LIFE CYCLE AND HOST PREFERENCES IN THE AGAPANTHUS BORER

(*Neuranethes spodopterodes*)

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Abstract

The species Neuranethes spodopterodes is a pest of the Agapanthus species. The larva feeds gregariously on the leaves and rhizomes and cause extensive damage to the plant.

This study shows the *N.spodopterodes* have 6 larval instars. The use of the head capsule width as a morphometric parameter and applying it to Dyar's rule gives significant results which allow for adequate determination of the larval instar. The *Agapanthus* borer has a life cycle that last approximately 84 days.

Some species of *Agapanthus* are the preferred host plant for oviposition by the female *N.spodopterodes*. *Agapanthus inapertus*, *Agapanthus caulescens* and *Agapanthus praecox* are the most infested with the *Agapanthus* borer eggs. Leaf sizes do not appear to be a factor that influences the female choice.

There appears to be no preference from the larva during feeding. The larva fed equally on all the six *Agapanthus* species including *A. africanus* ($p>0.05$). This shows that the chemical composition of these species is closely related. Since the larva has no feeding preference, this reinforces the fact that it is the female moth that chooses the host plant and that it abides with preference-performance hypothesis. Since *A. africanus* is subjected to feeding by the larva under laboratory conditions, it could potential imply that in the event of a range shift by the gravid female, that this species of plant is at risk of extinction.

The presence of a parasitoid wasp, *Trichogramma* in the egg of *N. spodopterodes* and that the level of parasitism is about 86% suggests that this parasitoid could potentially be used as a biological control agent of the Agapanthus borer.

Introduction

Prior to 2010, *Agapanthus* species in the Western Cape were free of attack by any lepidopteran species. Following the hybridization of *Agapanthus inapertus* with other *Agapanthus* species, and the distribution of these hybrids in the horticultural trade, the Agapanthus borer (*Neuranethes spodopterodes*) has spread very rapidly across South Africa (Picker & Krüger, 2013) from its natural range. This has a potential ecological impact on rare native species like *Agapanthus africanus* and *Agapanthus africanus* subsp *walshii* which now occur in the range of the Agapanthus borer.

In the first chapter, the life cycle of the Agapanthus borer will be assessed as well as the evaluation of the number of larval instar. The second chapter will answer the question "Which *Agapanthus* plants does the gravid female chooses to lay her eggs on and if her choice was influenced by the size of the leaves of the host plant?". Chapter 3 will deal with the feeding preference of the larva and how this correlates with the mother's choice of host plant. Finally, the last chapter explores any presence of a parasitoid species in the eggs of *N. spodopterodes* and if present, how effective would that parasitoids be as a biological control agent.

The main objectives of this project are:

1. To establish the duration of the life cycle of the *Agapanthus* borer.
2. Determine host preference using egg counts in a natural setting.
3. Find out a larval host preference.
4. Identify any possible parasitoids in the introduced range of the Agapanthus borer.

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Chapter 1

Duration of the life cycle of *Neuranethes spodopterodes* and instar determination using head capsule widths

Introduction

The moth *Neuranethes spodopterodes* (Hampson, 1908) (Noctuidae, Hadeninae) is a newly discovered pest of ornamental *Agapanthus* plants in Southern Africa (Picker & Krüger, 2013). However, not much is known about this insect. There are no published data on the duration of the life cycle or the number of larval instars of this moth. Picker and Krüger (2013) describe only details about the life cycle of this species.

The age structure of a population is an important factor to consider when carrying out ecological, taxonomic and environmental assessments. In order to execute those studies, it is crucial to ascertain the age composition of the juvenile stages of the population under study, which depends on the ability to discriminate among the larval instars (Di Veroli et al, 2008). The correct identification of each instar of a pest can be of practical significance in pest management. Since, the proper development of larvae can be easily influenced by external factors such as temperature, light, food availability and quality (Canteiro &Albertoni, 2011) as well as physiological stress, population densities, chemical interactions and sex of the individual (Esperk et al, 2007), it is therefore important to assess the age of the population under various regimes. In Lepidopteran, it is possible to distinguish among instars using measurements of the head capsule, even under different environmental condition (Stevens, 1993).

In determining the number of insect instars, the most commonly used tool is morphometry (Daly, 1985). Morphometric measurements of the head capsule are widely used in the identification of instars, but alternative ways for determining the age of insects can expand the options for the analysis of field and laboratory populations (for example, counting the number of molts that occur). The width of the head capsule is the most widely used dimension to determine larval instars since it remains constant during each instar but differs widely in size between instars (Daly, 1985; Silva et al, 2008). In identifying instars, the head capsule width of the larval stages of Lepidopteran species is a very efficient parameter (Beaver & Sanderson 1989; Russel & Bouzouane 1989; de Groot, 1998). Using the fully sclerotized head capsule as a morphometric tool confers an advantage since its growth is discontinuous and therefore, changes in size can easily be measured after each molt (Chapman, 1969).

In evaluating the reliability of head capsule measurements to discriminate instars, mathematical models were used: of which the more well-known is the geometric progression, called Dyar's rule (Dyar, 1890). According to Dyar's rule, the widths of the head capsules of an insect have geometrically progressive growth. Other applied statistical methods relies on the assumption that head capsule measurements are normally distributed for each instar (Fink 1984, Sokal &Rohlf 1995 in Hunt & Chapman 2001) and when plotted in a frequency distribution, a multimodal curve results with each peak representing an instar. Studies by Rodriguez-Loeches and Barro (2008) found that the measurement of head capsule of *Phoenicoprocta capistrata* (Lepidoptera, Arctiidae) larvae followed a geometric pattern consistent with Dyar's rule. Based on head capsule measurement and Dyar’s rule, studies have found three larval instars for *Apion ulicis* (Coleoptera) (Hoddle, 1990). Twine (1978) found that *Heliothis armigera* (Lepidoptera, Noctuidae) varied from 5 to 7 instars using the width of head capsules and that the growth rate was constant when using Dyar’s rule.

The objective of this section is to determine the number of larval instars of the Agapanthus borer using the measurements of larval head capsule width and the number of molts observed, and applying Dyar’s rule to the results to verify that no instars are missing and assessing the geometrical growth rate of the instars. Moreover, this section endeavours to assess the duration of each life stages of *N. spodopterodes*.

Materials and methods

Insect rearing

Larvae of the Agapanthus borer were collected from *Agapanthus* plants in flower beds in Rondebosch, Cape Town, South Africa (S33°57 and E18°27) from March to July. The larva were reared in petri-dishes in a constant temperature room set at 22°C with relative humidity of 60% and exposure to a photoperiod of 12:12 (L:D) hours. They were supplied with fresh *A. praecox* leaves *ad libitum*. When too big for the petri-dishes, large larvae were transferred into clear plastic containers with perforated lids for air flow. The *Agapanthus* leaves supplied were rolled up such that they form a tube-like structure whereby the larva would bore inside; therefore mimicking the natural feeding sites (hollow tube-like structure of the *Agapanthus* stem and tightly pressed leaves). All larvae were allowed to pupate and emerge as adult moths.

Eggs collection

Reared male and female moths were allowed to mate. They were placed together in a card-board box and provided with a mixture of sugar and honey water and *Agapanthus* leaves. The box was closed by fine mosquito netting and a flat opaque framework was placed on top to limit the amount of light entering the box. This ensured that the moths were active and undisturbed by the brightness of the constant temperature room lights. Egg batches were laid all over the sides of the card-board box and the netting as well as a few on the leaves. A single gravid female laid up to 150 viable eggs in small batches. The egg batches were gently scraped off and placed in clear plastic containers. The time taken for the eggs to hatch was recorded.

Instar determination

A total of 83 larvae of *N. spodopterodes* were measured from March to July. Fifty-eight larva were collected in the field and 25 hatched from eggs obtained from the laboratory matings. Pictures of the head of the larva were taken at different larval stages using a Leica EZ4 HD stereo-microscope integrated with a 3.0 mega-pixel CMOS Camera and viewed using the software Leica LAS EZ (Leica Microsystems). The width of the head capsules was measured to

0.01 mm using the electronic measurement option in the Leica software (Figure 1a). Visual observations of each molt were also recorded till pupation (Figure 1b). The number of observable molts was used to ascertain that the head capsule measurements before or after each molt corresponded with a specific instar.

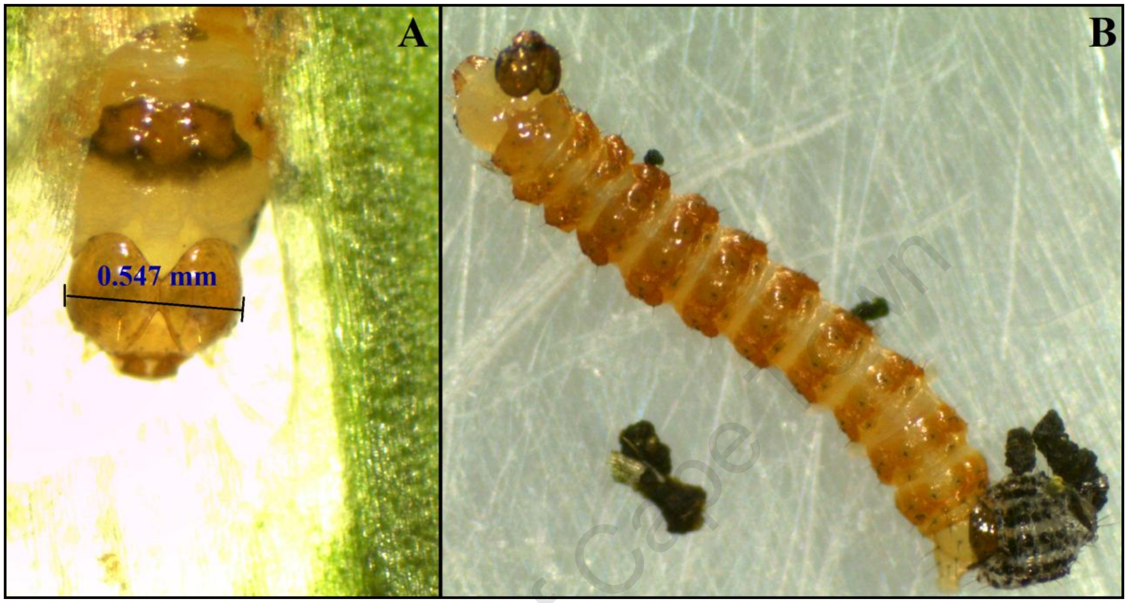

Figure 1. A. Head capsule width measurement. B. Larva molting.

