Biology and host preference of *Selitrichodes neseri:* a potential biological control agent of the Eucalyptus gall wasp, *Leptocybe invasa*

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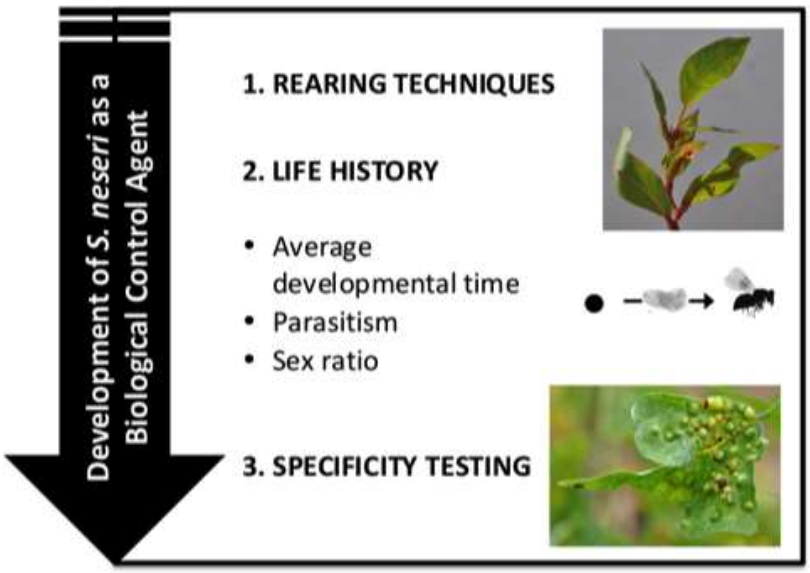
Highlights

- *Selitrichodes neseri* is a parasitoid of the Eucalyptus gall-wasp *Leptocybe invasa* described in 2012
- *S. neseri* has the potential to be a biological control agent for *L. invasa* given its life history characteristics
- *S. neseri* is shown to be specific to *L. invasa*
- Laboratory populations have shown parasitism levels of 70%
- Protocols are established here to rear *S. neseri*

Graphical abstract



ABSTRACT

Selitrichodes neseri (Hymenoptera: Eulophidae) is a parasitoid of the invasive gall-forming wasp *Leptocybe invasa* (Hymenoptera: Eulophidae), which has caused serious damage to *Eucalyptus* plantations in many parts of the world. *Selitrichodes neseri* is a recently discovered parasitoid considered to be a potentially important biological control agent of *L. invasa*. The aim of this study

was to provide the first basic data on the biology of *S. neseri*, which is essential for its application in biological control. *Selitrichodes neseri* was shown to be a biparental ectoparasitoid. Observation from dissected galls indicated that the parasitoid developed on late larvae, pupae and callow adults, although development did occur in a range of gall ages. Observed nominal parasitism in captivity ranged from 9.7 – 71.8 %. Adult *S. neseri* specimens, fed with honey-water and galled *Eucalyptus* leaves, survived an average of 26 days at 26°C. The average developmental time from oviposition to emergence was 19.3 days +/- 0.2 days. There was no pre-oviposition period. A single female produced a maximum of thirty-nine offspring, with a maximum of ten per day. Dissection of the ovaries showed that twelve ovarioles were present. The sex ratio of *S. neseri* observed in this study was 1: 3.43 males: females. Galls of native insects most closely related to *L. invasa* and to galls of similar morphology to *L. invasa*-induced galls, were not suitable for *S. neseri* oviposition. *Selitrichodes neseri* showed considerable potential as a biological control agent of *L. invasa* due to its relatively short developmental time, long adult life span when supplemented with carbohydrates, ability to utilize a range of gall ages and the fact that it has a high level of host specificity.

Keywords: host specificity; adult longevity; ectoparasitoid; parasitism rate; developmental time; *Eucalyptus*; galls

1. INTRODUCTION

Increasing global travel and trade is leading to a significant growth in the numbers of invasive organisms reaching new environments (Meyerson & Mooney, 2007). Specifically in plantation forestry, this represents a very serious threat to the sustainability of plantations that have been established with non–native species in various parts of the world (Wingfield *et al*., 2008, 2012). In this regard, the gall-wasp *Leptocybe invasa* Fisher & La Salle (Hymenoptera: Eulophidae), originating from Australia (Mendel *et al*., 2004) (Figure 1 A- D), is a recent example of such an invasion.

Leptocybe invasa is a threat in areas where susceptible *Eucalyptus* species and genotypes are planted commercially. Its galls affect the young growth of a variety of *Eucalyptus* species, potentially

Figure 1 A. L. invasa galls on Eucalyptus petioles, B., C., D. L. invasa larva, pupa and adult, E. Q. gallicola galls on Erythrina lysistemon, F., G. Detailed experiment of the S. neseri biology, H. S. neseri larva with an arrow indicating the mandibles I. S. neseri larva, J. S. neseri larva feeding on an L. invasa larva, K. S. neseri larva with meconium, L. The ovarioles of S. neseri, M. S. neseri female commencing oviposition, N. S. neseri female, O. S. neseri male

leading to tree deformation and stunted growth (Nyeko, 2005). In its native environment, populations of *L. invasa* remain low, at least in part due to natural enemies. It was only after the invasion of *L. invasa* into the Mediterranean region and the Middle East in 2000 that the species attracted attention, leading to the description and study of this previously unknown insect (Mendel *et al*., 2004). Thereafter its presence was noticed in Kenya (Mutitu, 2003), Uganda (Nyeko, 2005), South Africa (Neser *et al.*, 2007), Zimbabwe (Ministry of Environment & Natural Resources Management, December 2010) and Mozambique (Tree Protection News, December 2010). Worldwide, *L. invasa* has spread rapidly affecting commercial forestry plantations as well as small-scale farmers in Africa (Nyeko *et al*., 2009), South-East Asia (Thu *et al*., 2009), the Middle East (Mendel *et al*., 2004), Mediterranean region (Protasov *et al*., 2008), India (Kumari *et al*., 2010), China (Thu *et al*., 2009) and South America (Wilcken *et al*., 2010), becoming an important pest and serious threat in those regions.

Various management tactics have been explored to control *L. invasa*. Chemical control has been tested with varying success (Nyeko *et al*., 2007; Basavana Goud *et al*., 2010; Kulkarni 2010a, Jhala *et al*., 2010; Javaregowda *et al*., 2010). But its high costs and likely negative effect on other biological control agents, suggests that it is unlikely to be a feasible option at a large plantation scale. More feasible options include breeding and selection of resistant planting stock (Dittrich-Schröder *et al*., 2012; Nyeko *et al*., 2010) and/or biological control (Kim *et al*., 2008). Although there are risks associated with biological control (Babendreier, 2007; Barratt *et al*., 2010), it is generally considered an attractive alternative to other control methods due to its ecological and economic benefits (De Clerq *et al*., 2011).

