host range, when in reality it would never utilise all of its fundamental host range in the field. Open-field host specificity testing is a useful tool in uncovering the more confined ecological host range of a potential biocontrol agent, thereby potentially reducing the rate of false rejection of agents (Briese et al. 1995).

Briese *et al.* (2002) demonstrated a new two-phase testing method, in response to criticism of open-field host specificity testing. The first phase is an array involving target and non-target plants, while the second phase involves the removal of the target weeds by cutting those plants down, creating a no-choice situation with only the non-target plant. This demonstrates a field situation in which conditions have changed, and the target plant has been removed from the system, for example if the agent has depleted the supply of target plant material (Briese et al. 2002). The agents will either be forced to utilise the non-target plant, demonstrating that the non-target may fall within the agent's full ecological host range, or if the non-target hosts are unsuitable the agent population will crash.

Materials and Methods

Samples of South African *Tamarix* specimens with known phylogenetic identities based on the molecular identification performed by Mayonde *et al.* (2016; 2015) were harvested for cuttings. Hardwood cuttings were obtained from trees of *T. usneoides*, *T. ramosissima,* and *T. chinensis*, as well as from hybrids *T. usneoides* x *T. ramosissima*, *T. usneoides* x *T chinensis*, and *T. ramosissima* x *T. chinensis*. Cuttings and rooted plants were also obtained from researchers of the Ecological, Engineering and Phytoremediation Programme, University of the Witwatersrand (Wits). Cuttings were rooted as soon as possible after collection. Hardwood cuttings were transported to the USA by courier in the form of 15

cm long pieces of stem in sealed plastic bags, containing a weak fungicide solution. Some rooted, live plants which had already been rooted from cuttings at Wits were transported by courier as well as hand carried to the USA in plastic bags with roots wrapped in dampened paper towel. All cuttings and live plants from South African Tamarix plants were treated with insecticide and fungicide, and inspected by the South African Department of Water Affairs and Forestry in order to obtain a phytosanitary certificate, before importation into the USA.

For the purpose of comparison with the South African *Tamarix* plants, cuttings were collected from *Tamarix* trees in the USA. Cuttings were collected from populations of uncertain identity in Western Colorado, USA. It was assumed that all trees were hybrids of *T. ramosissima* and *T. chinensis* as there are no known populations of pure *T. chinensis* or *T. ramosissima* in western Colorado (Gaskin & Schaal 2002).

The South African imported and local USA cuttings were rooted and established in a greenhouse at Colorado State University and were later moved to the Palisade Insectary in Palisade, Colorado, for field work. The plants were irrigated by hand once per day until water began to drip-through the bottom of the pots. South African material was kept in pots and flower buds were removed by hand before flowering in order to prevent cross-pollination and hybridisation with plants growing in Colorado. After all the experiments were completed, all imported plant material was destroyed by burning.

A field trip to observe the impacts of *Diorhabda* on a putative population of *T. usneoides* on mine tailings in Arizona was conducted. Samples of foliage were collected from the putative *T. usneoides* trees and shipped to South Africa for genetic sequencing.

Study Sites

Field sites were chosen based on the abundance of adults of *D. carinulata*. In the summer of 2015, two experimental sites were set up in the area surrounding Grand Junction,

Colorado. The first site, Site A, was located east of Grand Junction along 45½ Road at the coordinates: 39°11'32.2"N 108°08'23.3"W. The second site, Site B, was located west of Grand Junction, along County Highway 201 in Garfield County, Colorado, at the coordinates: 39°22'31.0"N 108°58'52.0"W. At both sites, 4x4 Latin squares were laid out consisting of the following genotypes: *T. usneoides* (n=4), *T. chinensis* (n=4), *T. ramosissima* (n=4), and *T. ramosissima* x *T. chinensis* hybrid (n=4). Plants were spaced 1m apart, creating arrays of 4m x 4m (Figure 2.1). The size of the square was restricted to 4 plants x 4 plants as a result of the number of genotypes available, as well as the number of plants available per genotype. Each plant was placed on a drip tray for water retention and watered daily after every observation. Plants of the remaining two genotypes, *T. usneoides* x *chinensis* and *T. usneoides* x *ramosissima* were not used because too few of them had established successfully. Plants of similar size were selected for all of the experiments to avoid bias created by size discrepancies between the plants. Neither site produced enough data as beetle population numbers were extremely low in 2014 and 2015 due to unfavourable summer conditions, and low overwintering success in 2013 (D. Bean 2015 *pers. comm.*), therefore more controlled experimental methodology was employed (alternative experimental design, see below).

In 2016 a new experimental site, Site B2, was set up along County Highway 201, approximately 50m North West of the site known as Site B in 2015. A 4x4 Latin square was set up consisting of the following genotypes: *T. usneoides* (n=4), *T. chinensis* (n=4), *T. ramosissima* (n=4), *and T. usneoides* x *T. chinensis* (n=4). Plants of the genotype *T. ramisissima* x *T. chinensis* were not used as they had been used for the previous year's tests, and plants of the genotype *T. usneoides* x *T. ramosissima* were not used because they had lost all of their foliage during transportation, and therefore needed to regrow their foliage before they could be used.

The beetles at Site B2 began to move southwards along the West Salt Creek, leaving the initial location of the experimental plot almost completely devoid of adult *D. carinulata*. The experimental plants were moved southwards in order to keep pace with the moving beetle population. The plants were later moved a second time as a result of the shifting beetle population. At the third location the plants were repeatedly found to have been fed upon and completely defoliated by a large ungulate, most likely a cow or a deer. Eaten plants were replaced with fresh plants of the same accession, that is, they were replaced with plants cloned from the same parent plant. However, the experiment was discontinued almost one month after it had started, when all 16 plants were found to have been completely defoliated at once by the large ungulates, and any more replacement of plants would have been detrimental to other experiments.

A field experimental site, Site D2, was set up in July 2016 along the Salt Creek, on 7 2/10-Z 6/10 Rd, near Gateway, Colorado, at the coordinates: 38°31'28.1"N 108°58'54.7"W. The beetle population in the area was very low, nevertheless the site was set up because the area was available for use and because the location of the previous year's Site A had yielded an even lower beetle population. Two days after the plot had been set up, a flash flood occurred over the site, and washed several of the experimental plants into the stream channel, and downstream. All plants were recovered, and removed from the field to be used in other experiments and to replace eaten plants from Site B2.

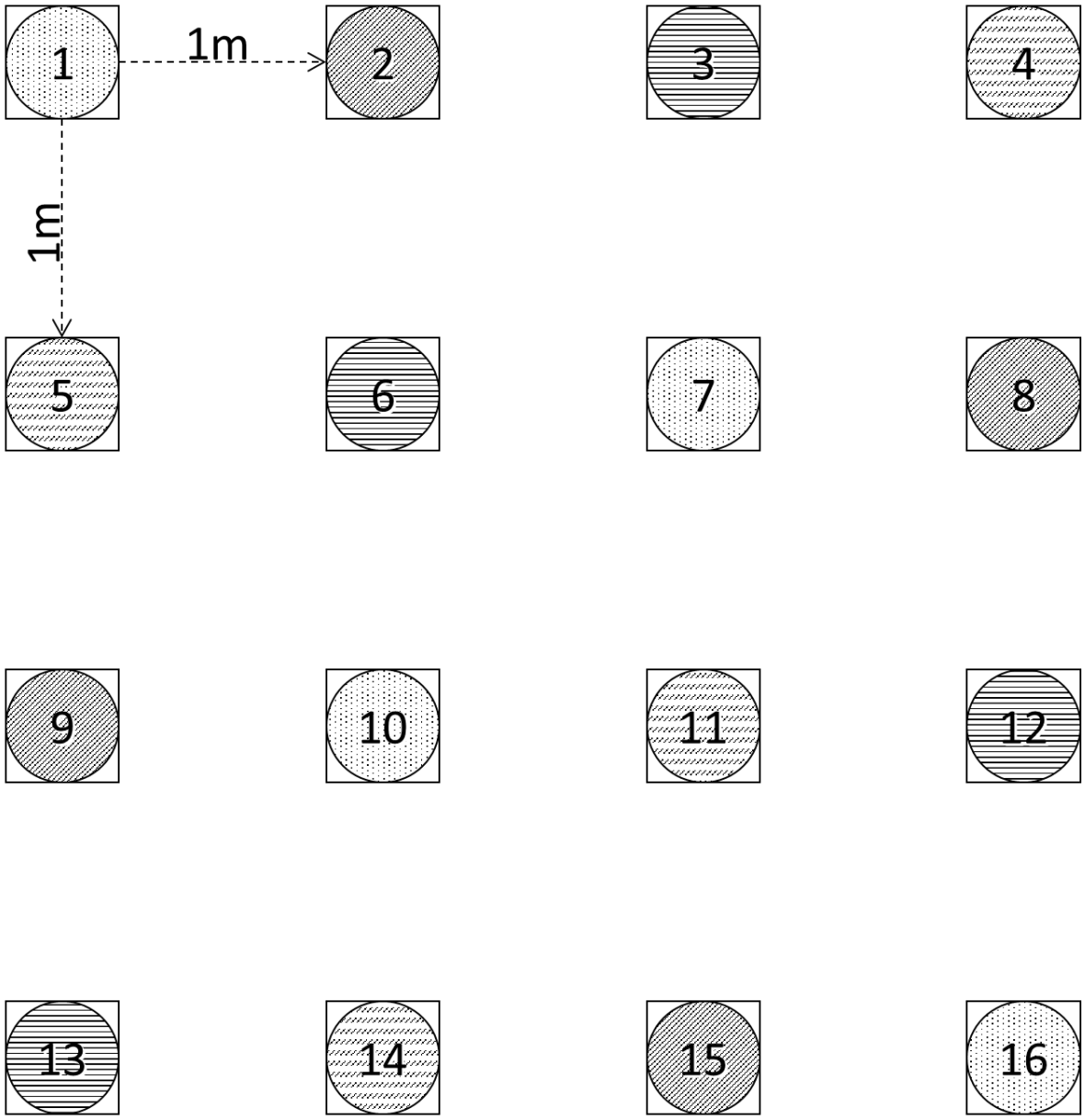


Figure 2.1: Generalised example layout of a 4x4 Latin square array for host specificity testing of *Diorhabda carinulata* on different *Tamarix* genotypes. Shading of the circles represents positions in which different genotypes used in the experiment would be placed. The layout was randomised for each experiment. Plants are numbered from 1 to 16 for data collection purposes.

Open Field Tests

At the selected sites, the potted *Tamarix* plants were set up in a randomly selected Latin square design (Briese et al. 2002), in which each genotype occurred only once in each row and each column (Figure 2.1).

The testing then followed the design used by Briese *et al.* (2002) comprising a two-phase experiment. The first phase was the exposure of the non-target (*T. usneoides* and its hybrids) and target plants to the biocontrol agents at field sites where the agent has established. The second phase involved removing the target weeds from the system in order to produce a no-choice scenario for the agent. Once the number of beetles present on the plants had reached a maximum and stopped increasing, the target weeds were cut down close to the soil surface, and left in the same positions they were in before they were cut. Therefore, the agents were given the opportunity to move from the dead target weeds to the non-target plants, as the target plants had been removed from the system (Briese et al. 2002).

The number of adults, egg clusters, and larvae on each plant were counted on alternating days. Additionally, the number of eggs in each cluster was counted, and it was noted whether the larvae were 1^{st}, 2^{nd} or 3^{rd} instars.

Outdoor Multichoice Cage Tests

Two outdoor caged experiments were conducted in August 2015 and a third was conducted in August 2016. For each experiment, a large (4.6m x 4.6m x 4.9m) mesh field cage was set up outside of the Palisade Insectary, Site C, for caged multiple-choice tests which somewhat simulated field conditions. Within the cage, a 4x4 random Latin square was set up for an experiment using the same experimental layout and design as was used for the open field sites. An adult choice experiment was conducted using the plants from Site A, which were removed from the field and placed into the cage. Four hundred unsexed adult *D. carinulata* individuals were released into the centre of the cage for each experiment.

Statistical Analyses

Data analysis was performed using STATISTICA Version 12 (StatSoft 2015). The data on adult counts were not statistically tested as these observations were of insufficient regularity to analyse. Data on oviposition in the outdoor caged multichoice test were analysed using a Kruskal-Wallis test performed on ranks of the data, as they violated the assumption of normality, and thus a parametric test could not be used.

Results

Open Field Tests

Potted *Tamarix* plants of four genotypes were laid out in a randomised Latin square array and exposed to adult *D. carinulata* beetles both in the field and in a large outdoor cage. A second phase of the experiment was to remove target plants by cutting them down in order to create a no-choice situation with the non-target *T. usneoides*. In the field experiment at Site B in 2015 no *D. carinulata* adults alighted on *T. usneoides* during the multi-choice phase. The beetles did not cause any defoliation of the plants, therefore movement between plants can not be attributed to defoliation. The field experiment also showed no increase in adults on *T. usneoides* after cutting (Figure 2.2), although time constraints prematurely ended the experiment and only one day could be recorded subsequent to the removal of target plants.