Pupation period and adult phase

The reared larvae were allowed to pupate till emergence into adult moths into clean plastic containers fitted with perforated lids. Colour changes and the duration of this stage were noted. Gender of the emerged moths from pupation was noted. The time taken for the moths to reach sexual maturity was noted as well as the time till they die. All pupae and moths were housed under the same environmental conditions as the larvae.

Statistical analyses

For the duration of each life stage, the number of individuals assessed, the mean, standard deviation, standard error, minimum and maximum were obtained for measurements and tabulated using descriptive statistics. To verify that no instars were missing, a linear regression of the natural log of the head capsule widths was plotted against the number of instars based on Dyar's rule. An analysis of variance was performed followed by Tukey's post hoc test to verify any significant differences in the width of the head capsule among instars and the duration of each life stage. The statistical software used for all analyses in this project was Statistica 7.

Results

Instar determination

Head capsule widths ranged from 0.37 to 3.65 mm, for all untransformed measurements. However, The frequency distribution showed six peaks, each corresponding to an instar (Figure 2). Therefore, there are six distinct larval instars in *N. spodopterodes*. The mean head capsule widths before and after each observable molt were used as arbitrary limits to each instar (denoted as arrows in Figure 2). The range for the widths of head capsule in 1st to 6th larval instars were 0.37-0.46, 0.57-0.67, 0.66-1.76, 1.22-2.44, 1.47-3.08 and 2.40-3.65 mm, respectively (Table 1).

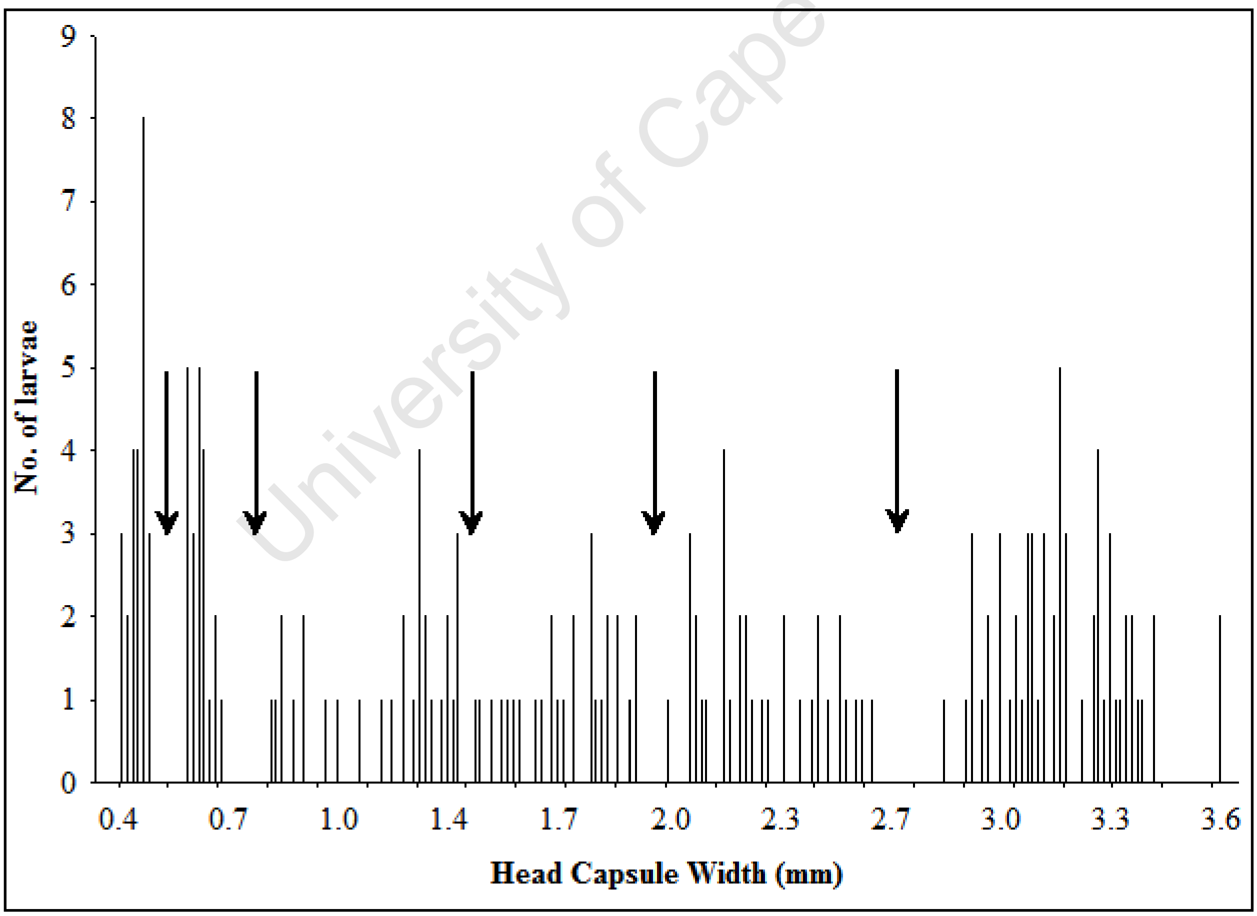

Figure 2: Frequency distribution of larval head capsule width (mm). (Arrows indicate arbitrary limits to each instar).

Analysis of variance revealed significant differences ($p<0.05$) among different instars with respect to head capsule width with exception of the head capsule width between the 1st and 2nd instars which did not differ significantly ($p=0.17$). Mean comparison indicated significant differences among all the instars with maximum (3.65 mm) and minimum (0.37 mm) head capsule width in 1st and 6th instars, respectively.

Table 1: Mean, Standard Error (SE), Minimum, Maximum, Standard Deviation (SD), and Dyar's growth ratio in head capsule width of *N.spodopterodes* larval instars. (n=192).

Instars	N	Mean ± S.E	Min.	Max.	S.D	Dyar's ratio
1	24	0.42 ± 0.01	0.37	0.46	0.03	1.45
2	20	0.61 ± 0.01	0.57	0.67	0.03	1.88
3	26	1.15 ± 0.05	0.66	1.76	0.26	1.55
4	38	1.78 ± 0.05	1.22	2.44	0.34	1.31
5	29	2.33 ± 0.07	1.47	3.08	0.39	1.36
6	55	3.17 ± 0.03	2.40	3.65	0.22	

Linear regression analysis showed a significant relationship between larval instars and head capsule width ($R^2 = 0.943$) which is congruent with Dyar's rule (Figure 3). The excellent fit indicates that no instar was overlooked. Dyar's ratio for successive instars was 1.45, 1.88, 1.55, 1.31 and 1.36 for first to last larval instars, respectively (Table 1) indicating a pattern of geometric progression which followed the equation: $y = 0.402x - 41.35$ ($R^2 = 0.943$; $n = 192$).

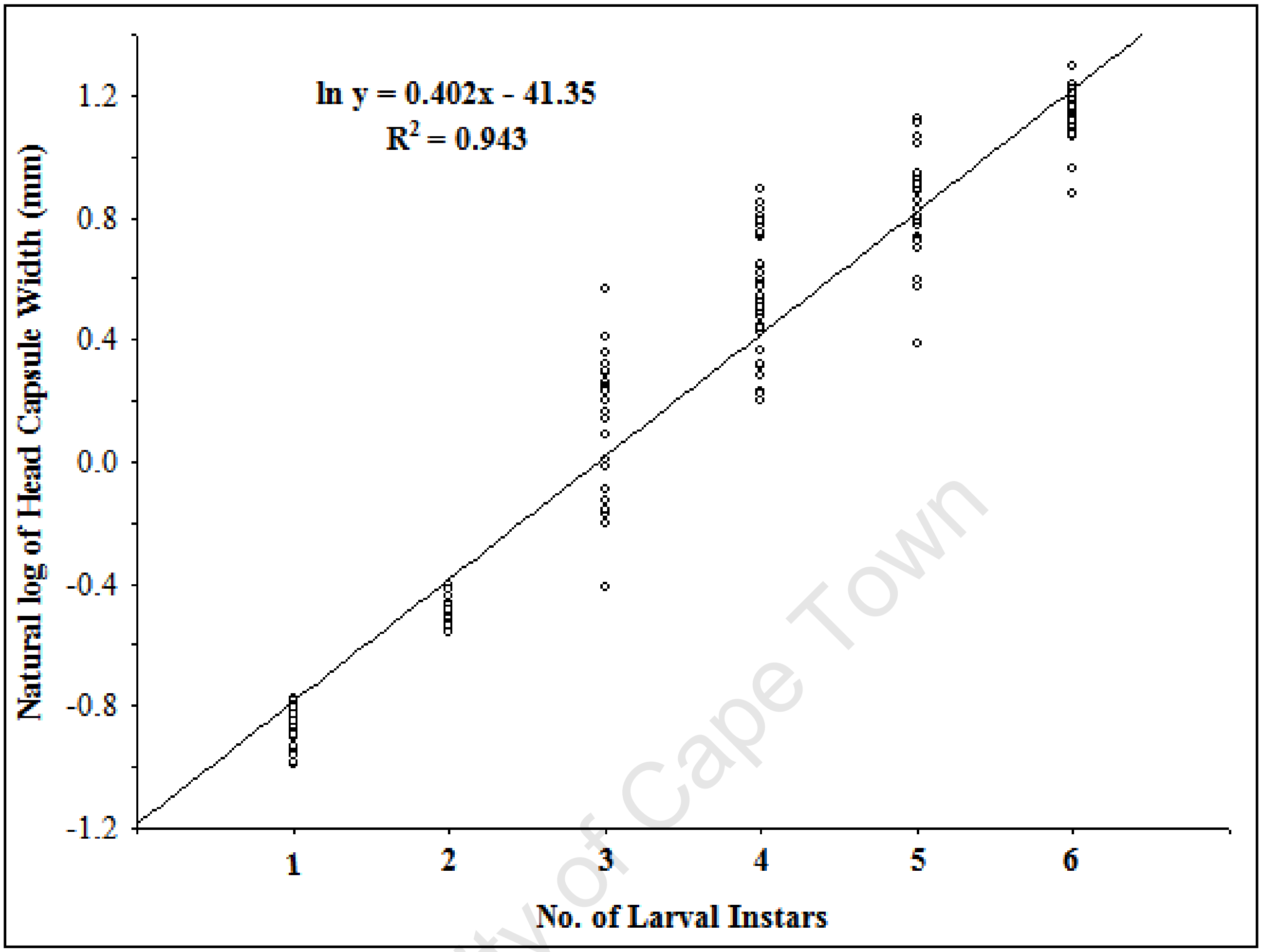

Figure 3: Relationship between head capsule width and instar of *N. spodopterodes*. A linear regression shows a straight line which fitted Dyar's rule.