Kim *et al*., (2008) described two Australian parasitoids of *L. invasa*, *Quadrastichus mendeli* Kim & La Salle (Hymenoptera: Eulophidae) and *Selitrichodes kryceri* Kim & La Salle (Hymenoptera: Eulophidae). These hymenopterans are both ectoparasitoids and have been successfully used in Israel to control *L. invasa* populations (Kim *et al.,* 2008). *Selitrichodes kryceri* is a biparental species whereas *Q. mendeli* is uniparental (Kim *et al.,* 2008). Both parasitoids were collected in Queensland, Australia. Under laboratory conditions parasitism rates for *S. kryceri* and *Q. mendeli* ranged from 3.2 – 67.4 % and 7.9 – 95.6 %, respectively (Kim *et al.,* 2008). A *Megastigmus* species (Hymenoptera:

Torymidae) from Australia, two *Megastigmus* species native to Israel and Turkey, and a species native to India have also been recorded (Doğanlar & Hassan, 2010; Protasov *et al*., 2008; Kulkarni *et al* 2010b). Neither the *Megastigmus* species from Israel nor from Turkey were successful parasitoids of *L. invasa* with the recorded parasitism rate observed being as low as 5.0 – 34.4 % (Protasov *et al*., 2008). Protasov *et al*. (2008) suggest that *L. invasa* may not be a preferred host for these *Megastigmus* species, explaining the low parasitism rate.

Initial efforts to establish some of these insects (*Quadrastichus mendeli* and *Selitrichodes kryceri*) in quarantine facilities in South Africa and Kenya have failed (Eston Mutitu, personal communication), and cultures of a *Megastigmus zvimendeli* obtained from Israel persisted but did not flourish in captivity, requiring alternative biological candidates to be sought. An unidentified *Megastigmus* species, close to *M. zebrinus* (O. Neser and G. L. Prinsloo, personal communication), possibly indigenous, has been reared from galls of *L. invasa* in parts of South Africa. This insect is widespread but its exact role as a parasitoid or inquiline is still unknown.

The discovery of a previously unknown parasitoid of *L. invasa*, has prompted research to assess the suitability of this insect as a biological control agent. This parasitoid wasp emerged with *Leptocybe invasa* (Hymenoptera: Eulophidae) from galls, collected on *Eucalyptus* spp. saplings in Queensland, Australia (S. Neser, unpublished). Its ease of laboratory rearing made this wasp an attractive biological control candidate for *L. invasa*. The aim of this study was thus to describe the biology of *S. neseri* and investigate its host preference in order to assess its suitability as a biological control agent against *L. invasa*.

2. MATERIALS AND METHODS

2.1 Collection of S. neseri *and original observations*

Unidentified galls on twigs, petioles and leaves were collected from *Eucalyptus* spp. saplings in Nanango, Queensland, Australia in April 2010 (S. Neser, unpublished). Ten specimens of a eulophid,

later described as *Selitrichodes neseri* Kelly & La Salle (Kelly *et al*., 2012), *Leptocybe invasa*, unidentified *Megastigmus* sp. and cecidomyiid specimens were reared from the galls. Subsequent observations showed that the unidentified eulophid laid eggs in *L. invasa* galls presented to them and could thus potentially be used as a biological control agent, and this lead to its taxonomic description (Kelly *et al*., 2012).

2.2 Plants infested by L. invasa

In order to study the biology of *S. neseri*, *Eucalyptus* plants with *L. invasa*-induced galls were required. The *Eucalyptus* clone GC540 (*Eucalyptus grandis x Eucalyptus camaldulensis* hybrid) was used as it is known to be very susceptible to *L. invasa*. Plants were grown in plastic pots (height 30cm, diameter 25cm), pruned to allow increased growth of young leaves and watered daily. Plants were maintained in the Forestry and Agricultural Biotechnology Institute (FABI) nursery at the University of Pretoria's Experimental Farm under shade netting (summer: daytime temperature range: 17.5°C – 28.5°C) (South African Weather Service, 26.10. 2010 – 28.02.2011). Plants displayed active growth throughout this period. Since 2008, *L. invasa* has been present in the FABI nursery allowing natural infestations of the GC540 plants by the gall wasp. Natural occurrence of *L. invasa* ensured that every plant had a range of gall ages. Controlled infestation and induction of galls by *L. invasa* was attempted by placing potted plants in cages (55 cm x 56 cm x 115 cm) and exposing them to different numbers of *L. invasa*, but this proved not to be successful.

2.3 Rearing of S. neseri *in quarantine*

All work with *S. neseri* was conducted in the FABI certified quarantine facility at the University of Pretoria's Experimental Farm. *Selitrichodes neseri* males and females were exposed to *L. invasa*-galled GC540 plants by enclosing the galled leaves in a gauze sleeve, releasing the *S. neseri* specimens and subsequently sealing the upper and lower parts of the sleeve. Sleeves remained on the plants for 14 days after which branches were picked and placed into labeled, unventilated plastic containers (ADDIS Flavour-tight seal Addisware Cake Saver (9.5l)). Paper towel, replaced daily, was placed in the containers (alternating between a layer of paper towel and a layer of leaves) to absorb

excess moisture. Daily emergences were recorded. The plants in the quarantine facility were watered every second day and macronutrients (Obaro (2:3:2 (22) and another formulation with micronutrients (Multifeed Classic (19:8:16 (43)) were applied every 2 months. Room temperature was 25.8°C +/- 0.03, relative humidity was 48% +/- 0.35 with a 12:12 light: dark cycle. In an effort to provide better light intensity and quality, plants were grown alongside light banks containing four plant growth tubes and eight fluorescent tubes and augmented by natural light via roof skylights in all rooms.

2.4 Characterisation of behaviour

Initial observations showed that *S. neseri* parasitized *L. invasa* but this needed to be confirmed. Furthermore, the possibility of *S. neseri* being an inquiline and gall-former needed to be excluded, especially since another species of *Selitrichodes*, *S. globulus* had been reported to be a primary gall former of *Eucalyptus globulus* in the USA (La Salle *et al.*, 2009). To determine whether *S. neseri* is a gall-former, four GC540 plants without *L. invasa* galls were exposed to *S. neseri* adults until all the *S. neseri* adults had died. Concurrently, *L. invasa* galls exposed to *S. neseri* for the duration of the *S. neseri* lifespan were dissected to observe the feeding behaviour of *S. neseri* larvae. It was thus possible to determine whether the *S. neseri* develops inside the *L. invasa*, alongside the *L. invasa*, feeds on the *L. invasa* or on gall tissue and to determine its role as a parasitoid, gall-former or inquiline.

2.5 Gall age preference and nominal parasitism rate

In order to produce plants with galls of known age (recorded as days of development under the conditions used), GC540 plants were pruned and placed in sealed, netted, walk-in cages (2.5 x 3m) outside, free of *L. invasa*, to allow development of uninfested plants. Approximately one month after pruning, new growth was present and the young leaves were of a suitable size (approximately 3 – 5 mm) for oviposition by the naturally occurring *L. invasa*. Subsequently, plants were placed in the nursery (spring- summer; October 2010 to February 2011) to allow oviposition by *L. invasa*. After 2-3 days, the plants were returned to the *L. invasa*-free cage. Individual plants were examined before returning them to the cage to ensure that *L. invasa* adults had not been accidentally introduced into the cage. The date of exposure of plants to *L. invasa* was recorded for each plant. Infested plants were

subsequently kept in sealed, netted cages until the *L. invasa*-induced galls were taken to be of the required age for exposure to *S. neseri*. After exposure to the parasitoid, the plants were taken into the quarantine facility for testing. The number of days from oviposition until emergence of the parasitoids was then recorded.