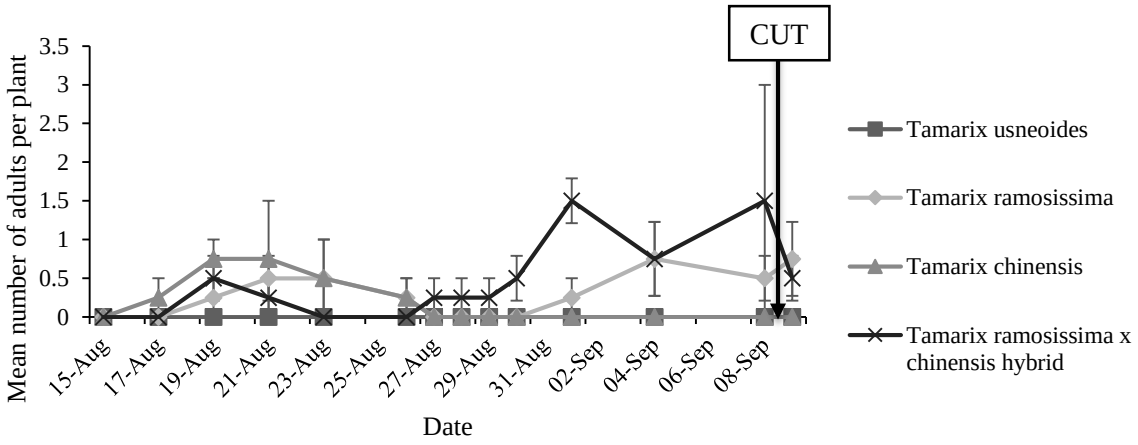


Figure 2.2: Mean number of adult *Diorhabda carinulata* beetles found over time on four different *Tamarix* genotypes in an open-field host-specificity test at Site B in Colorado, USA. For each genotype, n=4. "CUT" represents the point where the experiment becomes a no-choice experiment with *T. usneoides* by cutting down plants of the other three genotypes.

Outdoor Multichoice Cage Tests

After cutting, both cage experiments conducted in 2015 showed an increase in beetles on *T. usneoides*, (Figure 2.3 and Figure 2.4) although this difference could not be statistically tested. Therefore it is unclear from the outdoor cage tests what the preference of *D. carinulata* really is for *T. usneoides*. The preference of the beetles is especially unclear because only one of the three field tests conducted in 2015 successfully attracted *D. carinulata* beetles.

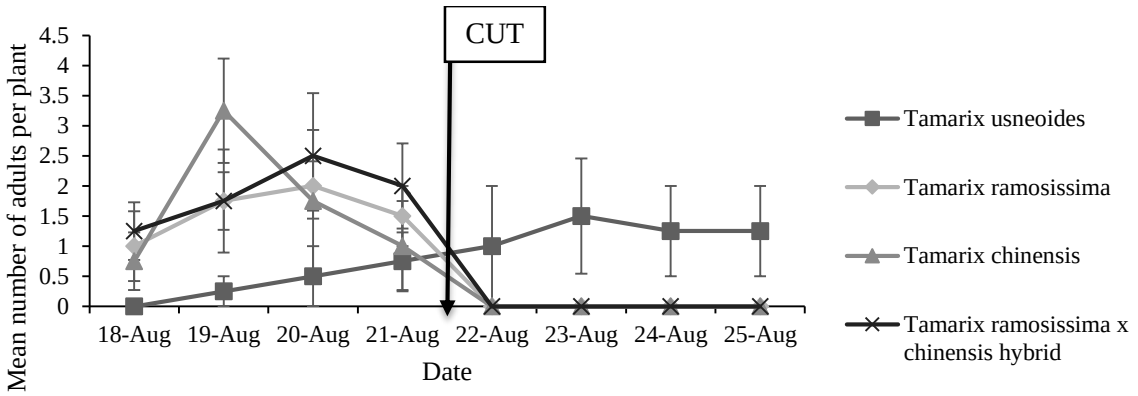


Figure 2.3: Mean number of adult *Diorhabda carinulata* beetles found over time on four different *Tamarix* genotypes in an outdoor caged, multi-choice host-specificity test at Site C in Colorado, USA in 2015. For each genotype, n=4. "CUT" represents the point where the experiment becomes a no-choice experiment with *T. usneoides* by cutting down plants of the other three genotypes.

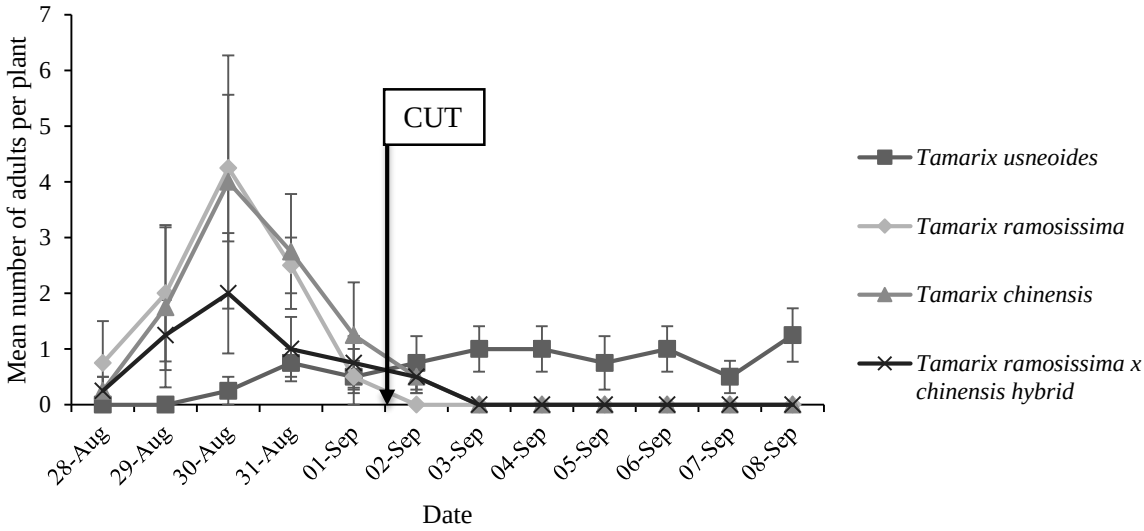


Figure 2.4: Mean number of adult *Diorhabda carinulata* beetles found over time on four different *Tamarix* genotypes in an outdoor caged, multi-choice host-specificity test at Site C in Colorado, USA in 2015. For each genotype, n=4. "CUT" represents the point where the experiment becomes a no-choice experiment with *T. usneoides* by cutting down plants of the other three genotypes.

In August 2016, a third cage experiment was set up at Site C, the Palisade Insectary grounds. This experiment was not turned into a no-choice experiment via cutting of the trees because the *T. usneoides* plants were already occupied by more *D. carinulata* adults than the

other plants, thus eliminating the need for the no-choice phase of the experiment. In this third experiment, the *T. usneoides* plants were larger than they had been for the previous two experiments, however they were equivalent in size to the plants of the other genotypes used. This regularity in size may account for the presence of *D. carinulata* on *T. usneoides* only in the third test and not in the previous two tests.

The number of adults on *T. usneoides* remained higher than the number of adults on any of the other genotypes in the experimental array for the majority of the duration of the experiment.

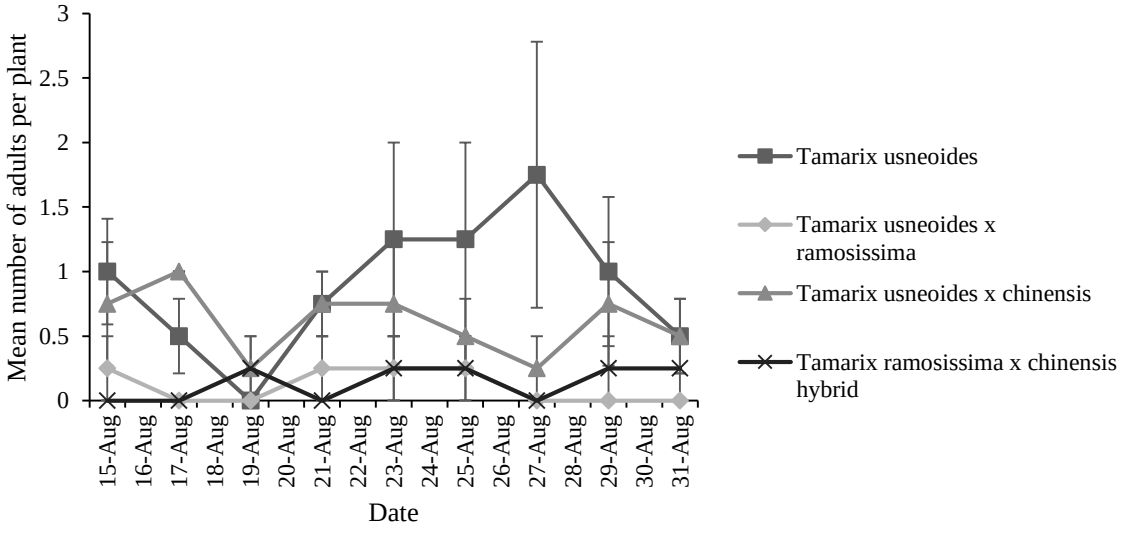


Figure 2.5: Mean number of adult *Diorhabda carinulata* beetles found over time on four different *Tamarix* genotypes in an outdoor caged, multi-choice host-specificity test at Site C in Colorado, USA in 2016. For each genotype, n=4.

Female *D. carinulata* individuals laid eggs on *T. usneoides*, *T. chinensis* x *T. ramosissima* hybrids, *T. usneoides* x *T. chinensis* hybrids, however they did not lay any eggs on *T. usneoides* x *T. ramosissima* hybrids (Figure 2.6). No significant difference was found between the numbers of eggs laid on plants of each genotype (Kruskal-Wallis test on ranks; $p>0.05$). No eggs were found on the cage walls, although the large size of the cage may have lead to a failure to notice any egg clusters.

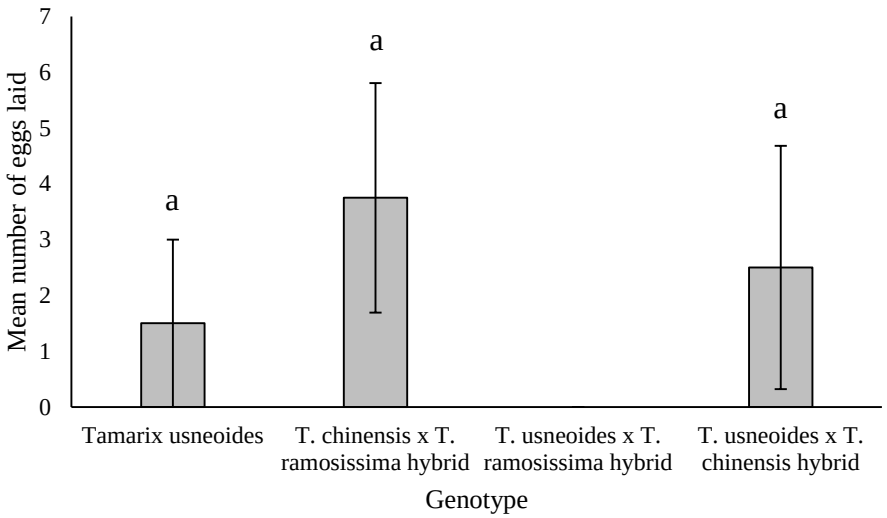


Figure 2.6: Mean number of eggs laid by *Diorhabda carinulata* beetles on different *Tamarix* genotypes in an outdoor, caged, multi-choice host-specificity test at Site C in Colorado, USA in 2016. For each genotype n=4. Error bars represent the standard error of the mean. No significant difference was found between the numbers of eggs laid on plants of each genotype (Kruskal-Wallis test on ranks; $p>0.05$).

Discussion

Open Field Tests

From the open-field plots laid out in 2015, only one plot out of three successfully attracted *D. carinulata* to the plants. The result of this test plot indicates that adult *D. carinulata* beetles do not preferentially select *T. usneoides* under multiple host choice field conditions.

The primary advantage of open-field host specificity tests is that they allow the agent to carry out its full range of host finding behaviours in an environment which is as natural as possible. Therefore, open-field testing presents the most realistic indication of how the potential agent would behave post-release. The realistic nature of open-field testing makes it a useful tool in biological control programmes, and its inclusion in the host specificity testing cycle would be valuable to many biological control programmes. The two-phase method used

in the current study also addresses a very common issue in biological control, namely the potential for agents to move on to a non-target plant once they have depleted the resource of the target plant in the field. (Briese et al. 2002).

Therefore, the open field host-specificity initially invites the conclusion that *D. carinulata* does not include *T. usneoides* in its realised host range through host selection behaviours. However, given that only one field plot produced data on adult selection behaviour, it would be imprudent to draw a conclusion based on the result of the field test alone. Therefore, somewhat more controlled tests, which allow the agents some freedom of movement to complete their host selection behaviours were conducted in the form of outdoor, caged, multiple choice tests.