Duration of the life periods

The average duration of the life cycle of *N. spodopterodes* at 22°C is 83.77 ± 7.92 days with a minimum of 57 days and maximum of 122 days. The average time taken for the eggs to hatch is 6.31 ± 0.58 days ranging from 6-8 days (Table 2). There is a progressive increase in the duration of the larval instars with increase in instars which agrees with Dyar's rule (Figure 4).

Table 2: Duration (in days) of the different life stages of *N. spodopterodes*.

Life stage	N	Mean ± S.E	Min.	Max.	S.D
Egg	36	6.31 ± 0.10	6	8	0.58
Larva (all instars)	83	46.78 ± 0.31	32	69	1.42
1st instar	31	5.10 ± 0.05	5	6	0.30
2nd instar	17	6.12 ± 0.15	5	8	0.60
3rd instar	17	7.71 ± 0.22	5	9	0.92
4th instar	14	7.14 ± 0.52	4	11	1.96
5th instar	25	8.52 ± 0.38	6	14	1.92
6th instar	31	12.19 ± 0.51	7	21	2.82
Pre-pupa	39	2.05 ± 0.04	2	3	0.22
Pupa	29	18.52 ± 0.63	11	27	3.39
Adult	35	10.11 ± 0.39	6	15	2.31

The mean duration of the larval stage from the first to the last instars is 46.78 ± 1.42 days with a range of 32-69 days (Table 2). The length of the larval stages did not differ significantly among the first five instars (1^{st} and 2^{nd} instars, $p = 0.39$; 2^{nd} and 3^{rd} instars, $p = 0.09$; 3^{rd} and 4^{th} instars, $p = 0.95$; 4^{th} and 5^{th} instars, $p = 0.18$). The 5^{th} and 6^{th} instars differed significantly in terms of duration ($p < 0.05$).

The pre-pupa stage lasts 2-3 days and the pupation period lasts from 11-27 days with an average of 18.52 ± 3.39 days. From the day of emergence till it dies, an adult moth has a lifespan of about 10.11 ± 0.39 days with a range of 6-15 days. The female moth appeared to live longer than the male but this was not quantified.

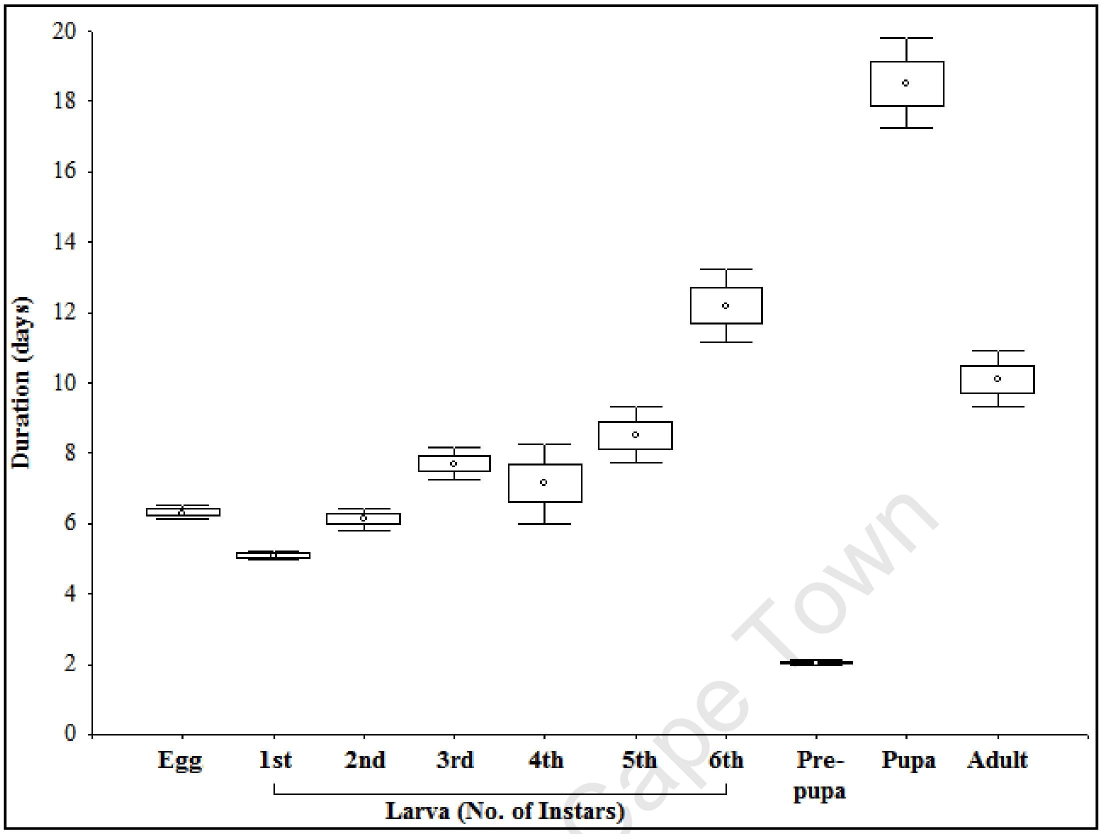

Figure 4: The box-plots of the duration of the life stages of *Neuranethes spodopterodes*. There is a progressive increase in the duration of the instars which fits Dyar's rule.

Discussion

Since there is not enough literature data on the morphometry of *N. spodoterodes*, comparisons of the head capsule width, the number of instars and the duration of the life cycle with other results cannot be performed. However, comparisons with species within the same family and subfamily are available.

From this study, *N. spodoterodes* appears to have at least six larval instars and the use of the head capsule width in line with Dyar's rule was successful in distinguishing them. Dyar's rule is regarded as a suitable criterion for the determination of instar and head capsule width as a reliable measurement for instar determination (Enrique, 2006; Garcia-Barros 2006; Agrawal & Kumar Pati, 2002; Francisco & Prado, 2001). A combination of the time of molting and the measurement of sclerotized part of the insect's body applied to Dyar's rule yielded positive results. Therefore, using the head capsule width and Dyar's rule method alone is not sufficient for larval instar determination.

The Agapanthus borer belongs to the subfamily, the Hadeninae. The number of instars in this subfamily ranges from 6-7 instars in some species. Sevastopulo (1948) found that the moth *Mythimna* (*Leucania*) *irregularis* (Lepidoptera, Hadeninae) had six instars similar to the number of larval instars in *N. spodopterodes*. Seven instars were reported in *Morrisonia confusa* (Lepidoptera, Hadeninae) (Wood & Butler, 1991).

The duration of Agapanthus borer life cycle was ~ 84 days at 22°C. The duration of each larval stage increased as the species progressed through the larval instars, with the pupal phase being the longest (~18 days).

It is thus possible, from the measurement of the head capsule width and visual observations of the molting time, to identify the larval instar of *N. spodopterodes* in laboratory and field conditions, as well as to obtain the average rate of growth of instars. However, caution is necessary when assessing the number of instars of field populations since the above study was carried out on a controlled laboratory population and therefore some differences may occur. Changing the development conditions of the moth may result in some differences in the duration of the life cycle.

Chapter 2

Host preference for oviposition by the female *Neuranethes spodopterodes*

Introduction

Oviposition preference in Lepidoptera is influenced by genetic, ecological and behavioural determinants. It influences a butterfly or moth choice of which plant is best for survival of the offspring (Thompson & Pellmyr, 1991). Females have a ranking of preferred host plants on which the number of eggs laid will decrease as her preference decreases. During the quest for finding a suitable host plant, a female moth is receptive to morphological traits, chemical stimuli and deterrents displayed by plants and these can be perceived from a distance (Mphosi &Foster, 2012). Plants have developed defense mechanisms against herbivorous insects. They produce chemicals that repel insects either by their smell or taste. Those chemicals may also be influential in host detection by the female moth as the chemicals present can help a female determine if the plant has already been utilized by another conspecific moth or if it is the correct host for utilisation (Spathe et *al*, 2012).

To recognise a plant best suited for its offspring survival, a gravid female uses a variety of cues. Host plant recognition process achieved by a female moth is similar in most Ledpidoteran species. This process is divided in two main factors (Hern et al., 1996; Renwick & Chew, 1994):

1. Pre-alighting behaviour characeterised bysearching for a suitable site, orientation and encountering any predators in selected site: Chemical cues in host plants help attract females (Stadler et. al, 1995; Pereyra & Bowers, 1988) and / or visual stimuli such as leaf shape and colour (Forsberg, 1987). A balance between positive and negative chemical signals is vital during host selection, and therefore more than one chemical substance will affect the female oviposition choice.

2. Post-alighting discrimination characterised by landing on the host plant, evaluation of the host and acceptance or rejection of such host plant. The transition between pre-and post-

alighting behaviour is when the female lands onto a potential host plant. Using sensory receptors in the forelegs, ovipositor and proboscis, a gravid female will evaluate the surface of the plant (Renwick &Chew, 1994). The best position suited for oviposition on the plant is then selected by the females upon acceptance of suitable host plant. The gravid female will then determine the egg load to assign to the selected plant based on the ranking of host preference (Thompson & Pellmyr, 1991).

In this chapter, the host preference of female *N. spodopterodes* is assessed based on the number of eggs laid on known *Agapanthus* species in a semi-natural environment. Leaf sizes will be used to determine if these visual cues affect the female host choice during oviposition. The position at which the eggs were laid will also be observed to estimate the best oviposition site on a plant. The number of eggs per plant species will show the intensity of infestation occurring on *Agapanthus* plants by the Agapanthus borer.

Materials and methods

Egg count

In order to assess the host preference of the Agapanthus borer, eggs were counted on a number of *Agapanthus* clumps at Kirstenbosch National Botanical Garden located on the foot slope of Table Mountain, Cape Town (S33°59 and E18°25) from April to June (Figure 5). Five known species of *Agapanthus* were accounted for in thus study, namely, *Agapanthus campanulatus*, *Agapanthus caulescens*, *Agapanthus coddii*, *Agapanthus inapertus* and *Agapanthus praecox* (Table A1). Out of the fourteen subspecies of *Agapanthus*, eleven can be found at Kirstenbosch National Botanical Garden (Table A2). For *A. campanulatus*, the subspecies recorded is *A. campanulatus* subspecies *patens*.