Seven categories of gall age, intended to correspond with a range of *L. invasa* from young larvae to pupae based on reported duration of development by Mendel *et al.,* (2004), were exposed to *S. neseri* specimens to determine a preferred age of *L. invasa* for development. These categories included (i) 30 days (n = 12), (ii) 53-57 days (n = 6), (iii) 59-66 days (n = 3),(iv) 68-74 days (n = 6), (v) 90 days (n = 6),(vi) 100 days (n = 6) and (vii) 110 days (n = 3), with *n* referring to the number of replicates per various age of *L. invasa* galls. Three female and two male *S. neseri* specimens were placed in a sleeve on these plants. Leaves were picked after 14 days, placed in plastic containers, *S. neseri* emergences monitored, and the sex of the offspring and the length of their developmental time recorded daily. Additionally, the numbers of *L. invasa* emerging from the galls were recorded. The nominal rate of parasitism for each plant/sleeve was calculated following the technique described by Kim *et al.* (2008), as the number of parasitoids emerged divided by the sum of the total number of emerged gall-formers and the total number of emerged parasitoids. The Kolmogorov-Smirnov test was used to test for normality among the treatments. A one-way ANOVA was used to test for significance between the percentage mortality/nominal parasitism of the seven categories of *L. invasa* galls.

2.6 Adult longevity

To determine the longevity of *S. neseri* adults, one female and one male were placed together in a ventilated glass vial (10 cm x 2cm) with the following treatments: (i) honey-water and a galled *Eucalyptus* leaf, (ii) honey-water and an ungalled *Eucalyptus* leaf, (iii) water and a galled *Eucalyptus* leaf, (iv) water and an ungalled *Eucalyptus* leaf, (v) galled *Eucalyptus* leaf, and (vi) ungalled *Eucalyptus* leaf. Treatments with or without galls were included to allow for the possibility of host-feeding by the females. Each treatment included 10 replicates. Honey-water and water were renewed daily whereas the galled and ungalled leaves were renewed every second day. Mortality of the male

and female specimens was recorded daily. A Kolmogorov test was used to test for normality, but despite transformation attempts, the data were found not to be normally distributed. Thus, the non-parametric, Kruskal-Wallis test was used to test for differences in longevity between treatments, followed by a series of post-hoc Mann-Whitney tests to determine the direction of the differences. However, results from the ANOVA are reported as these results were the same as the non-parametric tests but included all the treatments in the same analysis.

2.7 Developmental time, pre-oviposition period, number of offspring, sex ratio and potential fecundity

A single, newly emerged female and a single male *S. neseri* were exposed to an *L. invasa*-galled *Eucalyptus* branch (approximately 10-15 cm in length) and transferred to a new branch every 24 hours. Honey paper was placed on a part of the branch as a food source. The longevity of females was recorded and trials continued until 10 females had survived for 20 days. In total the oviposition pattern of 278 females was assessed. When the *S. neseri* male specimen died he was replaced with another. The survival day (e.g. day 7) of the female *S. neseri* specimen and the date the branch was to be removed (12 days after oviposition by *S. neseri* female) was recorded on a label per branch (Figure 1F). The removed branches were individually placed in a glass Petri dish (15 cm diameter and some with a 9cm diameter) lined with 2 pieces of paper towel and sealed with *PARAFILM "M"*Laboratory film (Pechiney Plastic Packaging) (Figure 1 G). Petri dishes were monitored daily until day 30 for emergence of offspring. The paper towel was renewed and Petri dishes wiped dry as required (every two to seven days). Data recorded per emerging *S. neseri* included (i) sex (ii) duration of development in gall (egg to adult emergence), (iii) oviposition day (i.e. the day in the female's life cycle when oviposition of these offspring occurred) and (iv) female longevity. Additionally, dissection of the ovaries of female *S. neseri* specimens (n = 30) was conducted to determine the number of ovarioles per female, as well as to determine whether female *S. neseri* specimens were pro-ovigenic or synovigenic.

2.8 Host specificity testing

The suitability of hosts for *S. neseri* was tested by exposing the parasitoid to the most likely possible non-target hosts present in South Africa. The possible non-target hosts tested were gall-forming arthropods that were phylogenetically similar to *L. invasa* (i.e. tetrastichine eulophids) and / or showed similar gall morphology (i.e. smooth and rounded, resembling those of *L. invasa*). The Chalcidoid database of the Agricultural Research Council – Plant Protection Research Institute (ARC-PPRI) (Anonymous, 2011) was consulted for recorded local gall-formers. The possibility that useful gall-formers, deliberately introduced in biocontrol programmes against alien invasive plants, could be affected was considered by the inclusion of such species (e.g. *Trichilogaster acaciae-longifoliae* and *Coelocephalapion camarae*). A total of 17 gall formers were selected and tested (Table 1). Of these, the *Erythrina* gall-former *Quadrastichus gallicola* Prinsloo & Kelly (Hymenoptera: Eulophidae) (Prinsloo & Kelly, 2009)(Figure 1E) was regarded as the most likely possible non-target host of *S. neseri*, because of the similarity of its galls to those of *L. invasa* (both being smooth and occurring on leaves, petioles and twigs) as well as its relatedness to *L. invasa* (both being gall-forming tetrastichine eulophids).

Various experiments were used to assess the suitability of *Q. gallicola* as a host for *S. neseri*. Firstly, varying numbers of male and female specimens of *S. neseri* (range = 1 male, 1 female to 10 males, 10 females) were placed in sleeves on eleven individual potted young *Erythrina lysistemon* plants, galled by *Q. gallicola* (and having galls of different developmental ages). The sleeves remained on the plants for approximately 18 days after which leaves were picked and placed in plastic cake savers. All emerging hymenopterans were examined to determine whether any *S. neseri* had emerged from the galls. Secondly, to accommodate the possibility that there was a pre-oviposition period an experiment was designed where *S. neseri* were moved between its known host (*L. invasa* on *Eucalyptus* (GC540)) and the possible non-target host (*Q. gallicola* on *Erythrina*). This experiment was conducted using a 3-plant sequence (*Eucalyptus* – *Erythrina* – *Eucalyptus*) and a 4-plant sequence (*Eucalyptus* – *Erythrina* – *Eucalyptus* - *Erythrina*) (Supplementary material - Table A). In the 3-plant sequence, female and male *S. neseri* specimens were exposed to an *L. invasa*-galled GC540 plant for

Table 1. Gall insect species tested, their respective host plants and the outcome of exposure to *Selitrichodes neseri*