Outdoor Multichoice Cage Tests

Outdoor multichoice tests were conducted in a large (4.6m x 4.6m x 4.9m) mesh cage. Each cage experiment used the same experimental layout as the field plots, in the form of a Latin square array. However, rather than exposing the experimental plants to an established population of *D. carinulata*, the beetles were instead collected and released into the cage for each experiment.

The two cage experiments conducted in the summer of 2015 both produced similar results, with *D. carinulata* initially occurring in lower numbers on *T. usneoides*, and then increasing after the removal of the target plants. This result indicates that while *D. carinulata* includes *T. usneoides* within its fundamental host range, it does not prefer *T. usneoides*. It is important to make the distinction between a complete absence of the agent on the non-target plant and a lower number of the agent on the non-target plant relative to the target plants. In this case, although the number of *D. carinulata* was lower on *T. usneoides*, it was not

completely absent. Therefore there is a considerable risk that *D. carinulata* would set upon *T. usneoides* even in the presence of target species if it were to be released in South Africa.

The outdoor cage experiment conducted in the summer of 2016 produced a very different result from that of the 2015 outdoor cage experiments. For the majority of the experiment, *D. carinulata* preferentially alighted on *T. usneoides*, which indicates that the beetles may have a preference for *T. usneoides*, which had not been observed previously. The beetles were also found to alight on the hybrids of *T. usneoides* which were included in this experiment, namely *T. usneoides* x *chinensis* and *T. usneoides* x *ramosissima*. The selection of the *T. usneoides* hybrid plants by *D. carinulata* is problematic because the widespread existence of hybrid plants of a target and a non-target weed creates a spectrum of genetic and morphological traits between the target and non-target plant, which may lead to the biological control agent being more readily able to shift its host range to include the non-target plant (Floate & Whitham 1993). Mayonde et al. (2016) identified that hybridisation of *Tamarix* species is common in South Africa. Thus conditions for the formation of hybrid bridges between *T. chinensis* or *T. ramosissima* and *T. usneoides* would already be in place should *D. carinulata* be released in South Africa. However, the relative lack of hybridisation between *T. usneoides* and the invasive *Tamarix* species in South Africa is likely to somewhat limit the occurrence of hybrid bridges involving *T. usneoides*. That being said, *D. carinulata* may not require the use of hybrid bridges to expand its host range if it already includes *T. usneoides* in its fundamental host range, which can be more fully assessed by no-choice host specificity testing.

In the multiple choice outdoor, caged host specificity test conducted in summer 2016, *D. carinulata* females oviposited on three out of the four genotypes used in the experiment. The oviposition on a plant by a potential biological control agent indicates that the agent has undergone part of the process of host acceptance (Bernays & Chapman 2007; Janz & Nylin

1997; Zwölfer & Harris 1971), and therefore that *T. usneoides* and its hybrid with *T. chinensis* may be included in the host range of *D. carinulata*. Oviposition by the potential agent on the non-target plant indicates that there may be a risk of the agent being able to successfully establish on the non-target plant, provided that the larvae that hatch from the eggs are able to complete their development to reproductively fertile adults (Janz & Nylin 1997; Zwölfer & Harris 1971). The lack of oviposition on the *T. usneoides* x *ramosissima* hybrid may indicate that the agent does not include this particular hybrid in its host range. However, this test is rather limited and is not necessarily a powerful indicator of a rejection of the hybrid by *D. carinulata*, which would require more thorough testing.

In the summer of 2016, none of the open field plots attracted *D. carinulata* to the experimental plants. This result was unexpected as the field population of *D. carinulata* was much higher in 2016 than in 2015. The relative lack of results found during the summer 2015 tests was initially attributed to exceptionally low field populations of *D. carinulata* during that season. It was suspected that the low numbers of beetles attracted to the experimental plots in the field was a direct result of the low numbers of beetles present on established stands surrounding the plots. Therefore it was expected that, if the field population of *D. carinulata* were to increase in the summer of 2016, more beetles would be attracted to the experimental plants, and thus more robust results would be obtained. This, unfortunately, proved not to be the case.

In the summer of 2016, the field population of *D. carinulata* at the experimental field site, Site B2 along County Highway 201, were much higher than they had been during the summer of 2015 (N. Louden 2016 pers. comm.), however the beetles showed no interest in the experimental plants. The population persistently moved southwards along the river channel, which may have been a contributing factor to the lack of results, however the experimental plot was moved along with the shifting population, therefore the movement of

the beetles is an unlikely cause of beetles not alighting on the experimental plants. Instead, the reason for the lack of beetle attraction to the experimental plants is likely a result of some feature of the experimental plants which differentiated them from the surrounding stands of *Tamarix*.

The most apparent difference between the experimental plants and the established stands of *Tamarix* was their size. The surrounding *Tamarix* trees were well-established and had been growing for many years, and most were in excess of 2m tall. In contrast, the experimental plants had only been growing for six months to a year from hardwood cuttings and were much smaller, approximately 0.5m in height, and with less dense foliage than the field established plants. Coupled with the large size of the established *Tamarix* trees would be a large volume of volatiles being emitted as well as volatiles emitted by the established *D. carinulata* beetles. The large volume of volatiles from the established trees and pheromones emitted by the beetles may have overshadowed the volatiles emitted by the experimental plants.

The butterfly *Melitaea cinxia* (Linnaeus 1758) (Lepidoptera: Nymphalidae) has been shown to use plant size of its host plant, *Plantago lanceolata* L. (Plantaginaceae) as an oviposition cue (Reudler Talsma et al. 2008). Heisswolf et al. (2005) found a similar case for the leaf beetle *Cassida canaliculata* Laich. (Coleoptera: Chrysomelidae), a specialist herbivore which preferentially selects larger plants for oviposition on its host plant *Salvia pratensis* L. (Lamiaceae). The reasons offered for the tendency of the leaf beetle to select larger host plants were that larger plants tend to have a higher nutritional content within their foliage, and that larger plants provide the newly hatched larvae with superior shelter from enemies, such as predators, than smaller plants do (Heisswolf et al. 2005). A similar situation may apply to *D. carinulata*, as females may have been preferentially attracted to the stands of larger plants for oviposition, thus overlooking the small, sapling-sized experimental plants. A

better result may have been achieved with larger experimental plants, however larger plants were not on hand because of the limited amount of time available for growing the plants. Additionally, larger plants would have presented a serious logistical problem when importing them into the USA. Another possible solution to the lack of beetle attraction may have been to set up the experimental plots in an area free from established stands of *Tamarix*, and then to release field-captured *D. carinulata* adults, similar to the experimental design employed by Thomas et al. (2010).

The overall narrative created by the results of the open field and outdoor cage tests is that *D. carinulata* is inclined to select *T. usneoides* as a host even in the presence of target species, including its native hosts, although no statistical pattern could be tested for as the data were minimal. Therefore, *T. usneoides* is included within the ecological host range of *D. carinulata*. *Diorhabda carinulata* thus carries some risk of non-target effects on *T. usneoides* if it were to be released in South Africa. However, the non-target effects of *D. carinulata* may be limited if the beetles are revealed to be unable to establish on *T. usneoides* by means of no-choice developmental testing. If no-choice testing reveals that *T. usneoides* is not suitable for the development of *D. carinulata*, it will make a case for its release as an agent in South Africa. Otherwise, *D. carinulata* may be deemed unsuitable for release, and other potential agents will need to be considered.

Chapter 3 - Laboratory Host Specificity Testing

Introduction

When considering a new organism for release as a biological control agent against an invasive weed, it is important to establish the full fundamental, or physiological, host range of the agent, which consists of all of the plants which the agent can utilise to successfully complete its life cycle (Cullen 1988). This is as opposed to the ecological host range, which includes only the plants which the agent will select under field conditions (Briese et al. 1995). The fundamental host range can be revealed using laboratory-based no-choice testing, in which the agent is given only a single plant, and its utilisation of the plant is measured (Wapshere 1989; Wapshere 1974; Zwölfer & Harris 1971). The ecological host range can be partially revealed through choice-testing in the lab, in which the agent is given the choice of two or more host plants, and feeding, alighting or oviposition response is measured (Wapshere 1974; Zwölfer & Harris 1971). However, in order to reveal the full ecological host range of the agent, open-field host specificity testing should be performed (Briese 2005; Briese et al. 1995; Clement & Cristofaro 1995).

It is important to be aware of the fundamental host range of the agent, because once the agent has been released, the potential exists for the ecological host range to expand to include species beyond that of its fundamental host range (Thomas et al. 2010). Laboratory-based host specificity tests are useful as they allow for control over the variables that may affect the agent's choice of a host plant, and because they provide a "worst case scenario" of the agent's host range.

A host plant is considered to be suitable for an agent if the plant facilitates the agent to complete its entire life cycle, for example from an egg to an adult. The successful completion of the agent's life cycle indicates that the host plant provides the correct physical or chemical stimulus to induce feeding, as well as providing adequate nutritional content to

the life stages of the agent to allow it to complete its life cycle, although it may not necessarily provide the correct ovipositional cue (Zwölfer & Harris 1971). The adults which emerge at the end of the life cycle also need to be reproductively fertile, and thus capable of maintaining the agent population (Wapshere 1974; Zwölfer & Harris 1971).

Materials and Methods

Samples of South African *Tamarix* specimens were harvested for cuttings and transported to the USA as described in Chapter 2.

Rooted plants at Wits were initially housed within an indoor growth room with natural light, irrigated by hand every second day and fertilised with a slow release N:P:K (7:1:3) general fertiliser once every two months. Infestations of spider mite were controlled using a general, non-systemic acaricide, and acaricide applications were discontinued at least one month before experiments were initiated. Over a year later, the plants were moved outside and irrigated using an automated irrigation system twice per day, and fertilised once every two months. Plants were deemed ready for experimentation when the height of their foliage reached 0.5m.

A culture of *Diorhabda carinulata* was started in South Africa in September 2015, using 500 adult beetles collected along County Highway 201 in Garfield County, Colorado, at the coordinates: 39°22'31.0"N 108°58'52.0"W. Beetles were initially kept in a quarantine facility at the Agricultural Research Council - Plant Protection and Research Institute (ARC-PPRI) in Tshwane, South Africa. The culture was kept under a 16-hour cycle of artificial light in order to prevent diapause induction. Beetles were fed bouquets foliage of *T. ramosissima* x *chinensis* collected from a site in Germiston, South Africa at the coordinates: 26°13'20.2"S 28°07'34.5"E. The culture was securely relocated to the newly-built quarantine facility at Wits at the end of October 2015. In the new facility, beetles were kept under a

combination of natural light and a 16-hour artificial light cycle. A second source of foliage was located near Brits, South Africa at the coordinates: 25°40'45.4"S 27°45'26.6"E. Beetles were kept in clear 5*l* plastic buckets, with ventilated lids, and separated according to generation. The field-collected initial generation was labelled P1, and subsequent laboratory generations were labelled F1, F2, F3, etc. The culture was restarted in October 2016 using 1000 adult beetles collected from the same source population as the 2015 culture, as the culture had collapsed.

No-Choice Experiments

A no-choice larval development test was conducted at the Palisade Insectary under 16-hour artificial light cycles using field-collected individuals in small cages (1m x 1m x 1m) in the laboratory using 74 3rd instar *D. carinulata* larvae collected in the field (Appendix C (a)). The larvae were weighed before being divided between one *T. usneoides* plant and one *T. ramosissima* x *T. chinensis* plant; therefore each plant received 38 larvae. The number of adults that emerged from pupae was counted for each plant, and each adult was weighed prior to feeding. Adults were allowed to emerge in a small tub without food, and after weighing they were moved to a tub containing food.

Further no-choice larval development tests were conducted in late 2015 to early 2016 under laboratory quarantine conditions at Wits using a beetle culture started from 500 beetles collected along County Highway 201 in Garfield County, Colorado, USA. Beetles were collected from the overwintering generation, and had already initiated reproductive diapause as they were collected from the foliage of the plants on 29 August 2015 after the critical day length had passed. However reproduction resumed once the beetles were introduced to a 16 hour artificial light cycle inside the quarantine facility.

No-choice egg-to-adult larval development tests were performed, using bouquets of different *Tamarix* genotypes placed inside the 5L plastic buckets; there were four replicates of *T. ramosissima*, five replicates of *T. usneoides*, and four replicates of *T. usneoides* x *T. ramosissima* hybrid (Appendix C (b)). Twenty *D. carinulata* eggs were placed onto the bouquet in each bucket. The number of larvae as well as the larval stage were noted. Once larvae reached 2nd instar, they were weighed every second day until pupation. Once larvae reached 3rd instar, sand was added to the bottom of the buckets to facilitate pupation. The number of adults that emerged was counted and each adult was weighed.

A no-choice egg-to-egg multi-generational test was conducted at the Palisade Insectary, Colorado, USA under a 16-hour artificial light cycle using field-collected individuals in small cages (1m x 1m x 1m) to determine whether *D. carinulata* adults raised on *T. usneoides* are capable of reproduction (Appendix C (c)). One live, potted target or non-target plant was placed into each cage. Two accessions of *T. usneoides* and one accession of *T. ramosissima x chinensis* were used. Fifty *D. carinulata* eggs were placed on each live plant, and larvae were monitored. Once the larvae had reached the 3rd instar, sand was added to the cages to facilitate pupation. Emerged adults were sexed, counted, weighed and moved into smaller 5*l* buckets, containing bouquets of the same plant accession they had been raised on, to measure egg production. The number of egg clusters laid as well as the number of eggs laid per cluster were counted.