52 clumps of *Agapanthus* plants were examined for eggs. The number of egg batches and number of eggs in each batch was recorded for each *Agapanthus* planting (Table A1-A2). A clump contained 20 plants of the same species and subspecies. The number of leaves present per plant was counted. The length and width of 10 leaves of each subspecies and species were measured to determine if leaf size affected preferences by the female moth (Table A3-A4).

Furthermore, in Silver Mine Nature Reserve in Muizenberg, 2 clumps (40 plants) of *Agapanthus africanus* subspecies *africanus* was assessed for eggs and the leaf sizes were obtained. This species is however not included in this study as it occurred in a different locality at a higher elevation.

Statistical analyses

The percentage of plants infested with eggs within a species and among species was calculated. The mean, standard error (S.E.), maximum and minimum egg count per *Agapanthus* species and subspecies was determined using descriptive statistics. The mean number of leaves per plant, the mean leaf length and mean leaf width were calculated. Boxplot graphs were used to show the egg occurrences and leaf sizes among each species and subspecies. Analysis of variance (ANOVA) and Tukey's post hoc test were performed to denote any significant differences occurring within and among species.

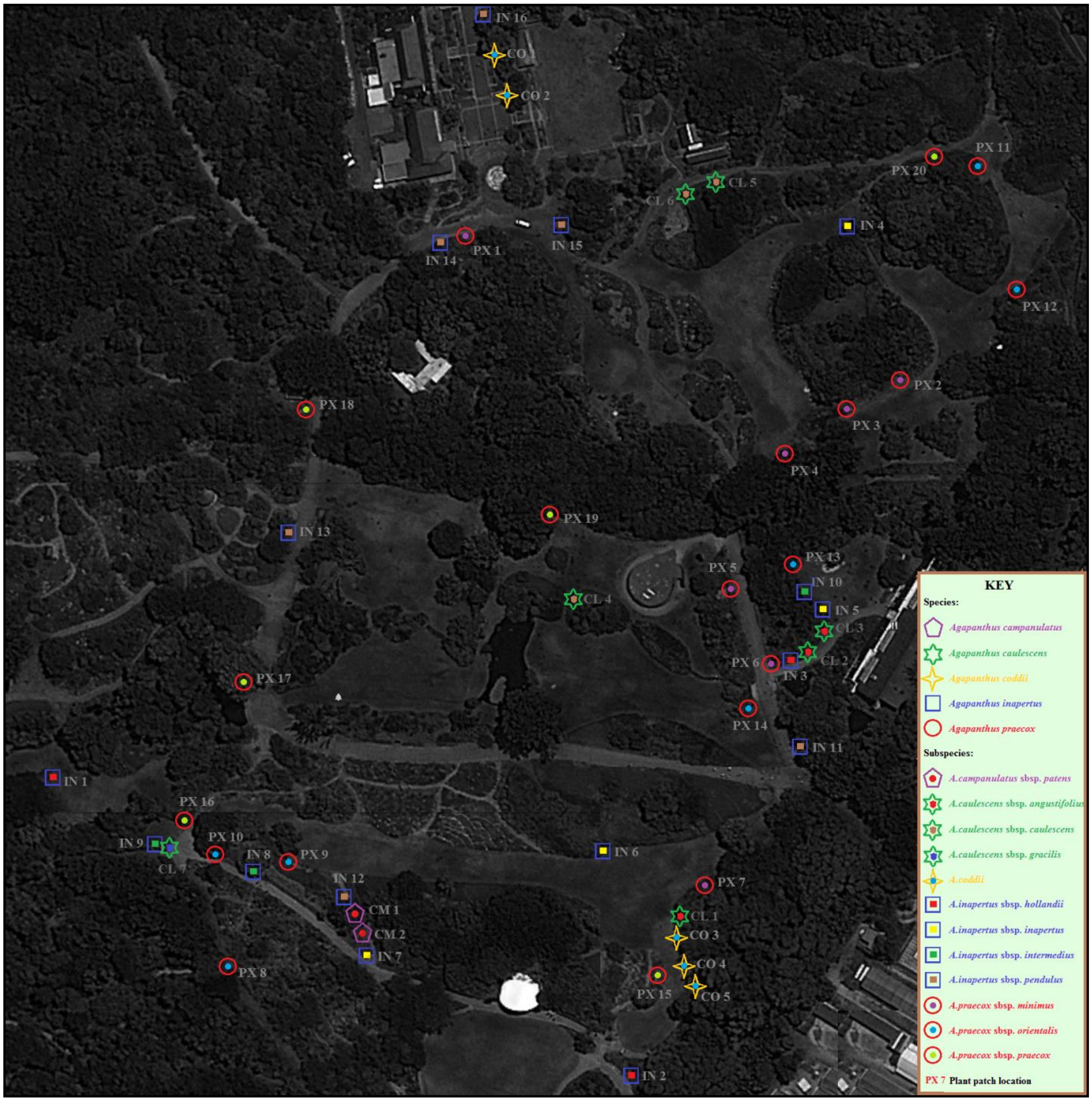

Figure 5: Aerial map of the study site showing the different clumps of Agapanthus species/subspecies at Kirstenbosch National Botanical Garden.

Results

A total of 1000 plants (9082 leaves) were assessed over the period of April-June. Five species of *Agapanthus* were covered (Table A1) as well as the subspecies of *Agapanthus caulescens*, Agapanthus *inapertus* and *Agapanthus praecox* (Table A2). The total number of eggs counted was 3281 eggs. *A. inapertus* recorded the highest egg count (1954 eggs) followed by *A. praecox* (894 eggs) and *A. caulescens* (412 eggs). All the other species recorded less than 16 eggs (Table A1).

Interspecific egg count

A graph of the mean number of eggs against species showed significant differences in the amount of eggs present in some species (ANOVA: $F= 35.94$, $n=995$, $df=4$, $p = 0.00$) (Figure 6a). The number of eggs occurring on *A.inapertus* (6.11 ± 0.30 eggs) showed significant differences when compared to all other species ($p<0.05$) with *A. inapertus* having the highest egg count indicating that the female Agapanthus borer moth preferred this species to the others during oviposition. *Agapanthus praecox* (2.23 ± 0.27 eggs) and *Agapanthus caulescens* (2.94 ± 0.46 eggs) egg count differed significantly from that on *Agapanthus coddii* (0.15 ± 0.54 eggs) which had a lower egg count than the other two species which implied that *A. coddii* was least favoured as an egg-laying host plant ($p=0.007$ and $p=0.001$ respectively). There was no significant difference when comparing *A. campanulatus* (0.15 ± 0.86 eggs) with *A. coddii* and *A. praecox* ($p>0.05$) indicating that the number of eggs occurring on *A. campanulatus* was similar to the egg counts on *A. coddii* and *A. praecox*. Moreover, significant differences of this species with A.caulescens and *A.inapertus* were noted suggesting that *A. campunulatus* was, like *A coddii*, least favoured during oviposition by the female moth ($p=0.04$ and $p<0.05$ respectively). When comparing egg dispersal on *A. praecox* and *A. caulescens*, no significant difference was seen indicating that there was no preference in oviposition between those two host plant species as their egg count was very similar to one another ($p=0.69$). The ranking for oviposition preference was such that *A. inapertus* was the most preferred followed by both *A. praecox* and *A. caulescens*. *A. coddii* and *A. campanulatus* were the least preferred oviposition choice by the female moth.

Interspecific leaf sizes

A. campanulatus appeared to have the longest leaves (54.42 ± 1.30 cm) and *A. caulescens* and *A. praecox* both exhibited the shortest leaves (40.44 ± 2.19 cm and 40.42 ± 2.35 cm respectively) (Figure 6b, Table A3) (ANOVA: F= 2.19, n=162, df=4, p = 0.07). No significant differences were noted amongst the leaves of all the *Agapanthus* species (p>0.05) suggesting that the leaf sizes of the different species were all within the same range. *A. praecox* and *A. caulescens* were found to have the same leaf length (p=1.00).

Among all the *Agapanthus* species, *A. coddii* recorded the broadest leaves (4.56 ± 0.26 cm) and *A. campanulatus* had the narrower leaves (2.58 ± 0.08 cm) (Figure 6c, Table A3) (ANOVA: F= 3.74, n=162, df=4, p = 0.01). There were some levels of significant differences in the leaf width in most of the *Agapanthus* species when compared to *A. coddii* (p < 0.05) suggesting that these other species all had narrower leaves than *A. coddii*; with the exception to *A.caulescens* (p= 0.16) indicating that these two species had the same leaf width. All other species do not differ significantly in terms of leaf width (p>0.05) indicating that these species had similar leaf width sizes when compared to one another.