Gall Formers		Other parasitoids (all Hymenoptera) that emerged from the galls	Host Plant	Interest in galls by *S. neseri*	*S. neseri* offspring
Order	**Species**				
Acari	*Aceria camdebooi (Eriophyiidae)*	*Tamarixia sp*. (Eulophidae)	*Celtis africana* (Celtidaceae)	None	None
	Aceria rhusi (Eriophyiidae)	None	*Searsia lancea* (Anacardiaceae)	None	None
Hemiptera	*Cissococcus fulleri* (Coccidae)	None	*Cissus rotundifolia* (Vitaceae)	None	None
	Pemphigus populitransversus (Aphididae)	None	*Populus deltoides* (Salicaceae)	None	None
	Pseudophacopteron electum (Psyllidae)	None	*Ekebergia capensis* (Meliaceae)	None	None
	Trichochermes insleyae (Psyllidae)	*Psyllaephagus* sp. (Encyrtidae)	*Ziziphus mucronata* (Rhamnaceae)	Some probing	None
Coleoptera	*Coelocephalapion camarae* (Brentidae)	None	*Lantana camara* (Lamiaceae)	None	None
	Unidentified Ceutorhynchinae (Curculionidae)	cf. *Meruana* sp. (Eulophidae)	*Portulaca oleracea (Portulaceae)*	None	None
Diptera	*Dasineura dielsi* (Cecidomyiidae)	Two *Aprostocetus* spp. (Eulophidae)	*Acacia cyclops* (Fabaceae)	None	None
		Eupelmus sp. (Eupelmidae)			
		Spp. indet (Torymidae)			
		Mesopolobus sp. (Pteromalidae)			
	Dasineura strobila (Cecidomyiidae)	*Torymoides* sp. (Torymidae)	*Leptospermum laevigatum* (Myrtaceae)	None	None
		Pseudotorymus sp. (Torymidae)			

	cf. *Lopesia sp.* (Cecidomyiidae)	*Systasis* sp. (Pteromalidae)	*Acacia caffra* (Fabaceae)	None	None
	Procecidochares utilis (Tephritidae)	*Torymoides* sp.(Torymidae)	*Ageratina adenophora* (Asteraceae)	None	None
		Pteromalus sp. (Pteromalidae)			
	Unidentified gall-midge (Cecidomyiidae)	*Aprostocetus* sp. (Eulophidae)	*Centella triloba* (Apiaceae)	None	None
	Zeuxidiplosis giardi (Cecidomyiidae)	*Neanastatus* sp. (Eupelmidae)	*Hypericum perforatum* (Hypericaceae)	None	None
Hymenoptera	*Quadrastichus gallicola* (Eulophidae)	None	*Erythrina lysistemon* (Fabaceae)	None	None
	Trichilogaster acaciae-longifoliae (Pteromalidae)	None	*Acacia longifolia* (Fabaceae)	None	None
	Unsure which of the emerged parasitoids is the gall former.	*Aprostocetus* spp. (Eulophidae)	*Syzygium cordatum* (Myrtaceae)	None	None
		Anselmellini genus indet (Eulophidae)			
		Megastigmus zebrinus (Torymidae)			
		Torymus sp. (Torymidae)			
		Genus indet (Encyrtidae)			

24 hours, then recaptured and released onto a *Q. gallicola*-galled *E. lysistemon* plant for a further 24 hours, and then recaptured and placed onto a *L. invasa*-galled GC540 plant. In the 4-plant sequence, the *S. neseri* individuals were recaptured from the second GC540 plant after 24 hours and placed onto another *Q. gallicola*-galled *E. lysistemon* plant. For both the 3-plant and 4-plant sequence, the *S. neseri* were left on the last plant until they had died. In all cases the plant and the *S. neseri* were enclosed in a sleeve. The sleeve remained on the last plant until the galled leaves were picked, approximately 14 days after exposure of the *L. invasa* to the last galled *E. lysistemon* plant. Picked leaves were placed in plastic containers and examined for the emergence of any *S. neseri* specimens. The experiment was only considered successful when *S. neseri* offspring emerged from the *L. invasa*-induced galls of the first and third plant, indicating the suitability of the *S. neseri* females used in the experiment to produce offspring. The absence of *S. neseri* specimens emerging from the *E. lysistemon* plants would indicate that the host was unsuitable for its development. Thirdly, the behaviour of the female *S. neseri*, when exposed to galls of (GC540) and to galls on *E. lysistemon* leaves was compared when female *S. neseri* were kept in a confined space to allow close observation of their behaviour on the galls.

For the remaining 16 potential non-target hosts tested, the galled leaves from these hosts were exposed to *S. neseri* to determine any interest in probing or ovipositing, or ability to develop on these hosts. A special effort was made to include galls on other species of Myrtaceae. For this, the smooth, bulging galls in the berries of *Syzygium cordatum* were regarded as particularly relevant, being caused by a eulophid similar to *L. invasa* and also yielding parasitoids in the genera *Quadrastichus* and *Megastigmus*, as do *L. invasa* galls in Australia (S. Neser and O. Neser, unpublished; Anonymous, 2011). Newly-collected galls were placed inside large glass Petri dishes (diameter of 15cm) on damp cotton wool. Ten female *S. neseri* were released inside the Petri dish, which was subsequently sealed with *PARAFILM "M"* Laboratory film (Pechiney Plastic Packaging). Strips of honey were placed with a fine paintbrush onto the inside of the lid of the Petri dish for the wasps to feed on, ensuring normal survival and oviposition. Additional water was not supplied, with moisture being available on the paper and as condensation. The behaviour of the female *S. neseri* was observed for 25 minutes

under a dissection microscope, specifically noting any sign of oviposition behaviour. *Leptocybe invasa* galls on *Eucalyptus* (GC540) were used as the control. The females remained inside the Petri dishes with the galled material for five days, after which, they were removed, to ensure that no emerging *S. neseri* could be confused with the females that had initially been released. The galls were observed daily for emergence of either the gall-formers, their parasitoids or of *S. neseri*. Galls were kept for at least a month, after which the galls were dissected to search for any signs of eggs or larvae. All insects emerging from the galled material were retained for identification.

3 RESULTS

3.1 Plants infested by L. invasa

The duration of the *L. invasa* life cycle in summer (egg to adult) in Pretoria, South Africa on plants kept outdoors under shade netting was 91.6 days +/- 5.4 days (daytime temperature range: 17.5°C – 28.5°C) (South African Weather Service, 26.10. 2010 – 28.02.2011).

3.2 Characterisation of behaviour

The four GC540 plants exposed to *S. neseri* specimens showed no gall formation. Dissections of *L. invasa*-induced galls exposed to *S. neseri* specimens showed two distinctly different types of larvae. *Leptocybe invasa* larvae were spherical in shape whereas those of *S. neseri* were elongate (Figure 1 H, I & J). In some instances, upon gall dissection (i) an elongate larva with dark gut contents was observed in a gall with the remains of an *L. invasa* adult or near adult (Figure 1 K), (ii) a *S. neseri* pupa was observed in the same gall as the remains of an *L. invasa* adult, and (iii) eggs of *S. neseri* were observed on a nearly fully developed *L. invasa* adult.