In September 2016, 1000 *D. carinulata* adults were imported into quarantine at Wits in order to restart the culture, which had reached very low numbers during the winter months. The adults were collected from near to the collection site of the beetles that were imported for the first culture. The adults imported in 2016 were also in reproductive diapause, however they began to reproduce after a few days under 16-hour artificial light.

No-choice egg-to egg multi-generational tests were conducted in the quarantine at Wits with the intention of expanding the dataset of the multi-generational tests conducted at the Palisade Insectary (Table 3.1c). Live, potted plants of *T. usneoides* and *T. ramosissima* were used; six replicates of *T. usneoides* and four replicates of *T. ramosissima* (Table 3.1c). *Tamarix usneoides* was used as this was the presumed non-target plant, and *T. ramosissima* was used as a control to compare with results obtained on *T. usneoides*, as it is the known host plant of *D. carinulata*. Other genotypes were not included as time constraints did not permit their inclusion.

For each multi-generation no-choice test, 50 eggs were placed on a square of paper towel, which was positioned in contact with the foliage of a caged, live plant. Upon hatching, the number of larvae as well as their developmental stage; 1st instar 2nd instar, 3rd instar, or adult was recorded every second day. Sand was added to the bottom of the cages to facilitate pupation. Emerged adults were sexed and weighed upon termination of each test. Eggs were removed and counted as soon as they had been laid, and this continued for two weeks, after which the experiments were terminated.

The suitability of each of the genotypes tested in the egg-to-egg trials as hosts for *D. carinulata* was assessed by calculating Maw's host suitability index (Maw 1976). This index evaluates the suitability of a host based on the mass, developmental time and survival rate of the insect utilising the host. It is calculated as follows:

$$Host\ suitability\ index = \frac{unfed\ adult\ mass\ \times \%\ pupation}{developmental\ time}$$

Paired-Choice Experiments

Paired-choice adult oviposition trials were conducted using live, potted plants of two different *Tamarix* genotypes in 0.5m x 0.5m x 1m cages (Table 3.2); four replicates of *T.*

usneoides paired with *T. ramosissima*, and four replicates of *T. usneoides* paired with *T. chinensis*. Fifty five adult *D. carinulata* individuals were released into the cages, of which 20 were male and 35 were female. The number of adults found on each plant was recorded along with the number of egg clusters laid on each plant, every second day until the 1st instars emerged approximately two weeks following oviposition, at which point the experiments were terminated.

Statistical Analyses

Data analysis was performed using STATISTICA Version 12 (StatSoft 2015). Data in the 3rd instar-to-adult development test conformed to assumptions of normality, and thus were analysed using a parametric t-test. All of the other data violated the assumptions of normality, and thus parametric tests could not be used. Data from the no-choice experiments, including adult weights, percentage survival, and female fecundity were analysed using Kruskal-Wallis tests performed on ranks of the data. Data from the paired-choice tests were analysed using Friedman tests with Kendall's coefficient of concordance. The Friedman test is a non-parametric equivalent to the repeated measures ANOVA, and Kendall's coefficient of concordance is a statistic, analogous to Pearson's Correlation Coefficient, which is used to determine whether individual repeated measures in the Friedman test agreed with one another (Field 2005). A value of 1 indicates full agreement of the repeated measures, whereas a value of 0 indicates no agreement. In the present study it was used to indicate whether beetles in paired-choice experiments remained on the same plant for the entire duration of each experiment, or whether they moved between the two plants frequently.

Results

No-Choice Experiments

In a larval development and adult emergence test involving wild-collected early 3rd instar larvae placed on one live specimen each of *T. usneoides* and a *T. ramosissima* x *chinensis* hybrid, total adult emergence was slightly lower (16 adults, 42.11%) on *T. usneoides* than on *T. ramosissima* x *chinensis* hybrid (17 adults, 44.74%). The mean mass of the adults which emerged in the *T. usneoides* trial (0.01089 g) was found not to be significantly different from the mean mass of adults which emerged in the *T. ramosissima* x *chinensis* trial (t-test; $t_{31} = 0.39$; $p>0.05$)(Figure 3.1).

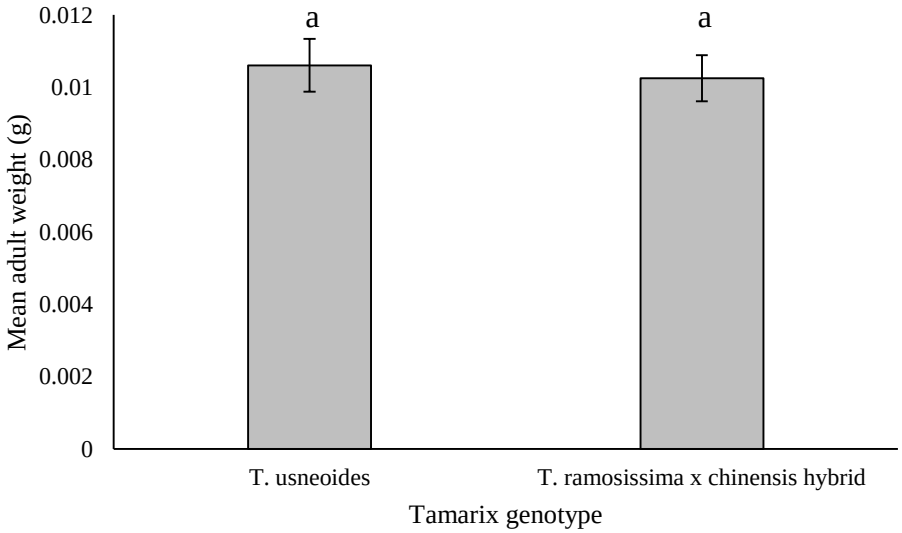


Figure 3.1: Mean mass of adult D. *carinulata* reared from early 3rd instar stage on live specimens of two different genotypes of *Tamarix* (n=32 larvae per genotype). There is no significant difference between the mass of adults reared on either genotype (t-test; $p>0.05$).

No-choice experiments using bouquets of different *Tamarix* genotypes were used test egg-to-adult development of *D. carinulata*. The percentage egg-to-adult survival of *D. carinulata* in each experiment refers to the percentage of 1st instar larvae which hatched from eggs that survived until adulthood (Figure 3.2). In the trials using *T. ramosissima*, no adults emerged from the pupae, therefore the survival rate was 0%. The reason for the lack of

survival on *T. ramosissima* is unknown and difficult to explain. The *T. usneoides* trial produced the highest egg-to adult survival rate of 36.73%, with 18 adults surviving (Figure 3.2). In the *T. usneoides* x *ramosissima* hybrid trials the survival rate was 24.07%, with 13 adults surviving. In the *T. ramosissima* trial and the *T. usneoides* x *ramosissima* trial, the majority of the mortality (66.67% and 55.56%) occurred during the 1st instar larval stage (Figure 3.2).

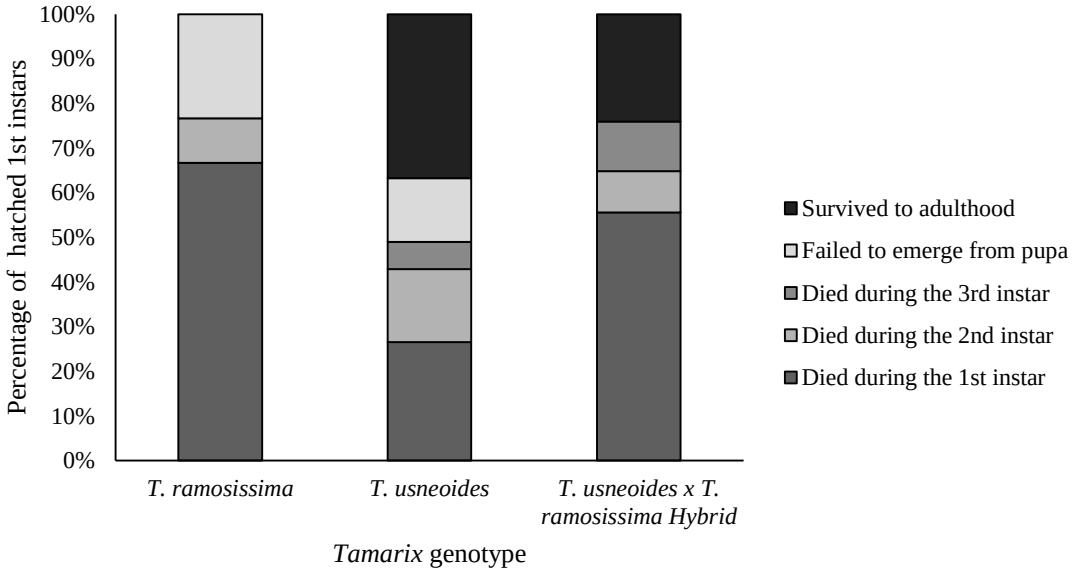


Figure 3.2: Percentage of *Diorhabda carinulata* 1st instars surviving to adulthood, as well as mortality at each life stage. Larvae were reared from eggs, on bouquets of *Tamarix* plant material. n = 4 for both *Tamarix ramosissima* and the *T. usneoides* x *T. ramosissima* hybrid. n = 5 for *Tamarix usneoides*.

Surviving adults in the bouquet egg-to-adult development trials were weighed. The mean weight of adults from the *T. usneoides* trial was 0.008025g ± 0.0004762g (Figure 3.3). The mean weight of the adults from the *T. usneoides* x *ramosissima* trial was 0.008956g ± 0.0009092g (Figure 3.3). No significant difference was found between the mean weights of the surviving adults in these two trials (Kruskal-Wallis test on ranks; $H_{1,22} = 0.657$; $p>0.05$).

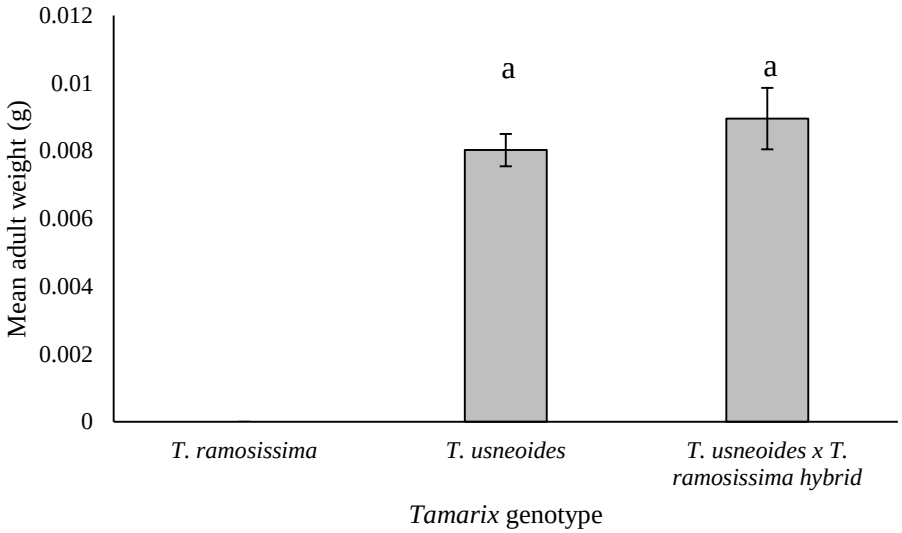


Figure 3.3: Mean weight of adult *Diorhabda carinulata* reared on bouquets of three different *Tamarix* genotypes. . n = 4 for both *Tamarix ramosissima* and the *T. usneoides* x *T. ramosissima*. hybrid. n = 5 for *Tamarix usneoides*. No beetles survived to adulthood on *T. ramosissima*. There is no significant difference between the remaining two mean weights (Kruskal-Wallis test on ranks; $H_{1,22} = 0.657$; $p>0.05$). Error bars represent the standard error of the mean

The survival rate of *D. carinulata,* reared from 1st instar to adult, was tested using live plant specimens of *T. usneoides* and *T. ramosissima* (Figure 3.4). The *D. carinulata* larvae had a higher survival rate on *T. usneoides* in all three life stages measured, which suggests that *T. usneoides* is a more suitable host than *T. ramosissima* for *D. carinulata* (Figure 3.4). The percentage survival to the 2nd instar stage was higher on *T. usneoides* (95.71% ± 4.29%) than on *T. ramosissima* (72.02% ± 4.34%) and the difference was significant (Kruskal-Wallis test on ranks; $H_{1,8} = 4.288$; $p<0.05$). The percentage survival to the 3rd instar stage was higher on *T. usneoides* (81.82% ± 9.24%) than on *T. ramosissima* (60.42% ± 4.87%) however the difference was not significant (Kruskal-Wallis test on ranks; $H_{1,8} = 3.000$; $p>0.05$) as a result of a high variance in the *T. usneoides* dataset. The percentage survival to the adult stage was higher on *T. usneoides* (67.56% ± 6.98%) than on *T. ramosissima* (43.78% ± 7.81%) and the difference was significant (Kruskal-Wallis test on ranks; $H_{1.8} = 4.136$; $p<0.05$).