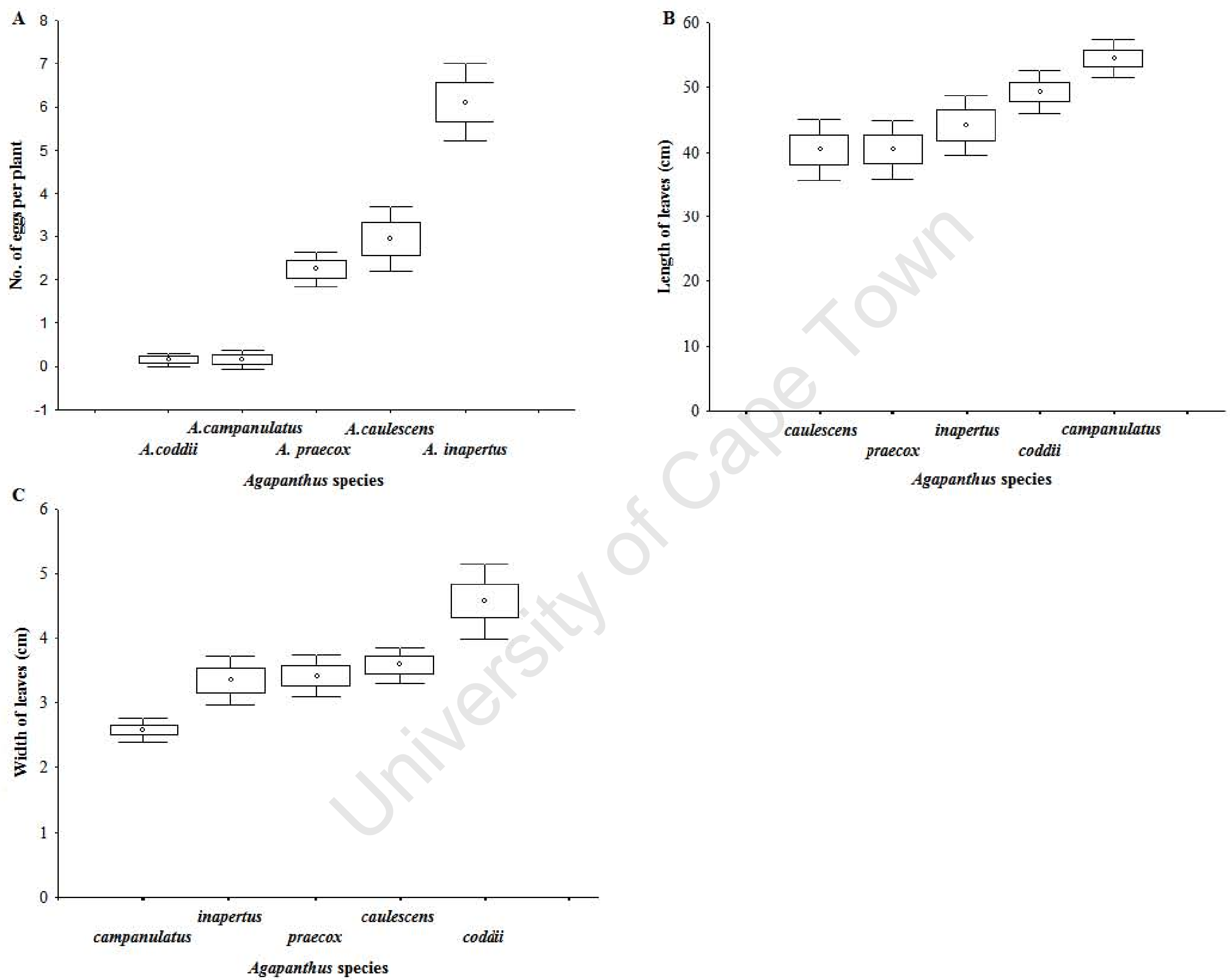

Figure 6. Boxplots for the number of eggs per plant (A), leaf length (B) and leaf width (C) against *Agapanthus* species.

Interspecific relationship between egg count and leaf sizes

There is no linear relationship between the leaf sizes and the number of eggs present in the different species of *Agapanthus* (Figure 7). Therefore, it can be seen that the leaf sizes of *Agapanthus* plants did not influence the female host choice during oviposition by the female *N. spodopterodes* moth.

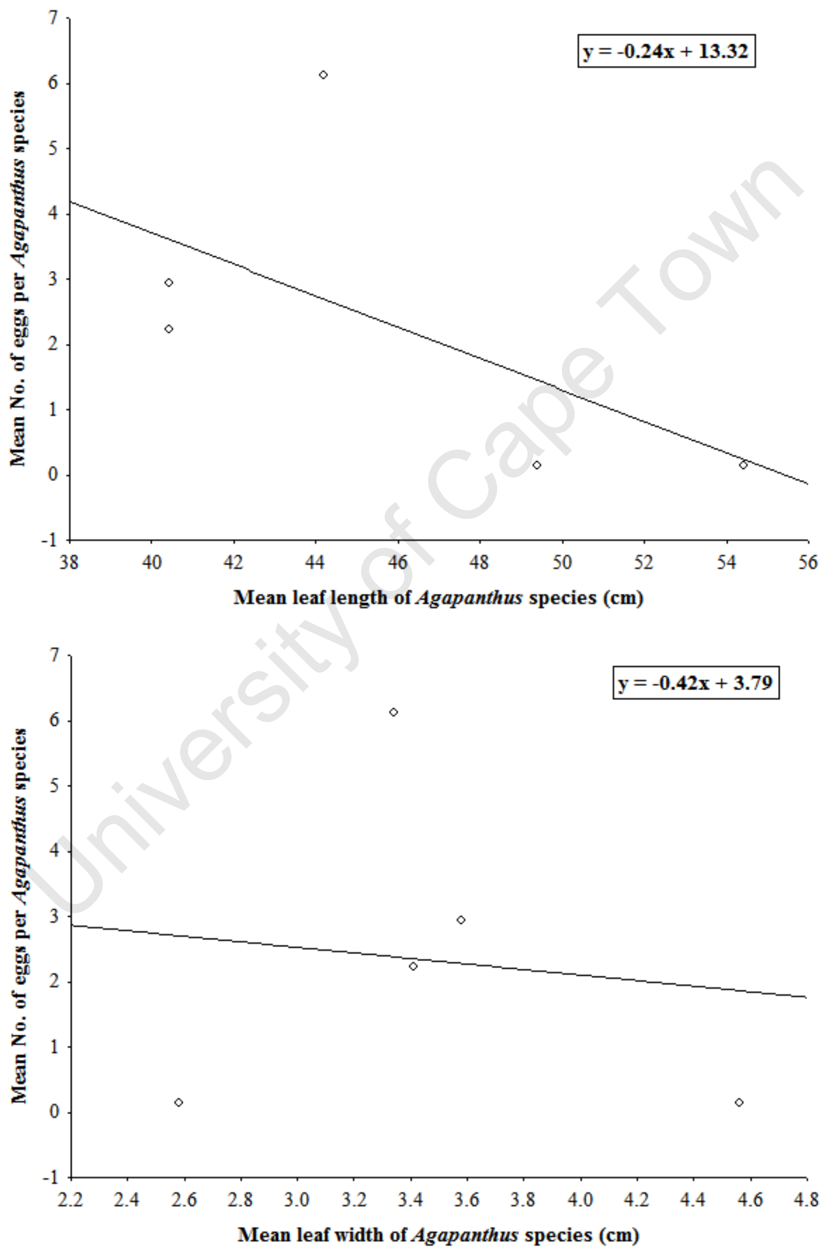

Figure 7: Linear relationship between leaf sizes and egg count on *Agapanthus*.

Intraspecific egg count and leaf sizes

A. caulescens:

The three subspecies of *A. caulescens* differed significantly in the number of eggs present on them (Figure 8a, Table A2) (ANOVA: F= 11.99, n=137, df=2, p = 0.00).The mean number of eggs on *A.c.angustifolis* (0.93 ± 0.37 eggs) differed significantly with those of the two other subspecies (p<0.05) with this species having a smaller egg count. The average egg count on *A.c.caulescens* (4.42 ± 0.64 eggs) and *A.c.gracilis* (4.55 ± 1.13 eggs) were similar (p=0.99) suggesting that the female preferred both these subspecies during oviposition.

The leaf length of the three *A. caulescens* subspecies did not differ significantly (p>0.05) (Figure 8b, Table A4) (ANOVA: F= 0.04, n= 27, df= 2, p= 0.96). The leaf width of *A.c.angustifolis* (2.91 ± 0.13 cm) differed significantly with those of the two other subspecies (p<0.05) (Figure 8c, Table A4) whereby *A.c.angustifolis* had the narrowest leaves. The average leaf width of *A.c.caulescens* (3.75 ± 0.04 cm) and *A.c.gracilis* (4.08 ± 0.28 cm) were similar and therefore, these two subspecies had the same leaf broadness (p=0.40) (ANOVA: F= 11.41, n= 27, df=2, p = 0.00).

A. inapertus:

The mean number of eggs on *A.i.intermedius* (0.35 ± 0.16 eggs) was similar to that of *A.i.hollandii* (1.07 ± 0.30 eggs) (p=0.94) indicating that these two species were least favoured during oviposition by the female moth. All the other subspecies differed significantly with one another in terms of the mean number of eggs present in each subspecies (Figure 8a, Table A2) (ANOVA: F= 46.20, n=316, df=3, p = 0.00). *A.i.inapertus* appeared to have the highest mean egg count (11.85 ± 1.05 eggs) followed by *A.i.pendulus* (7.67 ± 0.72 eggs) suggesting that the female moth preferred those two subspecies over the others for oviposition.