3.3 Gall age preference and nominal parasitism rate

There was large variation in nominal parasitism between and within the different gall age treatments, ranging from an average of 9.7% - 71.8%. Plants with galls 59 – 66 days and 90 days old showed the

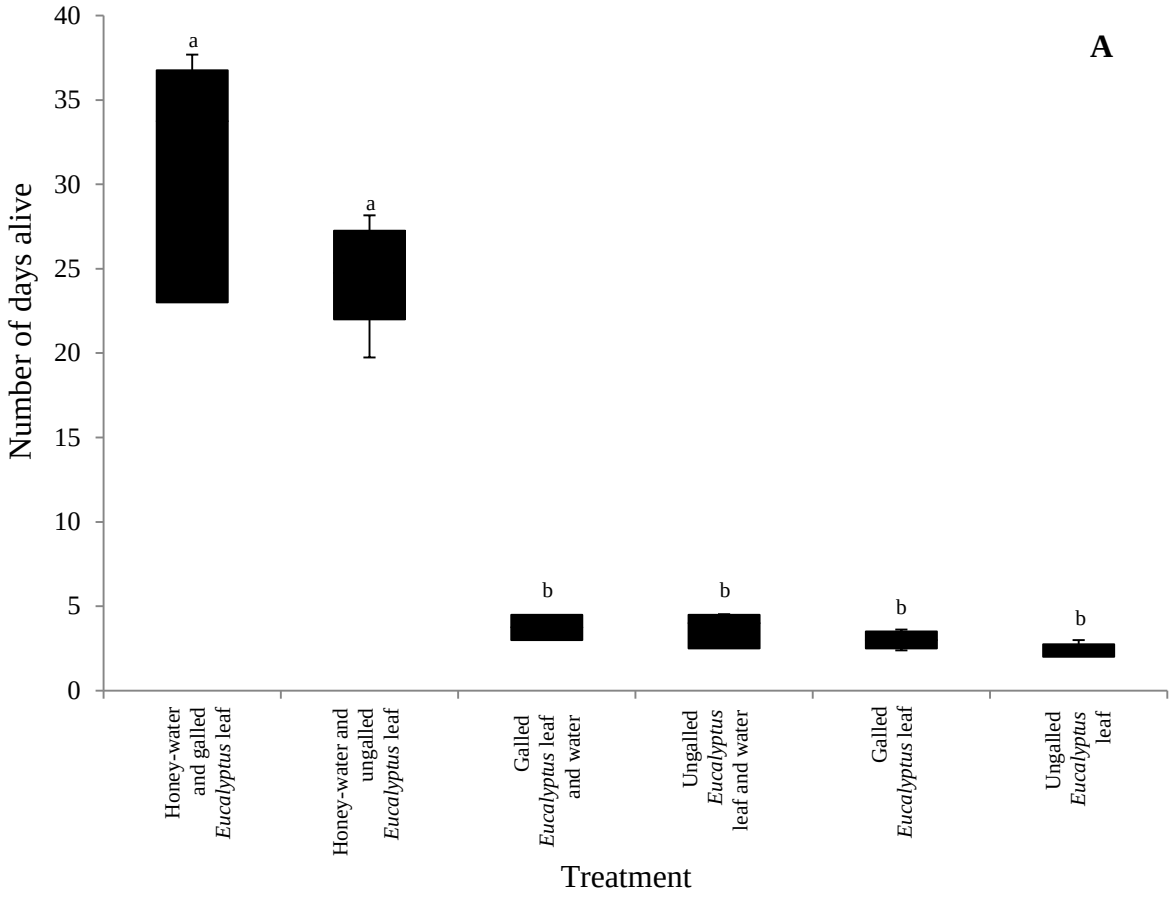
highest average percentage mortality of *L. invasa* (71.8% and 71.7 %, respectively), and plants with galls 30 days and 53 - 57 days old showed the lowest average percentage mortality (20.9 % and 9.7 %, respectively). However, the differences in nominal parasitism between galls of different ages was not significant ($F_{(6,27)} = 1.379$, $P = 0.259$).

3.4 Adult Longevity

There were significant differences in adult longevity between the diet treatments for both females ($F_{(5,54)} = 46.12$, $P< 0.001$) and males ($F_{(5,54)} = 82.38$, $P< 0.001$), with adults living significantly longer on diets containing honey water than those without honey water (Figure 2). The average longevity of males and females with honey-water ranged from 23.3 days – 26.1 days. The shortest average longevity was observed when *S. neseri* specimens had only ungalled *Eucalyptus* leaves with no food or water (2.7 days).

3.5 Developmental time, pre-oviposition period, number of offspring, sex ratio and potential fecundity

The average *S. neseri* developmental time in the gall (oviposition to adult emergence) was 19.3 days +/- 0.2 days (n = 359) (males: 19.9 days +/- 0.5 days (n = 81); females: 19.1 days +/- 0.3 days (n = 278)). The minimum developmental time was 12 days (n = 2) and the maximum developmental time was 31 days (n = 2). The maximum total number of offspring produced per female over her lifespan was 39 (34 female offspring and 5 male offspring) (Supplementary material - Table B, C & D). The maximum number of offspring recorded per day per female was 10. Of the total 278 females used in the study, only 49 females produced offspring possibly due to the galls not containing a suitable stage of host for successful development. On average (for those females producing offspring) each female produced 7.3 offspring (5.7 females and 1.7 males) showing a sex ratio of males to females of 1: 3.43. Dissection of the ovaries showed twelve ovarioles, six on each calyx arranged in three groups of two (Figure 1 L). This suggests that the maximum offspring a female is able to produce in rapid succession is twelve. A monitored female *S. neseri* produced offspring from day one to day 27 of her life span. There was no clear favourable period in the life of *S. neseri* to oviposit. The results showed that eggs were available to be laid on the day of emergence (i.e no pre-oviposition period), and that egg