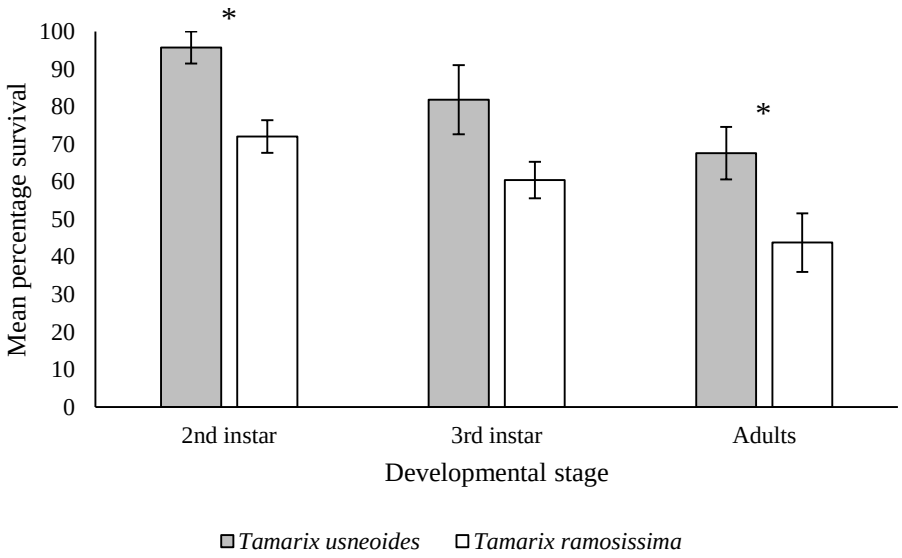


Figure 3.4: Mean percentage survival of *Diorhabda carinulata* through three developmental stages when reared on two different genotypes of *Tamarix*. Askerisks (*) indicate a significant difference between the mean percentage survival rate on the two genotypes used for each developmental stage (Kruskal-Wallis test on ranks; $p<0.05$). Absence of an asterisk indicates no significant difference between genotypes for that developmental stage. Error bars represent the standard error of the mean.

Adult *D. carinulata* beetles that survived from the 1st instar-to-adult rearing experiment were allowed to reproduce, and the number of eggs laid on the foliage in each test was counted (Table 3.1). The average number of eggs laid per female was used to estimate the fecundity of females reared on either genotype (Figure 3.5). The average number of eggs laid per female was higher on *T. usneoides* (57.66 ± 13.71) than it was on *T. ramosissima* (32.48 ± 22.35), however the difference was found not to be significant (Kruskal-Wallis test on ranks; $H_{1,9} = 1.500$; $p>0.05$).

Table 3.1: The number of eggs laid per female in a multi-generation egg-to-egg no-choice host specificity test. The number of eggs per female was approximated based on the total number of eggs laid on the foliage and the number of females which had emerged from rearing. on two different *Tamarix* genotypes.

	Tamarix usneoides					***Tamarix ramosissima***			
***Tamarix* accession**	GM169a	Tu 67	Tu 48	Tu 231	Tu 68	Tr 45	Tr via Isabel	Tr 171-16	Tr 43
No. Females	17	10	9	9	10	5	2	7	5
No. Eggs	435	924	248	543	824	55	199	63	52
Eggs Laid Per Female	25.59	92.4	27.56	60.33	82.4	11	99.5	9	10.4

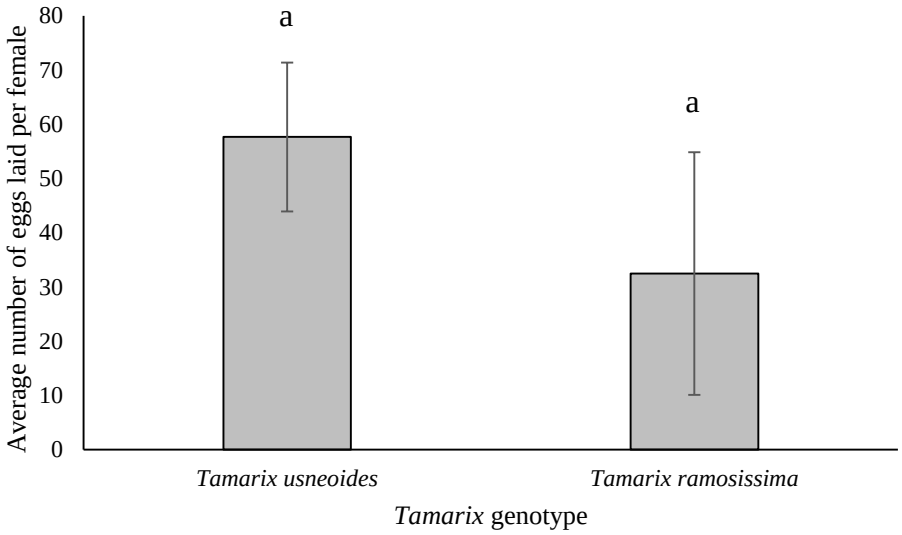


Figure 3.5: The average number of eggs laid by female *Diorhabda carinulata* beetles reared on two different genotypes of *Tamarix*, as a measure of fecundity; n = 6 for *T. usneoides* and n = 4 for *T. ramosissima*. There was no significant difference between the two groups (Kruskal-Wallis test on ranks; $H_{1,9} = 1.500$; $p>0.05$). Error bars represent the standard error of the mean.

The suitability of *T. usneoides* and *T. ramosissima* as hosts of *D. carinulata* was estimated using Maw's Host Suitability Index (Maw 1976). *Tamarix usneoides* yielded a higher percentage pupation and shorter development times than *Tamarix ramosissima* (Table 3.2). According to the index, the host suitability of *T. usneoides* was 2.33 times higher than

the host suitability of *T. ramosissima* (Table 3.2). Unfortunately the host suitability index for *T. chinensis* could not be calculated as the numbers of *D. carinulata* and *T. chinensis* available were not sufficient to test on this genotype.

Table 3.2: Host suitability of *Tamarix usneoides* and *T. ramosissima* for *Diorhabda carinulata* as characterised by the Maw's Host Suitability Index. The index is calculated as: (unfed adult mass * percentage pupation)/development time (Maw 1976). Relative suitability was calculated with the assumption that *T. ramosissima* represents the baseline suitability.

***Tamarix* genotype**	***T. usneoides***	***T. ramosissima***
Unfed adult mass (g)	0.009048	0.009048
% Pupation	66.41	42.86
Duration of Development (days)	22.75	34.25
Host suitability	0.02641	0.01132
Relative suitability (%)	233.3%	100%

Paired-Choice Experiments

Caged, paired-choice tests were conducted using live, potted plant specimens of a pair of *T. usneoides* and *T. ramosissima* (n = 4), and a pair of *T. usneoides* and *T. chinensis* (n = 4). The number of adults positioned on each plant was recorded thrice weekly, on Monday Wednesday and Friday. In the *T. usneoides* and *T. ramosissima* trials (Figure 3.6), the mean number of adults was higher on *T. ramosissima* (47.33 ± 5.71) than on *T. usneoides* (39.17 ± 1.70). However this difference was found not to be statistically significant (Friedman test; $\chi^2_{6,1} = 0.667$; P>0.05), and the value of the Coefficient of Concordance (0.11) indicated that beetles did not consistently select one plant over the other. In the *T. usneoides* and *T. chinensis* trials (Figure 3.7), the mean number of adults was higher on *T. usneoides* (65.83 ± 10.08) than on *T. chinensis* (31.67 ± 2.54). This difference was found to be statistically

significant (Friedman test; $\chi^2_{6,1} = 6.000$; $P<0.05$), and the Coefficient of Concordance (1.0) indicated that the beetles consistently preferred *T. usneoides* over *T. chinensis*.

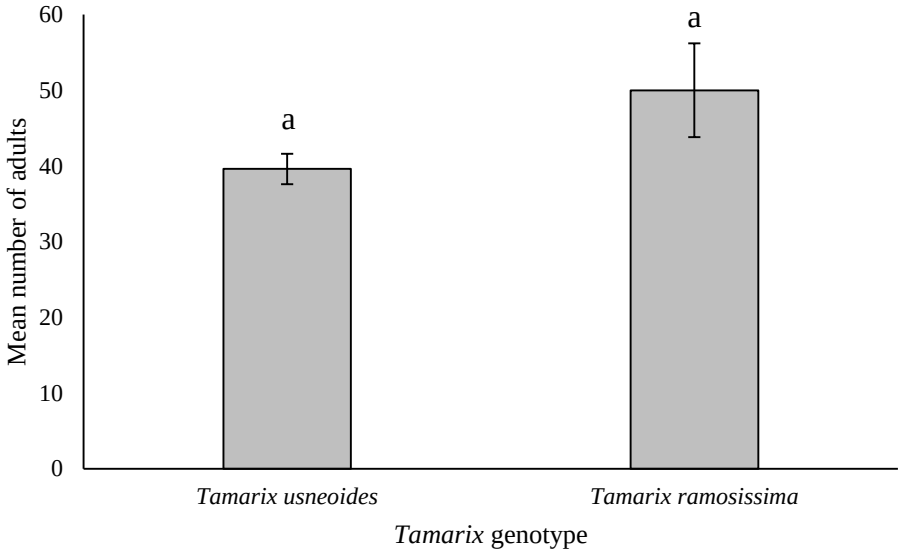


Figure 3.6: Mean number of adults recorded in a paired-choice test on live, caged *Tamarix usneoides* and *T. ramosissima* plants (n = 4). Number of adults was not significantly different between *T. usneoides* and *T. ramosissima* (Friedman test; $\chi^2_{6,1} = 0.667$; $P>0.05$). Error bars represent the standard error of the mean.

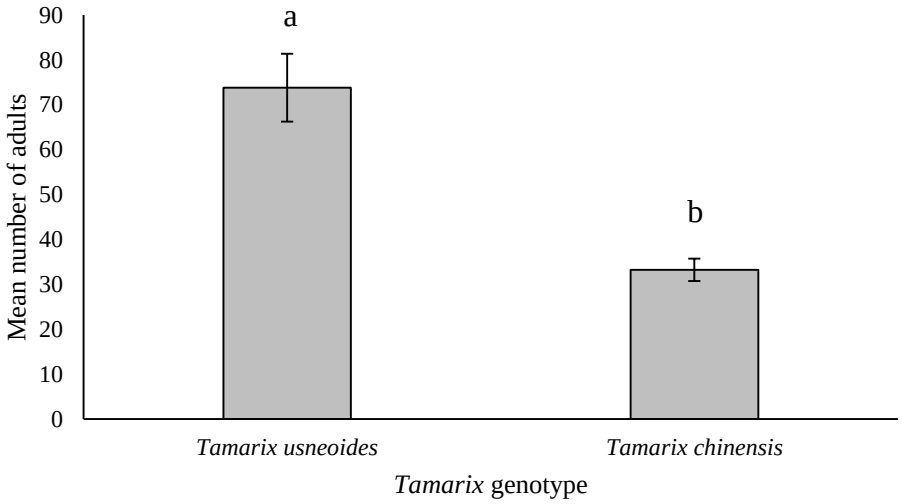


Figure 3.7: Mean number of adults recorded on in a paired-choice test live, caged *Tamarix usneoides* and *T. chinensis* plants (n = 4). Number of adults was significantly higher on *T. usneoides* than on *T. chinensis* (Friedman test; $\chi^2_{6,1} = 6.000$; $P<0.05$). Error bars represent the standard error of the mean.

In the caged paired-choice tests, the number of eggs oviposited by female *D. carinulata* beetles on each plant was also recorded. In the *T. usneoides* and *T. ramosissima* trials (Figure 3.8), the mean number of eggs was higher on *T. ramosissima* (97.33 ± 19.70) than on *T. usneoides* (54 ± 5.06). However this difference was found not to be statistically significant (Friedman test; $\chi^2_{6,1} = 2.667$; P>0.05), and the value of the Coefficient of Concordance (0.44) indicated that the beetles were moderately consistent in their selection of *T. ramosissima* for oviposition. In the *T. usneoides* and *T. chinensis* trials (Figure 3.9), the mean number of eggs was higher on *T. usneoides* (107.2 ± 17.06) than on *T. chinensis* (36.33 ± 7.33) This difference was found to be statistically significant (Friedman test; $\chi^2_{6,1} = 6.000$; P<0.05), and the value of the Coefficient of Concordance (1.0) indicated that *D. carinulata* consistently preferred to oviposit on *T. usneoides* rather than *T. chinensis*.