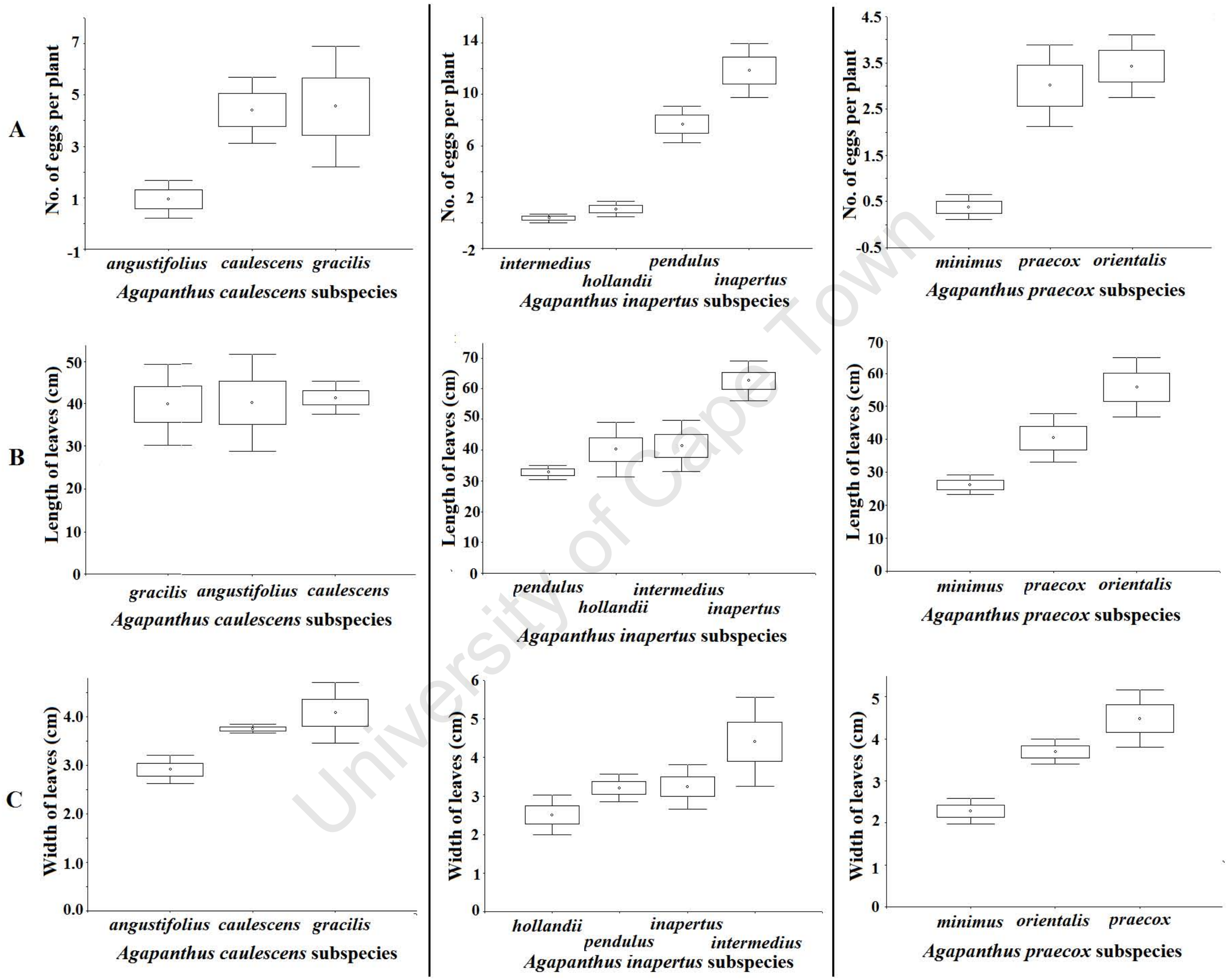

Figure 8: Boxplots of egg count (A), leaf length (B) and leaf width (C) of *A.caulesecens*, *A.inapertus* and *A.praecox* subspecies.

A.i.inapertus mean leaf length (62.5 ± 2.91 cm) differed significantly with all the other subspecies leaf lengths ($p<0.05$) and it was the subspecies with the longest leaves (ANOVA: F= 17.10, n=36, df=3, p = 0.00). The leaf length of the other three *A. inapertus* subspecies did not differ significantly ($p>0.05$) therefore these subspecies all had the same leaf lengths (Figure 8b, Table A4). The leaf width of *A.i.intermedius* was the broadest (4.41 ± 0.51 cm) and it differed significantly to that of *A.i.hollandii* (2.51 ± 0.23 cm) which had the most narrow leaves (Figure 8c, Table 6) (ANOVA: F= 6.15, n=36, df=3, p = 0.00). The average leaf widths of all the other *A. inapertus* subspecies did not differ significantly indicating that they all had the same leaf broadness ($p>0.05$).

A. praecox:

The mean number of eggs on *A.p.minimus* (0.38 ± 0.13 eggs) differed significantly with those of the two other subspecies (ANOVA: F= 27.27, n=397, df=2, p = 0.00) with this subspecies having the lowest egg count. The average egg count on *A.p.orientalis* (3.43 ± 0.34 eggs) and *A.p.praecox* (3.01 ± 0.44 eggs) were similar (p=0.63) suggesting that the female moth showed no preferences between these two subspecies during oviposition.

The leaf length and width of the three *A.praecox* subspecies differed significantly ($p<0.05$) (Figure 8b-c, Table A4) (ANOVA: Length: F= 21.25, n=74, df=2, p = 0.00; Width: F= 27.99, n=74, df=2, p = 0.00). *A.p.orientalis* had the longest leaves (55.8 ± 4.40 cm) and *A.p.praecox* had the broadest leaves (4.48 ± 0.33 cm). *A.p.minimus* had the smallest leaves both in terms of length (26.17 ± 1.46 cm) and width (2.27 ± 0.15 cm) (Table A4).

Percentage infestation

From Table A1 and A2, it can be seen that infestation of the plants with eggs was very pronounced in some species. *A. coddii* and *A. campanulatus* were the least infested (≤ 5% infested). *A. inapertus*, *A. caulescens* and *A. praecox* were the most infested species (53.12%, 40.71% and 32.75% respectively). Within *A. inapertus*, the subspecies *A.i.inapertus* was the most infested species (85%) followed by *A.i.pendulus* (70.83%). *A.c.caulescens* and *A.c.gracilis* were the most infested subspecies within *A. caulescens* with an infestation of 63.33% and 60.00% respectively. There are two *A. praecox* subspecies that were the most infested (>39%). These were *A.p.orientalis* and *A.p.praecox*.

Intraspecific relationship between egg count and leaf sizes

There is no linear relationship between the leaf sizes and the number of eggs present in the different subspecies of *A. caulescens*, *A. inapertus* and *A. praecox* (Figure 9). Therefore, it can be seen that the leaf sizes of the *Agapanthus* subspecies did not influence the female host choice during oviposition by the female *N. spodopterodes* moth.

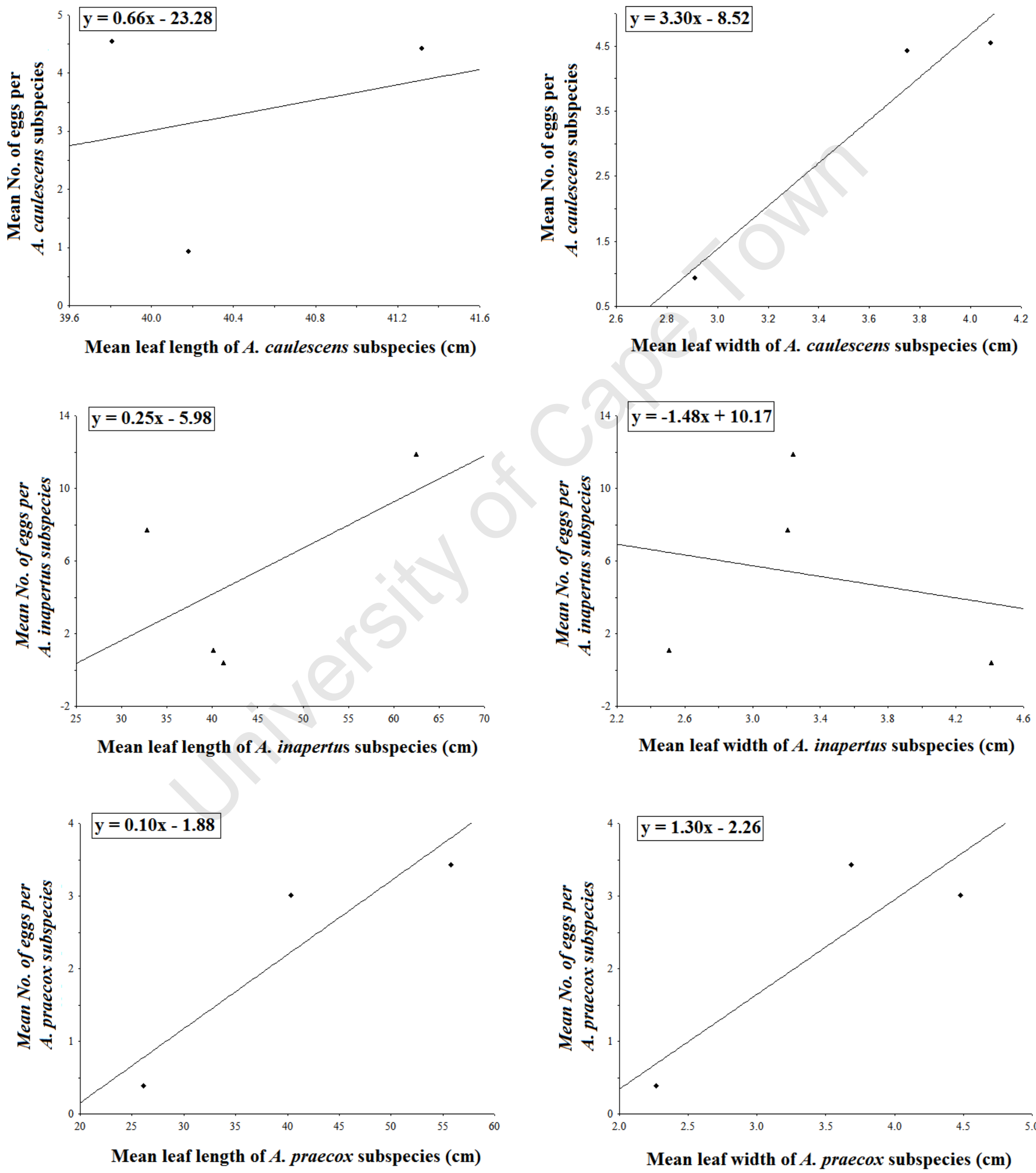

Figure 9: Linear relationship between leaf sizes and egg count in *Agapanthus* subspecies.

Discussion

When relating the leaf sizes to the number of eggs in each species of *Agapanthus*, there is no significant relationship between the leaf sizes and the eggs number. Therefore, leaf length and width of *Agapanthus* species and subspecies does not act as visual cues during host selection by the female *N. spodopterodes*. Other avenues act as stimuli to influence female oviposition choices.

Based on the female host choice for oviposition, this study demonstrated that *A.inapertus*, *A. caulescens* and *A. praecox* were the most attacked plants by the *Agapanthus* borer in ranking order of preference. Therefore, it can be inferred that these species are the most suitable for larval performance and survival due to their suitable nutritional values. Since *A. coddii* and *A. campanulatus* have some egg occurrences, it could mean that in the event of presence of conspecifics egg/larva already present on the three main host species, the female moth may opt to lay her eggs on those alternative plant species, even if these plant nutritional values may be lower, the larva still have a chance of survival. This assumption is reinforced by the fact that only one last instar larva was found per plant despite the presence of numerous hatched eggs on the same plant. Therefore to limit competition of resources by its offspring, the size of the egg load plays an important role in oviposition (Thompson & Pellmyr, 1991).

However, leaf sizes of the *Agapanthus* plant are not factors that attract the female *N. spodopterodes*. Secondary compounds like volatile or contact chemicals present in plants affects the female host preference (Mphosi & Foster 2012). *Agapanthus* plants contain several allelo-chemicals which may be volatile and perceivable by the female moth from a distance. Chemicals like chalconoids, saponins and sapogenins have been reported to occur in *Agapanthus* leaves, rhizomes and roots (Gonzalez et al, 1996 in Hutchings; Kamara et al, 2000; Van Wyk et al, 2005). These chemicals bear anti-bacterial, anti-fungal and anti-inflammatory properties. No previous studies have evaluated how these chemicals affect the female moth oviposition choices. It can be assumed, that these chemicals may play an important role in female oviposition preference.