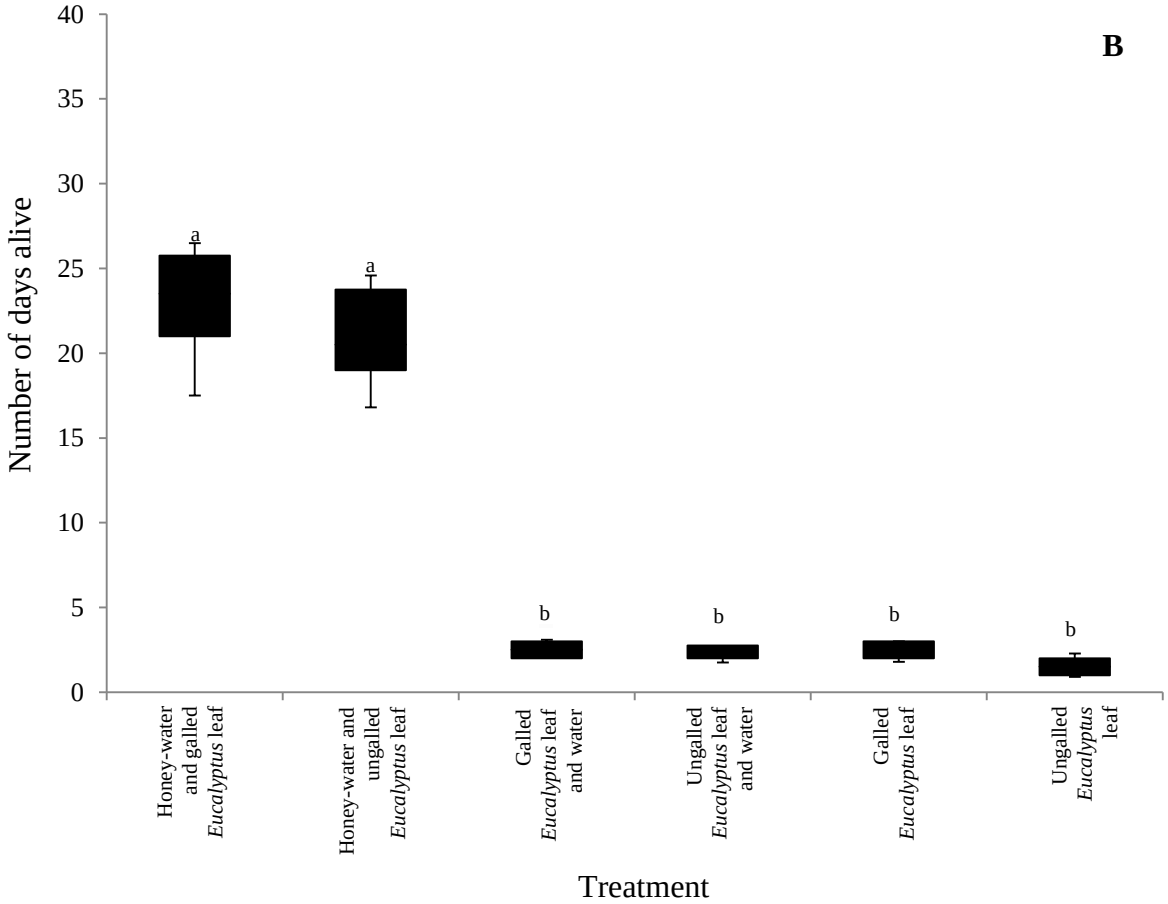


Figure 2. The longevity of Selitrichodes neseri females (A) and males (B) with six different treatments (Mean ± SE). Treatments with the same letter are not significantly different from one another ($P < 0.05$).

production is sustained. Comparison of the longevity of these female *S. neseri* specimens showed that, with the addition of honey paper, females lived up to 27 days, which agrees with the results obtained from the separate longevity study. The average longevity of the female specimens in these experiments was low (between 12 and 13 days).

3.6 Host specificity testing

Exposure of *S. neseri* male and female specimens to eleven *Q. gallicola*-galled *Erythrina lysistemon* plants yielded no *S. neseri* offspring. Similarly, from the five successful 3-plant experiments and two successful 4-plant experiments, no *S. neseri* emerged from the *Q. gallicola*-galled *E. lysistemon* plants (Supplementary material - Table A). While the females probed the galls on *E. lysistemon* by inserting ovipositors into occasional galls, this behaviour was atypical. Here ovipositors were withdrawn after a shorter duration than when oviposition was occurring on the normal host and no offspring emerged from the non-host galls.

Results from exposing the *S. neseri* females to the other 16 potential non-target hosts showed that no females oviposited in the galls, and likewise no interest was shown in these galls (Table 1). *Ziziphus mucronata* was an exception and probing with ovipositors was observed on two occasions (even though the primary gall-former was a psyllid). However, no typical oviposition behaviour was observed after probing. Behaviour on the *L. invasa*–induced *Eucalyptus* galls, used as the control, differed from the above. Females explored the galls and oviposition on the *L. invasa*-induced galls was consistently observed within the first 10 minutes. No *S. neseri* emerged from any of the plants tested for specificity (Table 1). In most exposures, the gall-dwelling insects were able to emerge from the plant material, indicating that the galls remained suitable for their occupants and parasitoids to complete their development. *Coelocephalapion camarae* Kissinger (Brentidae) on *Lantana camara,* and *Procecidochares utilis* Stone (Tephritidae) from the galls on *Ageratina adenophora*, together with the parasitoid wasps of a gall fly, emerged. An undescribed eulophid, provisionally placed in the tribe Anselmellini (O. Neser, personal communication), emerged from *Syzygium cordatum* berries, along with parasitoid wasps. Dissection confirmed the absence of *S. neseri* within the galled plant material.

Oviposition and emergence of *S. neseri* was observed only on *L. invasa*-induced galls on *Eucalyptus* and not on any other tested plant species.

4 DISCUSSION

This study generated detailed information pertaining to the biology of *S. neseri*. We confirmed that *S. neseri* is an ectoparasitoid throughout its development. It is well recorded that many eulophid gall-formers are attacked by eulophid parasitoids and secondary evolution of eulophid gall-formers has emerged several times from their parasitoid ancestors (Gauthier *et al*., 2000). .Eulophidae comprises mainly parasitoids, but is also characterized by differing biology within one family (Gauthier *et al.*, 2000). Members of the genus *Selitrichodes* are also known as gall-formers (Fisher *et al*., 2014). Although several other species in the genus *Selitrichodes* (*S. auriflavus, S. casuarinae, S. consobrinus, S. fasciativentris, S. flavus, S. globulus, S. giraulti, S. multifasciatus, S. quinqnigrimaculae, S. secus, S. tricolor, S. utilis, S, variegatus* and *S. varigatus (sic)*) have been described, little or no information is available regarding their biology (Kim *et al*., 2008; La Salle *et al*., 2009; Fisher *et al*., 2014).

The nominal parasitism rate observed for *S. neseri* ranged from 9.7% - 71.8%. A congener, *Selitrichodes kryceri*, showed a similar parasitism success (3.2% - 67.4%) under similar laboratory conditions (Kim *et al.*, 2008). Results of the gall age preference studies, suggest that *S. neseri* can develop successfully on a wide range of gall ages. Gall age is not necessarily an accurate reflection of the developmental stage of the host within the gall as the larvae/pupae are not necessarily in a similar stage of development. Dissections of compound galls, containing eggs laid in one batch, have shown that gall age does not always relate to developmental stage of the host. Further work is needed to confirm whether *S. neseri* equally parasitises different developmental stages of *L. invasa*. However, the results do indicate that given the right conditions, *S. neseri* can potentially obtain parasitism rates of over 70 % of hosts suitable for development at the time of exposure.

Longevity of *S. neseri* adults varied greatly depending on whether nutrients were available. This result has important implications for the release and successful establishment of *S. neseri* when it

is used for biological control. Release of *S. neseri* would require mass-reared adults to be transported from the rearing facility to various release sites. To ensure that the adults survive the transportation, increase their time in the field and thus maximise offspring produced (Eliopoulos *et al*., 2003), a source of carbohydrates and free water should be available during transport and possibly after release. These food sources could include small strips of honey paper during transport and naturally available food sources in the field, such as nectar from flowers or honeydew from sap sucking insects. The presence of sap-sucking insects, such as aphids and the psyllids *Blastopsylla occidentalis* Taylor and *Ctenarytaina eucalypti* (Maskell) (Hemiptera: Psylloidea) on *Eucalyptus*, may thus promote the survival and successful establishment of *S. neseri* in the field (Neser & Millar, 2007).

Understanding the potential fecundity and female adult longevity of parasitoid wasps is important for their optimal use as biological control agents (Eliopoulos *et al*., 2003). Results from this study showed that an *S. neseri* female may carry up to 12 mature eggs at one time. The very high proportion of females in this study producing no offspring may be as a result of super-oviposition, a suitable host being stung repeatedly, or the possibility that many galls exposed may not have contained suitable hosts, leading to no offspring maturing. Furthermore, *S. neseri* is able to oviposit immediately after it emerges and continues to develop eggs and oviposit until it dies. This indicates that *S. neseri* is a synovigenic species, which has also been recorded for other members of this family (Jervis *et al*. 2001). This further emphasises the importance of nutrient availability for *S. neseri* adults to increase their longevity, number of offspring and thus effectiveness as a biological control agent.