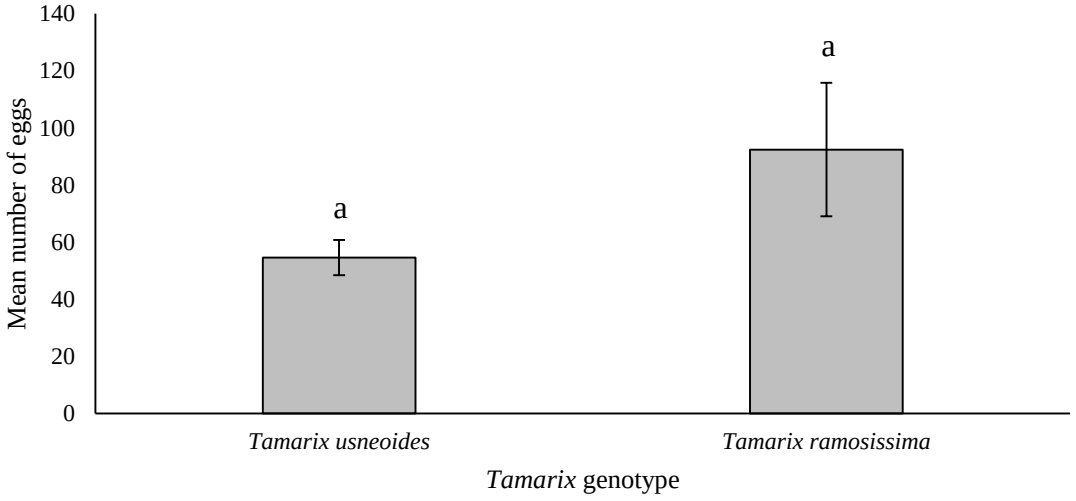


Figure 3.8: Mean number of eggs laid in a paired-choice test on live, caged *Tamarix usneoides* and *T. ramosissima* plants (n = 4). Number of eggs laid is not significantly different between *T. usneoides* and *T. ramosissima* (Friedman test; $\chi^2_{6,1} = 2.667$; P>0.05). Error bars represent the standard error of the mean.

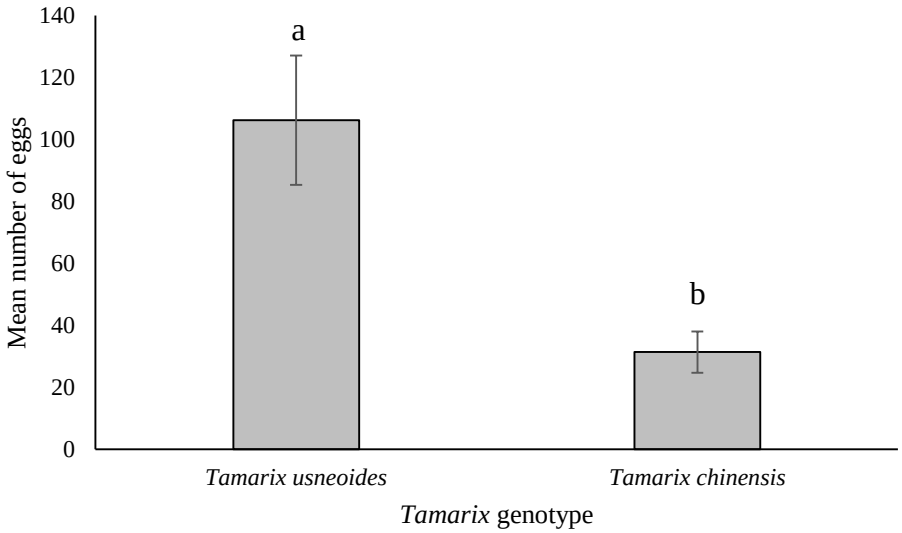


Figure 3.9: Mean number of eggs laid in a paired-choice test on live, caged *Tamarix usneoides* and *T. chinensis* plants (n = 4). Number of eggs laid is significantly higher on *T. usneoides* (Friedman test; $\chi^2_{6,1} = 6.000$; $P<0.05$). Error bars represent the standard error of the mean.

The overall pattern in the results indicates that *T. usneoides* is a more suitable host for *D. carinulata* than *T. ramosissima*, and that *D. carinulata* does not significantly prefer *T. ramosissima* or *T. usneoides* for feeding and oviposition. Additionally, *D. carinulata* preferentially utilises *T. chinensis* over *T. usneoides*.

Discussion

In laboratory no-choice tests, *D. carinulata* has repeatedly shown that it is able to successfully survive and develop on *T. usneoides* as well as on hybrids of *T. usneoides* with *T. ramosissima*, a native host of *D. carinulata*. All plants tested were of similar size and condition. This indicates that *D. carinulata* includes *T. usneoides* within its fundamental, or physiological host range (Cullen 1988). Therefore *T. usneoides* and *T. usneoides* x *T. ramosissima* are both suitable hosts for *D. carinulata*. The success of *D. carinulata* in completing its developmental cycle on *T. usneoides* and *T. usneoides* x *T. ramosissima* indicates that these genotypes provide the correct physical or chemical stimulus to induce

feeding, as well as providing sufficient nutritional content to the life stages of *D. carinulata*, thus allowing the beetle to complete a single life cycle (Zwölfer & Harris 1971).

The no-choice development tests have also shown that *D. carinulata* which have been reared from eggs to adults on *T. usneoides* are able to reproduce and lay eggs, and that the number of eggs laid per female is not significantly different for females reared on *T. usneoides* and on *T. ramosissima*. This indicates that *D. carinulata* would be able to establish on *T. usneoides* post-release (Zwölfer & Harris 1971), as they would be able to maintain stable population growth. Additionally, Maw's host suitability index (Table 3.2) indicated that *T. usneoides* is a superior host for *D. carinulata* as a result of a higher percentage pupation and faster generation time. The combination of a faster generation time and higher percentage pupation means that *D. carinulata* has a higher intrinsic rate of population increase, or rate of population increase per individual, on *T. usneoides* than on *T. ramosissima*. This indicates that, in the field, *D. carinulata* will be able to complete more generations per season on *T. usneoides*, possibly leading to more damage being inflicted on *T. usneoides* than on *T. ramosissima*.

The higher intrinsic rate of population increase of *D. carinulata* on *T. usneoides* relative to *T. ramosissima* is surprisingly different from the results obtained by Milbrath et al. (2006a), who found that the intrinsic rate of population increase for the tested *Diorhabda* species (*D. elongata sensu lato* at that time) was lower on *T. aphylla* than it was on *T. ramosissima* and *T. chinensis*. Work reported here shows the opposite of the expected result, which was that *T. usneoides* would produce similar results to *T. aphylla*. The lower success of the *Diorhabda* beetles on *T. aphylla*, as well as the fact that *T. aphylla* is nevertheless exotic to the USA and Mexico, were used to justify the release of *Diorhabda* beetles in the USA even though it was expected to have some non-target effects (DeLoach et al. 2003). In contrast, *D. carinulata* has shown greater success in the laboratory on *T. usneoides*, which at

the same time is a native species with economic value, making it nearly impossible to advocate for the release of *D. carinulata* as a biological control agent against *Tamarix* in South Africa.

Diorhabda carinulata has also shown that it will select *T. usneoides* as a host for settling and for oviposition in paired-choice caged host specificity tests with its native hosts *T. chinensis* and *T. ramosissima*. *Diorhabda carinulata* has even been shown to consistently prefer *T. usneoides* over *T. chinensis* for both alighting and oviposition. This finding is troubling when paired with the finding that *D. carinulata* is able to successfully complete its life cycle on *T. usneoides*. Thus *T. usneoides* is highly likely to fall within the ecological host range of *D. carinulata* (Cullen 1988).

Previous biological control agents which have shown limited non-target feeding effects in laboratory settings have sometimes been released citing the unlikelihood of the agents being able to establish on the non-target plant due to being unable to maintain a population. For example, the biocontrol effort against bugweed, *Solanum mauritianum* Scopoli (Solanaceae), faces similar problems to biocontrol against *Tamarix*, as there are native as well as economically important non-target plants in the genus *Solanum* in South Africa, however, biocontrol agents have been released against *S. mauritianum* in South Africa. For example, *Gargaphia decoris* Drake (Tingidae) displayed some non-target feeding and oviposition, but was nevertheless released based on the unsuitability of the non-target hosts to sustain a population of *G. decoris* under field conditions (Olckers 2000). On the other hand, the majority of potential agents against *S. mauritianum* have been rejected, for reasons of broad host ranges in laboratory choice and no-choice tests (Olckers 2011). Similarly, *D. carinulata* has displayed a broad host range in laboratory choice tests, coupled with the high host suitability of *T. usneoides*.

The utilisation of *T. usneoides* by *D. carinulata* represents a potential new association because the beetle has never encountered *T. usneoides* in its native habitat as the distribution of *Diorhabda* in Africa only extends as far south as Senegal (Tracy & Robbins 2009). The lack of any previous contact between *Diorhabda* and *T. usneoides*, as well as the lack of any *Diorhabda* beetles within the native range of *T. usneoides* initially made this genus attractive as a possible source for biological control agents. Beetles of the genus *Diorhabda* extend as far south as Senegal (Tracy & Robbins 2009), approximately 5 000 km from the northern limit of the distribution of *T. usneoides* in Namibia. The new association of *D. carinulata* with *T. usneoides* would not be the first of its kind, as *D. carinulata* has previously also shown a new association with *T. parviflora*, a tree species which occurs to the west of the native distribution of *D. carinulata* (Dudley et al. 2006).

A possible reason for the unexpected success of *D. carinulata* on *T. usneoides* is that the native hosts of *D. carinulata* may have evolved a form of defence, such as chemical antifeedants or toxins against the feeding damage caused by the leaf-feeding beetle (Ryan & Byrne 1988). Plants which have co-evolved with specialised insect herbivores often produce specialised chemical defences against those insects, while the insects simultaneously evolve to withstand the chemical defences produced by their host plant (Cates 1980). Often, these chemical defences are toxic to the insect herbivore, and can result in a lower population growth for the insect (Ryan & Byrne 1988). *Tamarix usneoides* has not encountered leaf-feeding by *D. carinulata* in its evolutionary history, due to geographical separation, and thus may not have evolved any specialised defence against the beetle. Therefore *D. carinulata* may be able to feed on the leaves of *T. usneoides* unimpeded by chemical defences.

Another possible explanation for the association of *D. carinulata* with *T. usneoides* is that *T. usneoides* may not truly be indigenous to Southern Africa. Given the large geographic distance between *T. usneoides* and its nearest relatives in Northern Africa (Hulten & Fries 1986), it remains possible that the species was introduced into Southern Africa by human dispersal.

Chapter 4 - Climatic Suitability of *Diorhabda carinulata* for South Africa

Introduction

Climate suitability modelling is an important tool to be used when considering a potential biological control agent for release, because in order to be successful, the agent needs be suited to the climate in the area in which it is to be introduced. During a collaborative, international biological control programme, a number of experts were surveyed as to what the primary constraints to agent effectiveness were in their biological control programmes (Moran 1984). The foremost reason given by the experts for the limited success of their biological control programmes was climatic incompatibility of the agent with the area of introduction, which was implicated in 44% of the unsuccessful projects (Moran 1984). Therefore, climatic compatibility of the agent is highly important to the success of a biological control programme, and climate modelling provides a means of predicting climatic suitability.

A common application of climate modelling in the context of biological control is the use of climate matching to determine areas of the native range of the weed in which the climate is similar to the areas where the weed has become invasive. This climate matching aids in the search for new potential agents, as it narrows down the areas in which potential agents, which are climatically suitable, may be found (Robertson et al. 2008; Senaratne et al. 2006). Climate models are also used as a means to predict areas in which an agent is likely to establish in relation to the weed against which it is to be released. For example, Coetzee et al. (2007) used the modelling programme CLIMEX to predict the potential range of the sap-sucking mirid *Eccritotarsus catariensis* (Carvalho) (Heteroptera: Miridae), used as a

biological control agent against water hyacinth, *Eichhornia crassipes* (Mart.) Solms (Pontederiaceae), an invasive aquatic weed.

The "Compare Locations" function of the CLIMEX software package is a climate model which makes use of physiological parameters of the study organism as a guide to start building the model, which is then validated and adjusted by comparing the output of the model to the native distribution of the organism (Sutherst et al. 1999; Sutherst 2003). The main activity undertaken when building a CLIMEX model is the matching of the known geographic distribution to the distribution produced by the model when the model parameters of the species are adjusted (Kriticos et al. 2005; Sutherst et al. 1999; Sutherst 2003). Therefore, both the physiology and geographical distribution of the organism under investigation need to be ascertained prior to building a CLIMEX model.

Herrera et al. (2005) found that the optimal temperature for the development of "*Diorhabda elongata*" was between 35°C and 39°C. The study by Herrera et al. (2005) was conducted before the taxonomic revision of the *Diorhabda* beetles released in the USA as biocontrol agents, and the species used is now referred to as *D. carinulata*. The lower and upper thresholds for the successful development of all immature stages were found to be 18°C and 40°C respectively. However, *Diorhabda* beetles are known to be able to survive a much broader range of temperatures in the field. The beetles are not continuously exposed to high temperatures in the field as they were in the experiments (Herrera et al. 2005). Additionally, *Diorhabda* beetles are known to be able to overwinter and survive through cold winters during which temperatures drop well below 0°C for extended periods of time, through the use of microclimates, for example insulation by leaf litter, as well as through diapause behaviour (Herrera et al. 2005; Lewis et al. 2003).