Moreover, flower coloration, shape and sizes of a plant may also affect the female choice for laying eggs (Thompson & Pellmyr, 1991). Studies have found that pollen coloration varied across the different species of *Agapanthus* (Zonnevald & Duncan, 2003). However, eggs were already laid before the opening of flowers (Picker & Krüger, 2013), indication that flower or pollen coloration and sizes were not selection cues by the female *N. spodopterodes* moth.

Post alighting factor showed that after a female have selected the host plant, the eggs were all laid on the underside of the leaves. This confers protection to the eggs against dessication from direct sunlight and conceals the eggs from potential predators. The epidermal cuticle is generally softer on the underside of leaves and this makes it easier for the newly hatched larva to bore their way into the leaves. Therefore, it can be seen that female moth abides by the "mother knows best" theory and that her oviposition behaviour is such that it optimizes larval performance and survival (Nylin et al, 2000). This suggests that the oviposition strategy of N.spodopterodes is that of a specialist (Futuyma & Moreno, 1988).

Furthermore, no eggs were noticed in *A. africanus*. This suggests that the moth may have not reached the area where this species thrive since it can only be found in higher and cooler elevations. In the event of *N.spodopterodes* shifting to a higher elevation due to the recent changes in the climate, *A. africanus* may become infested with the Agapanthus borer. Further analysis of the chemical composition of all the *Agapanthus* species will allow for a proper assessment of the chemical attractants used by the gravid female N. spodopterodes to select a host plant and to determine if *A. africanus* is indeed at risk of becoming a potential host plant to the Agapanthus borer.

Chapter 3

Feeding preference by the larva of *Neuranethes spodopterodes*

Introduction

Performance of larval offspring in Lepidoteran is greatly influenced by the oviposition host plant choice made by the female parent since early larval instars have a very limited dispersal range (Schoonhoven et al. 2005). Therefore, nutritional quality of the selected host plant is vital in larval survival. The preference-performance hypothesis (Jaenike, 1990) predicts that eggs are laid by the mother on host plants with high nutritional quality that will optimize larval performance such that they will grow faster and therefore, have a better chance of survival (Mayhew 1997). Several studies have put this hypothesis in evidence (Mphosi & Foster, 2010). but a clear correlation between performance and preference is not always evident. Ohsaki and Sato (1994) have demonstrated that females may prefer to oviposit on less nutritive plants. However, this behaviour is influenced by other ecological factors which overrides the unsuitability of the host plant such as less nutritious plant are less attractive to predators and parasitoids (Björkman et al. 1997; Sadek et al. 2010), therefore ensuring an enemy free space for optimal larval survival.

Phytophagous insects can be defined from their array of host plants. Monophagous insects feed on a single host plant and also include species that feeds on a number of plants within the same genus. Polyphagous insects feed on a larger number of hosts from several families. Oligophagous insects feed on hosts from different genera but within the same family (Bernays & Chapman, 1994). In polyphagous insects, host plants may differ in their suitability, and therefore a gravid female has elaborated a hierarchy ranking of host prefence correlating with larval fitness (Nylin & Janz, 1996). Different oviposition strategies may exist within species, namely generalists and specialists (Futuyma & Moreno, 1988). Specialists specialize on one host species or genera while generalists oviposit on a number of hosts. The oviposition strategy of a specialist is advantageous when the high nutrional quality host plant is abundant, thus offspring are supplied with a diet that optmizes their fitness. Additionally, specialists are more skilled at differentiating between good and poor host plant resulting in a decrease in poor oviposition choices (Bernays & Chapman, 1994; Nylin et al., 2000). Due to poor selection skills, a generalist oviposition strategy is associated with poor nutritional quality of host plant and therefore leading to "bad motherhood" (Janz & Nylin, 1997; Mayhew, 2001).

Upon hatching, *N.spodopterodes* larva bores its way into *Agapanthus* leaves where they remain to feed for some time. Upon exhaustion of food supply, the larva travels downwards to the innermost leaves to continue feeding. The last instars larva feed deeper into the innermost leaves and into the rhizome. (Picker & Krüger, 2013). The larva is found to remain on the same plant on which its egg was laid. Host shifting have not been recorded for this species since the mother have chosen the optminal host plant that maximizes larval fitness (Jaenicke, 1990). This type of preference is known as innate preference whereby the larva does not choose which host to feed on but rather it is the female moth that chooses for its offspring.

In this section, larva of *N.spodoterodes* will be given a choice of the six species of *Agapanthus* whereby the preferred feeding host species will be assessed in order to demonstrate a feeding hierarchy that correlates with the oviposition choices of the female moth.

Materials and methods

Larva

N. spodopterodes larvae which hatched from eggs from the rearing experiment at 22°C (Chapter 1) were used in the food choice experiment. The head capsule widths of the larva were measured to ensure that all larvae used were in the same instars. In this experiment, only 4^{th} instar larvae were assessed.

Food choice experiment

Leaves from all six *Agapanthus* species were used. The subspecies of *Agapanthus* used were selected on the basis of the plant on which the most eggs occurred (Chapter 2). The following subspecies were selected: *A. caulescens gracilis*, *A. inapertus inapertus, A. praecox orientalis*. For the other *Agapanthus* species, only one subspecies were available; notably *A. africanus africanus* and *A. campanulatus patens*. *A. coddii* has no subspecies and therefore the species itself was used in the feeding trials.

The leaves were cut in a small rectangular shape and weighed such that all the six species had the same starting weigh. The leaves were numbered so the leaf species were easily identifiable. The leaves were randomly placed into an enclosed petri-dish containing a 4^{th} instar larva. For the control experiment, a second petri-dish was set up similarly to the first petri-dish but with the larva removed from it in order to assess any reduction in the weight of the leaves due to water evaporation. The containers were placed in a constant temperature room at 22°C with 12 hours photoperiod for 24 hours. Following this, all the leaves were re-weighed and any loss in mass was tabulated. The experiment was repeated until a total of twenty 4^{th} instar larva feeding trials were conducted.

Statistical analyses

Calculation of the mass of leaves eaten was found by subtracting the final weight from the initial weight. A boxplot graph was used to show the feeding preference by the Agapanthus borer to each *Agapanthus* species. Analysis of variance (ANOVA) and Tukey's post hoc test were performed to denote any significant differences occurring among species feeding preferences.

Results

The larva appeared to feed on *A. caulescens* (0.18 ± 0.03 g), *A. praecox* (0.16 ± 0.02 g), *A. inapertus* (0.16 ± 0.03 g), *A. campanulatus* (0.15 ± 0.02 g), *A. coddii* (0.13 ± 0.03 g) and *A. africanus* (0.07 ± 0.02 g) leaves in ranking order from most preferred to least preferred food choice.

The amount of leaves per Agapanthus species consumed by the 4th instars larva were similar in most species (Figure 10) (ANOVA: $F = 2.51$, $n_1 = 6$, $n_2 = 120$, $df = 114$, $p = 0.03$). The mass of leaves consumed on *A. africanus* and *A. caulescens* differed significantly, whereby the larva preferred to feed on *A. caulescens* and fed poorly on *A. africanus* ($p=0.02$). However, *A. africanus* was equally consumed by the larva as the remaining four species ($p>0.05$).

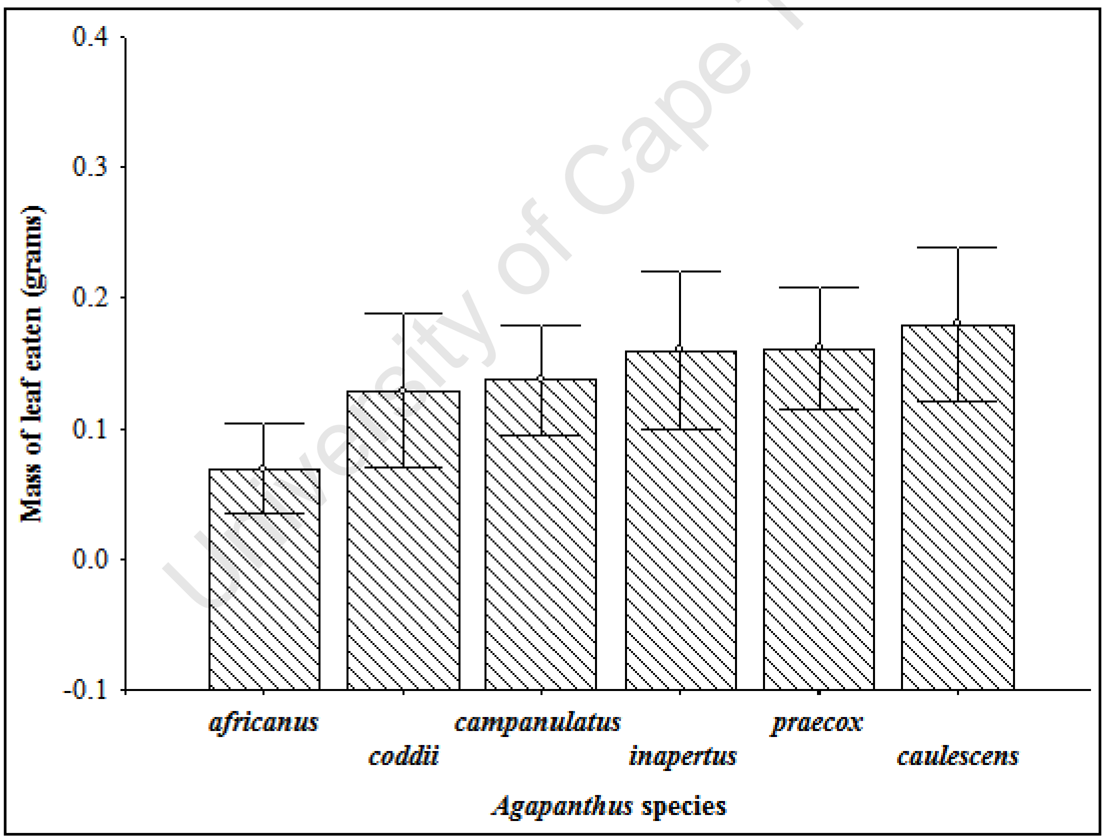

Figure 10: Mass leaf eaten of the six *Agapanthus* species by 4th instar *N.spodoterodes* larva.

However, since no significant differences were found among the six choices of *N. spodopterodes*, it could be assumed that the larva did not have a specific feeding preference.