The sex ratio observed in this study (1: 3.43 males: females) was substantially different to that observed in the mass reared colony (1:1.3 males: females). This is possibly because male and female *S. neseri* were paired in the study thus increasing the probability of mating, which would result in a greater proportion of females due to the haplodiploid mode of reproduction. In addition, more effort was made in the study to choose suitable galls as compared to the galls used in the rearing colony. Various studies on parasitic wasps have shown that quality/size of their hosts is correlated with males or females being produced and thus males typically emerge from small hosts and females emerge from larger hosts (Ode & Heinz, 2002).

Host specificity tests of potential biological control agents are necessary to assess possible non-target effects (Barratt *et al*., 2010). Host specificity testing of *S. neseri* against 17 possible non-target hosts, which were phylogenetically related or showed similar gall morphology to *S. neseri*, showed that these hosts were not suitable for the development of *S. neseri*. Although the potential non-target hosts tested were specifically relevant to South Africa, the fact that *S. neseri* did not parasitize other gall formers that were phylogenetically similar or showed similar gall morphology, suggests that similar results might be expected from host specificity tests in other countries.

Although caution needs to be exercised and the potential risks must be assessed when introducing an invasive arthropod for use in biological control, care must be taken not to discard promising biological control agents due to policies and regulations (De Clercq *et al.*, 2011). In this regard, *S. neseri* shows considerable promise as a biological control agent against *L. invasa*. This is due to its relatively long adult life cycle (if supplemented by a food source), its ability to utilize most developmental ages of *L. invasa*, its short developmental time and clear host specificity. Based on the work presented here and that of Kelly *et al*. (2012), permission for the release of *S. neseri* was obtained from the South African government in June 2012, and the first releases made in July 2012. Releases of *S. neseri* in other countries where *L. invasa* is present are likely to occur in the near future. Post-release studies on its establishment, spread and impact on *L. invasa* populations, will provide further information on its effectiveness as a biological control agent.

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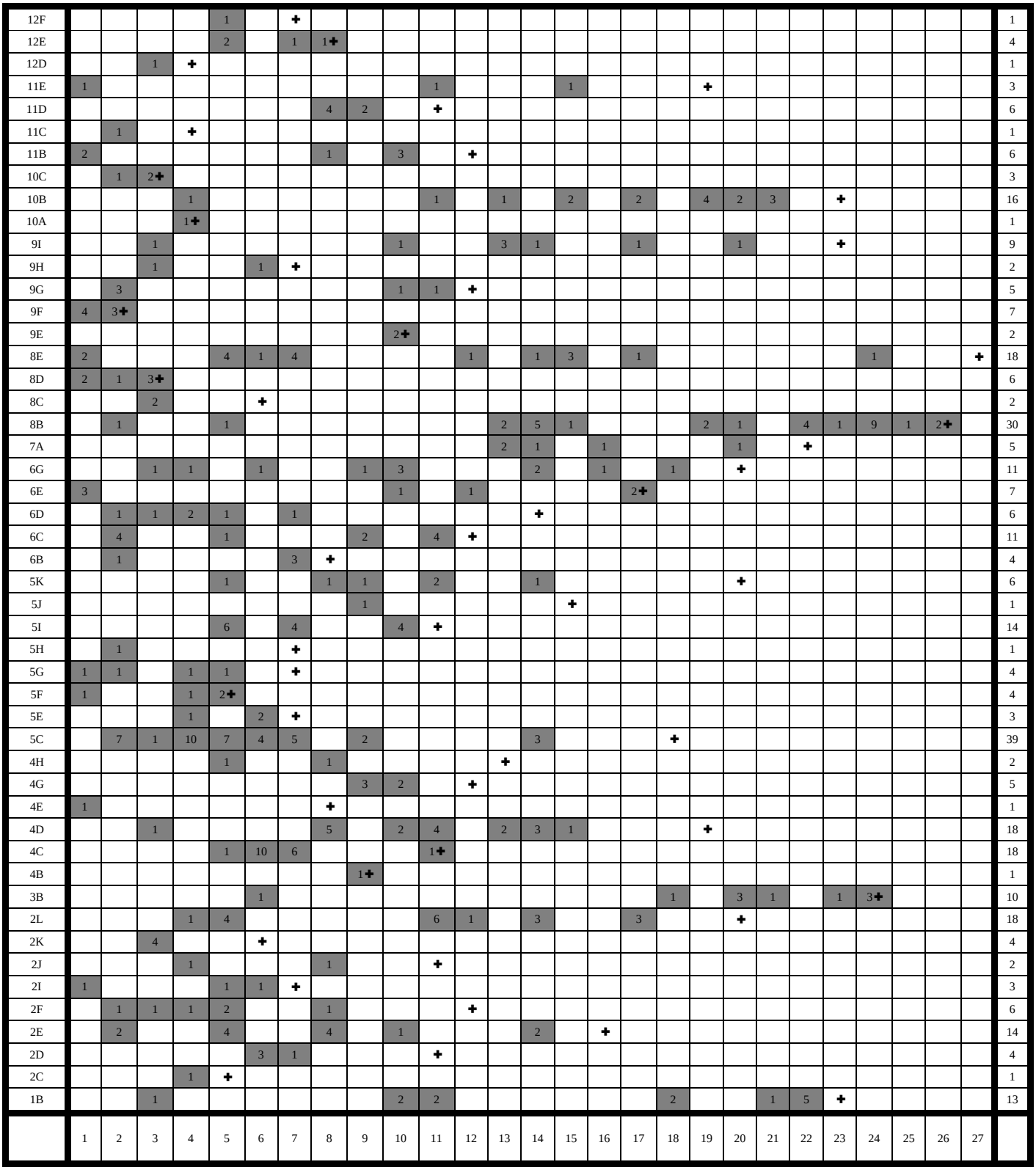
Supplementary Material

Table A. The results of the specificity testing of *Selitrichodes neseri* against *Quadrastichus gallicola*