Diorhabda beetles enter reproductive diapause, followed by a period of inactivity for overwintering, which is triggered by the length of the day, or photoperiod, dropping below a

certain threshold (Bean et al. 2007; Lewis et al. 2003). This threshold is known as the critical day length (CDL). The CDL is, more specifically, the photoperiod at which 50% of the beetle population has entered diapause. Four species of *Diorhabda* have been released in the USA for biological control of *Tamarix* spp. (DeLoach et al. 2003; Tracy & Robbins 2009). For the *Diorhabda* species released in the USA, the threshold ranges are between 14 hours 14 minutes, and 15 hours 8 minutes depending on the ambient temperature (Bean et al. 2007); at higher temperatures the day length needs to be shorter in order to induce diapause. At latitudes south of 36°20' N in the USA, the beetles are only able to complete a single generation in the summer and struggle to establish because the photoperiod is shorter and beetles are induced to diapause too early, which leads to their metabolic reserves becoming depleted causing death before they can exit diapause (Bean et al. 2007).

Thus, the CDL would be a highly influential factor if the release of *D. carinulata* in South Africa were to proceed. *Diorhabda carinulata* beetles have more recently been shown to have evolved a shorter CDL within 10 generations in the field (Bean et al. 2012). A decrease in the CDL allows the beetles to remain reproductively active for longer in the season (Bean et al. 2007), which allows the agent to do more damage to the target weed, *Tamarix* spp. (Bean et al. 2012). The reduced CDL also allows *D. carinulata* to expand its range further south and establish in areas where previously the CDL occurred too early for the beetle to establish (Bean et al. 2012).

Methods

A CLIMEX Compare Locations model was created using DYMEX Version 4.0.2.0 (CSIRO). The "desert species" template was used as the basis for the parameters. The values used for parameters in the model were initially based on physiological data reported in Herrera et al. (2005). The model was set to require a CDL of 14.5 hours for diapause

induction (Bean et al. 2007). The model was then modified by adjusting the species parameters until the output distribution matched the latest known distribution of the beetles in the USA according to the Tamarisk Coalition (2015). A second model was created which removed the CDL requirement of the beetle in order to assess the climatic suitability of South Africa regardless of the length of summer days.

Table 4.1: Values used for the CLIMEX parameters of *Diorhabda carinulata* which created the best fit to its introduced range in the USA.

Parameter Description	Abbreviation	Value
Lower temperature threshold	DV0	-10
Lower optimal temperature	DV1	0
Upper optimal temperature	DV2	35
Upper temperature threshold	DV3	50
Degree days to complete a generation	PDD	325.5
Lower moisture threshold	SM0	0
Lower optimal moisture level	SM1	0.001
Upper optimal moisture level	SM2	0.2
Upper moisture threshold	SM3	0.3
Diapause induction daylength	DPD0	14.5
Diapause induction temperature	DPT0	1
Diapause termination temperature	DPT1	10
Diapause development days	DPD	90
Diapause summer or winter indicator	DPSW	0
Heat stress threshold	TTHS	40
Heat stress accumulation rate	THHS	0.001
Soil moisture wet stress	SMWS	0.3
Wet stress accumulation rate	HWS	0.1

Results

A CLIMEX Compare Locations model was built to predict the suitability of the South African climate to the introduction of *Diorhabda* beetles. The CLIMEX model that was built

to include day length induced diapause produced a map indicating that no area of South Africa would be suitable for *Diorhabda*.

However, the model which excluded day length induced diapause showed large areas of high climatic suitability, as indicated by the Ecoclimatic Index (Figure 4.1). The CLIMEX model predicted that *Diorhabda* would be able to establish mostly in the arid western part of the country. Areas of high suitability for *Diorhabda* also extended north into Namibia and Botswana (Figure 4.1).

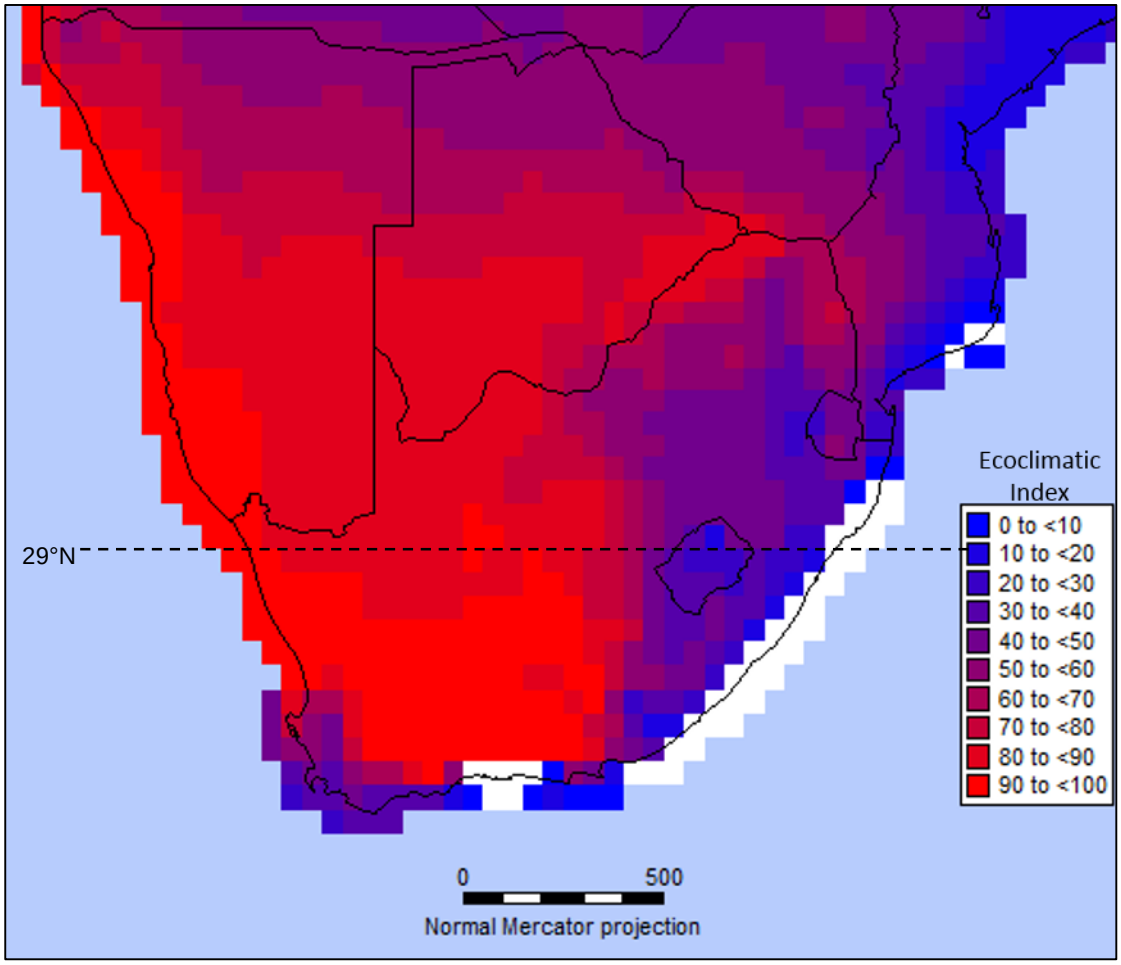


Figure 4.1: Results of a CLIMEX Compare Locations model for the potential distribution of *Diorhabda* spp. in South Africa. Ecoclimatic Index is an index of climatic suitability with 100 indicating a highly suitable area and 0 indicating an unsuitable area. The dotted line indicates the latitude 29°N, south of which *D. sublineata* from southern Texas, USA would be likely to establish if it were to be released in South Africa.

Discussion

According to the CLIMEX Compare Locations model constructed to include the daylength-induced diapause of *Diorhabda* spp., the beetles will not be able to establish in South Africa, as the day length at all latitudes remains below the CDL for most of the year. Thus the beetles will be prevented from completing even a single generation anywhere in the country (Bean et al. 2007; Lewis et al. 2003).

Diorhabda carinulata in the USA has been found to have evolved a shorter CDL, which results in an extended period of reproductive activity prior to entering diapause (Bean *et al.* 2012). If the CDL of *D. carinulata* could be reduced, then it would be able to establish in South Africa. One possibility is that lab breeding could be used to reduce the CDL of the *D. carinulata* population to be released. However, lab breeding for a reduced CDL is likely to take a very long time, and will use resources that might be better spent in scoping for and testing alternative agents for release against *Tamarix* in South Africa.

Although the range of *D. carinulata* has extended as far south as 34°N in the USA (Tamarisk Coalition 2015), a latitude equivalent to Cape Town, South Africa, the CLIMEX model predicted no suitability for *Diorhabda* spp. in Cape Town. The lack of suitability shown in Cape Town may be a result of the Mediterranean climate experienced in the region, with winter rainfall and dry summers. The photoperiod is not the only factor considered by the model in the establishment of *D. carinulata* in Cape Town, and the CLIMEX model reflects the other factors involved.

If the population to be released in South Africa originates from a low latitude, it will be more suited to South African day lengths (Dalin et al. 2010). Therefore it may be beneficial to collect *Diorhabda* beetles with a reduced CDL from a lower latitude, for example from southern Texas. *Diorhabda sublineata* has established at a latitude of approximately 29°N along the Rio Grande in southern Texas (DeLoach et al. 2012).

Therefore, hypothetically, *D. sublineata* may establish in South Africa, potentially everywhere south of 29°N (Figure 4.1). This potential range would include the entirety of the Eastern and Western Cape provinces, as well as a large portion of the Northern Cape province, which covers a large area of the range of both *T. usneoides* and the *Tamarix* spp. invasive in South Africa (Mayonde et al. 2016). Host specificity would need to be retested on the new species of *Diorhabda*, for example *D. sublineata*, to be released. The study reported here initially intended to include *D sublineata* as well as *D. carniata* and *D. elongata* for host specificity testing, however *D. carinulata* was the only species available for testing. The testing had already been prearranged to take place in Colorado and other *Diorhabda* species were not accessible as a result of the moratorium on transport of *Diorhabda* beetles across state borders.

The distribution map produced by the CLIMEX suitability model showing predicted areas of high suitability for *D carinulata* displays geographic overlap with the current known distribution of *T. usneoides* (Mayonde et al. 2016). The distribution of *T. usneoides* is mainly centred in the Northern Cape province of South Africa, which is in the arid western half of the country (Mayonde et al. 2016). The distribution of *T. usneoides* also extends northwards into Namibia (Mayonde et al. 2016). Thus there is a considerable risk that if *D. carinulata* or another species of *Diorhabda* were successfully introduced and established in South Africa, any establishment on the non-target *T. usneoides* would be supported by a high climate suitability. Additionally, there is a relative lack of the target *Tamarix* species in the Northern Cape province as compared to the abundance of *T. usneoides*, therefore increasing the chances of non-target effects There is also a risk that the agent would spread northwards to *T. usneoides* populations in Namibia, although this risk is mitigated by the fact that summer day length decreases with increasing proximity to the equator, thus *Diorhabda* populations would be less viable in the lower latitudes of Namibia.

As far as *D. carinulata* is concerned, this *Diorhabda* species is unlikely to establish in South Africa as a result of the limited number of daylight hours experienced at these latitudes. Future efforts should be focused on scoping and testing of new potential agents against *Tamarix* in South Africa, rather than attempting to decrease the CDL of *D. carinulata*.

Chapter 5 - Conclusion

Biological control is a method of controlling an invasive species using an agent which is a natural enemy of the target species. In classical weed biological control insect herbivores are often used. Weed biological control often has benefits over other control methods, such as chemical control or manual removal, in the form of cost and labour savings and longevity of the solution.

A study was undertaken to investigate the suitability of introducing *D. carinulata* into South Africa for biological control of invasive *Tamarix* spp. The genus *Tamarix* is a group of riparian plants which have become invasive in South Africa, the USA, and other regions such as Australia. The native range of *Tamarix* is completely restricted to the old world, with the majority of species occurring naturally throughout Europe and Asia. In regions where *Tamarix* has invaded, the tree grows in dense patches along river banks, extirpating native species and depleting the water supply (Brotherson & Field 1987; DiTomaso 1998; Shafroth et al. 1995). *Tamarix* species readily hybridise with one another in introduced ranges, while in their native ranges hybridisation is extremely scarce (Gaskin & Schaal 2002; Mayonde et al. 2016). In South Africa, two *Tamarix* species have become invasive, *T. ramosissima* and *T. chinensis*. These two species hybridise with one another as well as with the native *T. usneoides* (Mayonde et al. 2016; Mayonde et al. 2015). To date, a single chemical control programme has been implemented in an attempt to suppress the spread of *Tamarix* in South Africa, with the use of a broad-spectrum herbicide in the Namaqualand region of the Northern Cape province (D. Muir 2016 pers. comm). The application of herbicides to *Tamarix* infestations in the Namaqualand region is of concern to researchers at Wits University as *T. usneoides* is co-present with exotic *Tamarix* species in said region. Information regarding outcomes, impacts and public perception of the programme is not available at present.