Experiment	Replicate no.	Tree type	Sequence tree exposed to *S. neseri*	No. of *S. neseri* released			No. of *S. neseri* offspring emerged		
				Male	Female	Total	Male	Female	Total
3 -tree	1	*Eucalyptus*	1	10	10	**20**	1	2	**3**
		Erythrina	2	5	8	**13**	0	0	**0**
		Eucalyptus	3	3	8	**11**	1	6	**7**
	2	*Eucalyptus*	1	10	10	**20**	0	1	**1**
		Erythrina	2	10	8	**18**	0	0	**0**
		Eucalyptus	3	3	5	**8**	3	13	**16**
	3	*Eucalyptus*	1	10	10	**20**	0	1	**1**
		Erythrina	2	6	10	**16**	0	0	**0**
		Eucalyptus	3	0	4	**4**	0	2	**2**
	4	*Eucalyptus*	1	10	10	**20**	4	1	**5**
		Erythrina	2	7	7	**14**	0	0	**0**
		Eucalyptus	3	3	5	**8**	1	3	**4**
	5	*Eucalyptus*	1	10	10	**20**	0	1	**1**
		Erythrina	2	7	9	**16**	0	0	**0**
		Eucalyptus	3	4	5	**9**	3	5	**8**
4-tree	1	*Eucalyptus*	1	3	10	**13**	0	2	**2**
		Erythrina	2	3	9	**12**	0	0	**0**
		Eucalyptus	3	1	5	**6**	0	1	**1**
		Erythrina	4	0	3	**3**	0	0	**0**
	2	*Eucalyptus*	1	10	10	**20**	0	2	**2**
		Erythrina	2	8	8	**16**	0	0	**0**
		Eucalyptus	3	2	6	**8**	1	0	**1**
		Erythrina	4	2	2	**4**	0	0	**0**

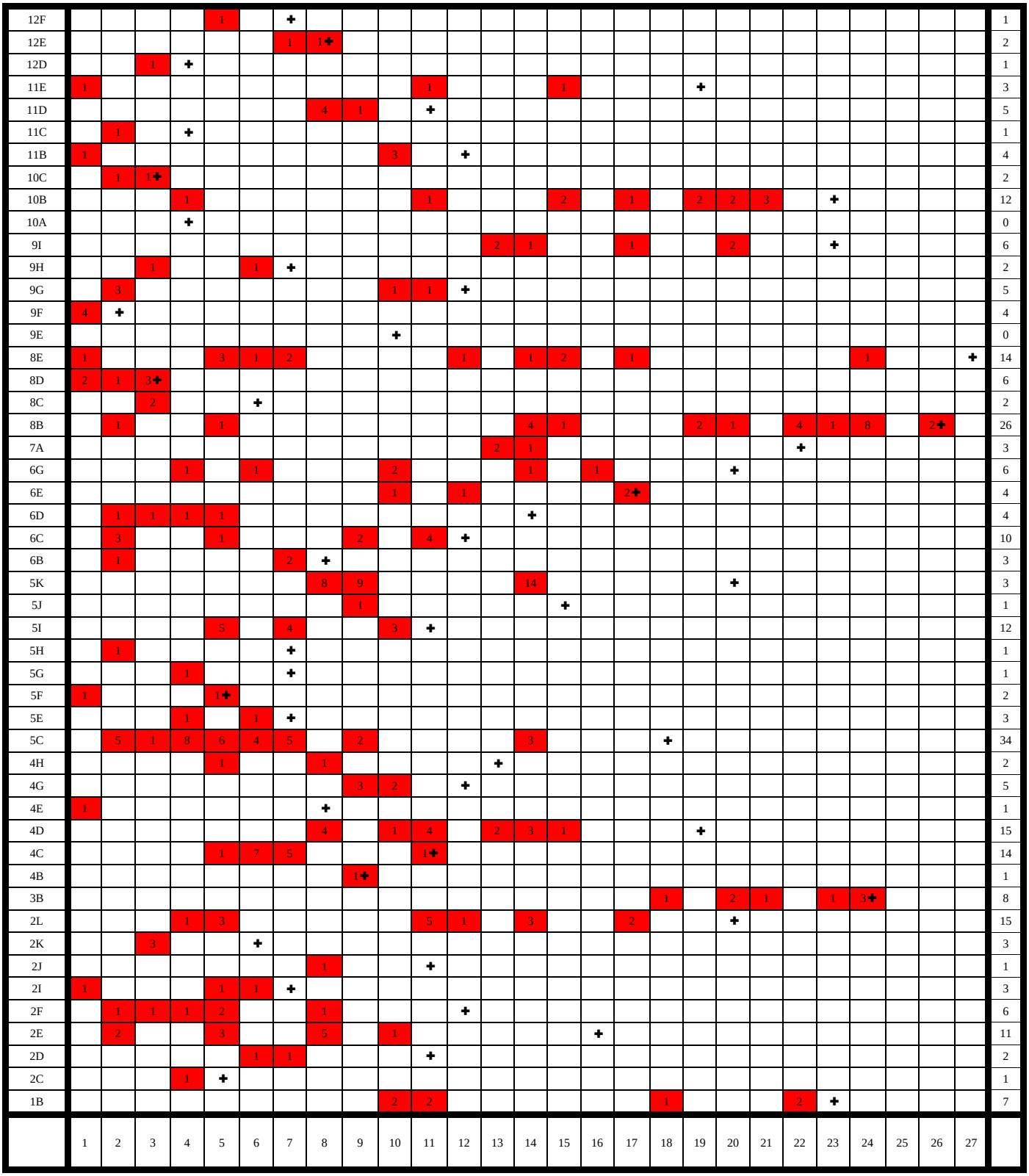
Table B. The total number of offspring produced per female per life cycle day, and the cumulative number of offspring for all females per life cycle day. Numbers in blocks denote numbers of emerged offspring per day



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	
12F					1		✚																					1
12E					2		1	1✚																				4
12D			1	✚																								1
11E	1										1				1				✚									3
11D								4	2		✚																	6
11C		1		✚																								1
11B	2							1		3		✚																6
10C		1	2✚																									3
10B				1							1		1		2		2		4	2	3		✚					16
10A				1✚																								1
9I			1							1			3	1			1			1			✚					9
9H			1			1	✚																					2
9G		3								1	1	✚																5
9F	4	3✚																										7
9E										2✚																		2
8E	2				4	1	4					1		1	3		1							1			✚	18
8D	2	1	3✚																									6
8C			2			✚																						2
8B		1			1								2	5	1				2	1		4	1	9	1	2✚		30
7A													2	1		1				1		✚						5
6G			1	1		1			1	3				2		1		1		✚								11
6E	3									1		1					2✚											7
6D		1	1	2	1		1							✚														6
6C		4			1				2		4	✚																11
6B		1					3	✚																				4
5K					1			1	1		2			1						✚								6
5J									1						✚													1
5I					6		4			4	✚																	14
5H		1					✚																					1
5G	1	1		1	1		✚																					4
5F	1			1	2✚																							4
5E				1		2	✚																					3
5C		7	1	10	7	4	5		2					3				✚										39
4H					1			1					✚															2
4G									3	2		✚																5
4E	1							✚																				1
4D			1					5		2	4		2	3	1				✚									18
4C					1	10	6				1✚																	18
4B									1✚																			1
3B						1												1		3	1		1	3✚				10
2L				1	4						6	1		3			3			✚								18
2K			4			✚																						4
2J				1				1			✚																	2
2I	1				1	1	✚																					3
2F		1	1	1	2			1				✚																6
2E		2			4			4		1				2		✚												14
2D						3	1				✚																	4
2C				1	✚																							1
1B			1							2	2							2			1	5	✚					13

✚denotes female death

Table C. The total number of female offspring produced per female per life cycle day, and the cumulative number of offspring for all females per life cycle day. Numbers in blocks denote numbers of emerged offspring per day



✚denotes female death

Table D. The total number of male offspring produced per female per life cycle day, and the cumulative number of offspring for all females per life cycle day. Numbers in blocks denote numbers of emerged offspring per day