When considering a new biocontrol agent for release it is important to consider four factors: the efficacy of the agent, the fundamental host range of the agent, the ecological host range of the agent, and whether the area of introduction is climatically suitable for the agent. The fundamental, or physiological host range of an agent is the full range of hosts which an agent can use to complete its life cycle (Cullen 1988). In the present study, fundamental host range has been tested using laboratory-based, no-choice host-specificity testing (Chapter 3). The ecological host range includes only hosts which an agent will behaviourally select to use when given a choice (Cullen 1988; Marohasy 1998). It is important to test ecological host range under field conditions as well as in the laboratory in order to reduce false negatives induced by any artificial range expansion that may have occurred in the laboratory (Briese 2005; Briese et al. 2002; Clement & Cristofaro 1995; Marohasy 1998). In the present study, ecological host range has been tested using choice-based host specificity testing both in the laboratory and in the field (Chapter 2; Chapter 3). Climatic suitability refers to the ability of an agent to survive, establish, and spread under the abiotic climatic conditions present in its area of introduction (Byrne et al. 2004). In the present study, climatic suitability of *D. carinulata* was predicted using a climate model (Chapter 4).

The outdoor field cage experiments, which offered the beetles a multiple-choice situation (Chapter 2) indicated that *D. carinulata* selects *T. usneoides* as well as hybrids of *T. usneoides* with *T. ramosissima* and *T. chinensis* as hosts when its natural host selection process is relatively unconstrained. The laboratory paired-choice experiments (Chapter 3) further indicated that *D. carinulata* will select *T. usneoides* as a host even when presented with a native host such as *T. ramosissima* or *T. chinensis*. The laboratory no-choice experiments (Chapter 3) showed that *T. usneoides* is able to effectively complete its life cycle on *T. usneoides*, as well as being able to successfully reproduce when reared on *T. usneoides*. Thus, combining the results of the outdoor field cage experiments (Chapter 2) as well as the

lab choice and no-choice experiments (Chapter 3) leads to the conclusion that *D. carinulata* includes *T. usneoides* in both its ecological and fundamental host ranges. It is therefore concluded that *D. carinulata* is not host specific to the target *Tamarix* species and native *T. usneoides* would be negatively impacted. Mayonde et al. (2016) found areas of overlap between *T. usneoides* and introduced species in the Northern Cape, Western Cape and Eastern Cape provinces of South Africa, and therefore there is the potential for transfer of *D. carinulata* on to *T. usneoides*.

Zachariades et al. (2002), rejected the leaf-feeding butterfly *Actinote thalia* (Lepidoptera: Nymphalidae: Acraeniae) as a biological control agent against *Chromolaena odorata* (L.) R.M. King and H. Robinson (Asteraceae: Eupatorieae), citing an unacceptably broad host range. The butterfly presented a similar problem as *D. carinulata* does in the present study, as two non-target native plants *Mikania capensis* DC. (Asteraceae: Eupatorieae) and *M. natalensis* DC. (Asteraceae: Eupatorieae) both were found to be superior hosts (107% and 110% respectively) for the butterfly as compared the target weed, *C. odorata*, using the Maw's host suitability index (Zachariades et al. 2002). In the present study (Table 3.2), *T. usneoides* was found to be a far superior host, with a 233.3% relative suitability over *T. ramosissima*. *Diorhabda carinulata* did not oviposit on *T. ramosissima* during the multichoice tests (Chapter 2), therefore this element could not be included in the calculation of relative suitability. However, the relative suitability is far higher in the present study than that found by Zachariades et al. (2002), therefore it should be considered sufficient evidence to reject the agent.

The climate suitability model produced for *D. elongata sensu lato* (Chapter 4) found that the agent would be unable to complete even a single generation in South Africa, as a result of the daylength, or photoperiod, being insufficiently long to prevent the beetles from

entering diapause. Therefore, *D. carinulata* would not be able to successfully establish and maintain a population in South Africa if it were to be released.

This study represents a three-tiered approach to host specificity testing: the open field tests, the laboratory choice and no-choice tests, and the climatic suitability matching. Although the results of all three approaches discouraged the potential for the introduction of *D. carinulata* as a biocontrol agent against *Tamarix* spp., the framework may be useful in other biocontrol programmes as it adds to the body of research on the testing of both fundamental and ecological host ranges. Therefore, it is recommended that *D. carinulata* be rejected as a biological control agent against invasive *Tamarix* spp. in South Africa, on the basis of an unacceptably broad host range as well as incompatibility with the South African climate.

Further research should seek a more host-specific and suitable biocontrol agent against *Tamarix*. Possible candidates include other *Diorhabda* species to be sourced from lower latitudes, either in the USA, or from their native range. A strong possible candidate is *D. sublineata* as it occupies low latitudes relative to the other *Diorhabda* species. *Diorhabda sublineata* was originally to be tested in the study presented here, however logistical and legal constraints made it inaccessible for this purpose. As the experiments had been arranged to be carried out in Colorado, *D. carinulata* was the only species readily available for testing, and testing on another species would have required relocation of the experimental setup, or a permit to transport *Diorhabda* across state borders.

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Appendices

Appendix A: *Tamarix* accessions (clones) used in an open-field host specificity test for *Diorhabda carinulata*. Different accession codes indicate different parent plants from which cuttings were cloned for experimental use. Plant # corresponds to the position of the plant in a 4 x 4 randomised Latin square array.

	Site A		Site B		Site B2	
Plant #	**Genotype**	**Accession Code**	**Genotype**	**Accession Code**	**Genotype**	**Accession Code**
1	*T. ramosissima* x *chinensis*	GM 069	*T. chinensis*	GM 136	*T. chinensis*	Tree 53
2	*T. chinensis*	GM 132	*T. ramosissima*	GM 068	*T. ramosissima*	Tree 45
3	*T. usneoides*	08-03-4	*T. usneoides*	09-03-5	*T. usneoides*	Tree 56
4	*T. ramosissima*	GM 068	*T. ramosissima* x *chinensis*	GM 150	*T. ramosissima* x *chinensis*	Miracle Waters 3
5	*T. usneoides*	08-03-4	*T. ramosissima* x *chinensis*	GM 150	*T. ramosissima* x *chinensis*	Miracle Waters 9
6	*T. ramosissima*	GM 068	*T. usneoides*	09-03-6	*T. usneoides*	Tree 68
7	*T. chinensis*	GM 136	*T. chinensis*	GM 136	*T. chinensis*	Tree 53
8	*T. ramosissima* x *chinensis*	GM 069	*T. ramosissima*	GM 068	*T. ramosissima*	Tr via Isabel W.
9	*T. ramosissima*	GM 068	*T. ramosissima*	GM 068	*T. ramosissima*	Tree 45
10	*T. usneoides*	08-03-4	*T. chinensis*	GM 136	*T. chinensis*	Tree 53
11	*T. ramosissima* x *chinensis*	GM 069	*T. ramosissima* x *chinensis*	GM 069	*T. ramosissima* x *chinensis*	Miracle Waters 3
12	*T. chinensis*	GM 136	*T. usneoides*	08-03-4	*T. usneoides*	Tree 48
13	*T. chinensis*	GM 132	*T. usneoides*	09-03-6	*T. usneoides*	Tree 68
14	*T. ramosissima* x *chinensis*	GM 069	*T. ramosissima* x *chinensis*	GM 069	*T. ramosissima* x *chinensis*	Miracle Waters 10
15	*T. ramosissima*	GM 068	*T. ramosissima*	GM 068	*T. ramosissima*	Tr via Isabel W.
16	*T. usneoides*	08-03-3	*T. chinensis*	GM 136	*T. chinensis*	Tree 53

Appendix B: *Tamarix* accessions (clones) used in an outdoor-caged host specificity test for *Diorhabda carinulata*. Different accession codes indicate different parent plants from which cuttings were cloned for experimental use. Plant # corresponds to the position of the plant in a 4 x 4 randomised Latin square array.

	Cage 1		Cage 2		Cage 3	
Plant #	**Genotype**	**Accession Code**	**Genotype**	**Accession Code**	**Genotype**	**Accession Code**
1	*T. usneoides*	08-03-3	*T. ramosissima* x *chinensis*	GM 069	*T. usneoides* x *ramosissima*	Eiland 16
2	*T. ramosissima* x *chinensis*	Salt Creek	*T. chinensis*	GM 132	*T. usneoides* x *chinensis*	GM 193-57
3	*T. ramosissima*	GM 068	*T. usneoides*	08-03-4	*T. ramosissima* x *chinensis*	Tree 61
4	*T. chinensis*	GM 136	*T. ramosissima*	GM 068	*T. usneoides*	GM 169a
5	*T. ramosissima* x *chinensis*	Whitewater	*T. usneoides*	08-03-4	*T. ramosissima* x *chinensis*	Miracle Waters 3
6	*T. chinensis*	GM 136	*T. ramosissima*	GM 068	*T. usneoides*	Tree 56
7	*T. usneoides*	08-03-4	*T. chinensis*	GM 136	*T. usneoides* x *chinensis*	Tree 57
8	*T. ramosissima*	GM 068	*T. ramosissima* x *chinensis*	GM 069	*T. usneoides* x *ramosissima*	LNR 14
9	*T. chinensis*	GM 132	*T. ramosissima*	GM 068	*T. usneoides*	Tree 68
10	*T. ramosissima*	GM 068	*T. usneoides*	08-03-4	*T. ramosissima* x *chinensis*	Miracle Waters 9
11	*T. ramosissima* x *chinensis*	Salt Creek	*T. ramosissima* x *chinensis*	GM 069	*T. usneoides* x *ramosissima*	LNR 14
12	*T. usneoides*	09-03-5	*T. chinensis*	GM 136	*T. usneoides* x *chinensis*	GM 193-57
13	*T. ramosissima*	GM 068	*T. chinensis*	GM 132	*T. usneoides* x *chinensis*	Tree 57
14	*T. usneoides*	08-03-4	*T. ramosissima* x *chinensis*	GM 069	*T. usneoides* x *ramosissima*	Eiland 16
15	*T. chinensis*	GM 136	*T. ramosissima*	GM 068	*T. usneoides*	Tree 56
16	*T. ramosissima* x *chinensis*	Horsethief	*T. usneoides*	08-03-3	*T. ramosissima* x *chinensis*	Miracle Waters 10

Appendix C: Genotypes and accessions (clones) of *Tamarix* used in no-choice host specificity tests of *Diorhabda carinulata* beetles on *Tamarix* spp. Tests were conducted either at the Palisade Insectary, Colorado, USA, or at the Wits Insectary, Johannesburg, South Africa (SA). Generation P1 refers to field-collected beetles, whereas F generations are lab-reared.

No-choice experiment	***Tamarix* genotype; accession used**	***D. carinulata* generation**
(a) 3rd instar to adult emergence (live plants, USA)	*T. usneoides*; 09-03-1	P1
	T. ramosissima x *chinensis*; Horsethief	P1
(b) Larval development, egg to adult (bouquets, South Africa)	*T. ramosissima*; GM068	F3
	T. ramosissima; GM171-16	F3
	T. ramosissima; Tr via Isabel	F3
	T. ramosissima; Tree # 45	F3
	T. usneoides x *ramosissima*; DWAF	F3
	T. usneoides x *ramosissima*; LNR 14	F3
	T. usneoides x *ramosissima*; Eil 16	F3
	T. usneoides x *ramosissima*; LNR 7	F3
	T. usneoides; Tree 48	F4
	T. usneoides; Tree 56	F4
	T. usneoides; Tree 68	F4
	T. usneoides; Tree 67	F4
	T. usneoides; GM 169a	F4
(c) Multi-generational development, egg to egg stage (live plants, USA and South Africa)	*T. usneoides*; Tree 68	P1
	T. usneoides; Tree 56	P1
	T. usneoides; GM 169a	F1
	T. usneoides; Tree 67	F1
	T. usneoides; Tree 48	F1
	T. usneoides; Tree GM231	F2
	T. ramosissima; Tree 45	F1
	T. ramosissima; Tr via Isabel W.	F1
	T. ramosissima; GM171-16	F1
	T. ramosissima; Tree 43	F2

Appendix D: Genotypes and accessions (clones) of Tamarix used in paired-choice host specificity tests of Diorhabda carinulata beetles on live, potted specimens of Tamarix spp. All beetles used were from lab reared generations.

Choice experiment	***Tamarix* genotypes; accessions used**		***D. carinulata* generation**
Oviposition in cages, adult to egg (live plants)	*T. usneoides*; Tree 48	*T. ramosissima*; Tree 45	F2
	T. usneoides; Tree 56	*T. ramosissima*; GM 171-16	F2
	T. usneoides; GM 095	*T. ramosissima*; GM 171-16	F2
	T. usneoides; GM 113	*T. ramosissima*; Tree 43	F3
	T. usneoides; GM 111	*T. chinensis*; Tree 53	F2
	T. usneoides; GM 231	*T. chinensis*; Tree 18	F2
	T. usneoides; GM 052	*T. chinensis*; GM 136	F3
	T. usneoides; GM 087	*T. chinensis*; GM 132	F3