Discussion

The Agapanthus borer larva did not appear to make any specific choices when picking out the highest quality host plant. This suggests that the leaves of all the *Agapanthus* species were equally palatable to the larva further inferring that they all were high quality leaves which optimized fitness and survival of the larva. Therefore in a natural setting, this could explain why once a female moth oviposited on a specific *Agapanthus* species, the larva remained at the same feeding site since the mother had already made the host choice for the larva. Furthermore, this reinforces the preference-performance hypothesis (Jaenicke, 1990), whereby the female moth makes the best oviposition choices to ensure that her offsprings are on the highest quality plant for its survival and fitness.

Since the larva fed on all six species of *Agapanthus*, it can be assumed that the chemical composition of the various species did not differ significantly as they all yielded in a high performance of all the twenty assessed larvae (all of which pupated in the rearing experiment (Chapter 1). Therefore secondary compounds present in *Agapanthus* species did not appear to adversely affect larval performance. However, leaves of *A. africanus* and A. *caulescens* may exhibit some differences in chemical composition affecting the overall palatability whereby *A. caulescens* will be favoured by the larva which will only feed on *A. africanus* once *A. caulescens* is completely consumed if a dual choice test was performed between those two leaves only.

The implications of this feeding pattern suggest that the rare species *A. africanus* and subsequently *A. africanus* subspecies *walshii* may be at risk of *N. spodopterodes* infestation in the event that the female moth shifts its range to a higher elevation. Since the larva was found to feed on *A. africanus* in laboratory setting, if the environmental conditions are favourable, the Agapanthus larva have the potential to feed on this species in nature causing irreversible damage which may lead to the extinction of the species *Agapanthus africanus* in South Africa.

Chapter 4

Parasitoids of *Neuranethes spodopterodes* eggs

Introduction

In response to herbivory, defense mechanisms are induced in host plants which affect the performance of the herbivore which can occur as plants modify their chemical composition (Schoonhoven et al. 2005). Not only the herbivore and its conspecifics are affected by this change (Denno et al. 1995), but other herbivores as well. Indirectly the trophic level is also affected by the change in plant chemistry, for example, volatile chemicals act as selection cues for third trophic level like parasitoids and predators (Dicke et al. 2003). Plants are repeatedly attacked either simultaneously or in succession by several herbivores (Vos et al. 2001). These attacks on a single plant may alter plant-herbivore-parasitoid interactions (Dicke et al. 2009). The type of interaction is coined indirect defense since the volatiles do not affect the herbivore itself but is used to signal third trophic level that it is under attack by herbivores.

The evolution of such chemical signalling cues occured from direct defence whereby volatile chemicals from synthesis of toxic substances in plants were used by parasitoids and predators of herbivorous species to locate a plant under stress from herbivory (Turlings et al, 1995). A study on indirect defense of damaged corn by *Spodoptera* larva demonstrated that the distressed plant attracted the parasitoid wasp, *Cotesia* (Turlings et al., 1995) which laid its egg in a *Spodoptera* larva on which it feed till it caused the death of its host. Infested plants and mechanically damaged plants produce different volatiles. A study by Turlings et al (1995) demonstrated that by placing plant with cutted stems in dilute larval regurgitate, a specific plant response is obtained as the plants became more attractive to parasitoids which responded to the presence of the compound volicitin. Volicitin is present in the regurgitate of larvae of the genus *Spodoptera* (family Noctuidae) and of the tobacco hornworm, *Manduca sexta* (family Sphingidae) (Alborn et al., 2003).

Parasitoids are insects that lay their eggs inside or on the outside of the eggs or bodies of other insects. Upon hatching, the parasitoid larvae (the only parasitic stage of a parasitoid) consume its host from the inside ultimately leading to the host death at which time the parasitoids emerge as free-living adults (Quicke, 1997). Parasitoids are host and stage specific insects. Most parasitoids are smaller in comparison to their host and only a single host is necessary for them to complete their life cycle (Alphen & Visser, 1990).

Biological control is the use of bio-agents like predators or parasitoids for natural management of insect pests (Debach & Schlinger, 1964). The use of parasitoids as biological control agents have been intensively studied in recent years. For example, the parasitoid *Diaeretiella rapae* was introduced in Australia to control aphids populations with much success (Baker et al, 2003).

In this study, *N. spodopterodes* eggs were collected to observe the presence of any parasitoids which could potentially act as biological control agent in the management of the Agapanthus borer in order to reduce its impact on *Agapanthus* plants.

Materials and methods

Egg collection

Egg batches of the Agapanthus borer were collected from several *Agapanthus* plants from February to April in Kirstenbosch National Botanical Garden (S33°59 and E18°25) and in Rondesbosch, Cape Town (S33°57 and E18°27). The eggs were kept in closed vials and allowed to hatch. Colour changes in the eggs were noted.

Parasitoid count and identification

The vials were frozen when parasitoids emerged. The vials were washed using ethanol 70% and the content was poured in a petri-dish. Using a compound microscope, the number of parasitoids present per eggs batches was counted and tabulated. Using a Leica EZ4 HD and Leica Microsystems LAS EZ imaging software, pictures of the parasitoid was taken for proper identification to be made.

Statistical analysis

Using basic statistics, the mean number of parasitoids and the percentage parasitism was calculated in the Agapanthus borer eggs using Statistica 7.

Results

Parasitoid identification

The parasitoid was identified to belong to the genus *Trichogramma* (Hymenoptera, Trichogrammatidae) (Figure 11). Parasitized eggs changed colour from healthy pink coloration to black which coincided with the pupal stage of the *Trichogramma*.

Figure 11. The parasitoid, *Trichogramma* specimen of *N. spodopterodes* eggs. © Mike Picker

Parasitoid count

A total of 36 egg batches were collected. Five of the eggs batches were empty. All the other 31 eggs batches were parasitized with the *Trichogramma*. Each egg batches contained 9.36 ± 2.08 eggs. The total number of parasitized eggs was 337 eggs and 1456 parasitoids emerged from those eggs with on average 40.4 ± 8.62 parasitoids per egg batches. The minimum number of parasitoids emerging from an egg batch was 8 parasitoids and the maximum was 246 parasitoids. Out of 36 egg batches, 31 were parasitized indicating that Trichogramma infested more than 86.1% of all egg batches collected and out of 390 collected eggs, 337 were parasitized representing 86.4% of all the eggs collected.

Discussion

Trichogramma species are facultatively sociable insects (Rabinovich 1971). They are polyphagous egg parasitoids (Smith 1996). They are widely used as bio-agents against a wide range of Lepidopteran eggs (Corrigan & Laing 1994). *Trichogramma* specimens have been found to be synovigenic (Jervis et al. 2001). Females lay eggs shortly after emergence (Chassain & Boulétreau 1991). Sexual dimorphism occurs within this species with males being generally smaller than females (van den Assem et al. 1989). Males tend to emerge before females from the host (Charnov et al. 1981).

The short life cycle of Trichogramma wasps and their abibity to be reared on factitious hosts allow these wasps to be produced on a big scale at avery fast rate (Li, 1994). *Trichogramma* is widely used in the control of agricultural pests worldwidely. The use of Trichogramma pretiosum in citrus to control *E. aurantiana* (Lima), the citrus fruit borer have been demonstrated (Molina, 2003). In Brazil, egg parasitism of *Anticarsia gemmatalis*, a soybean herbivore, by *T. pretiosum* exceeds more than 90% (Zachrisson 1997).

The high level of parasitism inflicted on *N. spodopterodes* eggs by the parasitoid *Trichogramma* is very promising as a potential biological control agent in managing the impacts of the Agapanthus borer on the horticultural trade of *Agapanthus* plants. Bearing in mind that only one or two Agapanthus borer larva survives till pupation, the presence of this parasitoid greatly reduces the egg performance and therefore reduces the number of hatchlings considerably and consequently since the mortality rate till pupation due to competition of resources is very high, this optimizes the probability that no Agapanthus larva may reach pupation.

Conclusion

This study shows the *N.spodopterodes* have 6 larval instars. The use of the head capsule width as a morphometric parameter and applying it to Dyar's rule gives significant results which allow for adequate determination of the larval instar. The *Agapanthus* borer has a life cycle that last approximately 84 days.

Some species of *Agapanthus* are the preferred host plant for oviposition by the female *N.spodopterodes*. *Agapanthus inapertus*, *Agapanthus caulescens* and *Agapanthus praecox* are the most infested with the *Agapanthus* borer eggs. Leaf sizes do not appear to be a factor that influences the female choice.

There appears to be no preference from the larva during feeding. The larva fed equally on all the six *Agapanthus* species including *A. africanus* ($p>0.05$). This shows that the chemical composition of these species is closely related. Since the larva has no feeding preference, this reinforces the fact that it is the female moth that chooses the host plant and that it abides with preference-performance hypothesis. Since *A. africanus* is subjected to feeding by the larva under laboratory conditions, it could potential imply that in the event of a range shift by the gravid female, that this species of plant is at risk of extinction.

The presence of a parasitoid wasp, *Trichogramma* in the egg of *N. spodopterodes* and that the level of parasitism is about 86% suggests that this parasitoid could potentially be used as a biological control agent of the Agapanthus borer.

However, in depth studies is required to determine the duration of the life cycle between males and females moths as many Lepidopteran studies have found that the gender of an individual affects the overall duration of the various life stages. The study conducted on the female host plant was restricted to one locality only. It is therefore important to expand the range of this study to the greater Western Cape region to assess the impact of the *Agapanthus* borer at a larger scale. Focus should be made in the female oviposition choice in determining if the rare *Agapanthus africanus* subspecies are at risk from this moth. The chemical composition of all the species and subspecies should be assessed in order to get more insight in how the female *N. spodopterodes* chooses which plant to lay her eggs on. The food choice experiments on the larva should be carried such that all subspecies of *Agapanthus* are included each at each instars since this study only took into account 4th instar larva. Proper identification of the parasitoid larva is needed in order to implement appropriate biological control. Assessing the development rate of the parasitoids will be helpful. The effect of the secondary compounds on larval performance should be explored. Only after all these objectives have been concluded that a better understanding of how to control the spread of the translocated moth, *N. spodopterodes* will be achieved.

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Appendix

Table A1: Number of plant sampled per *Agapanthus* species showing the intensity of the egg infestation.

Species	Total No. of plants sampled (No. of clumps)	No. of plants with egg batches	Percentage of infested plants (%)	Total No. of eggs found	Mean number of eggs per plant (± S.E.)	Minimum and Maximum No. of eggs per egg batch
A. campanulatus	40 (2)	2	5.00	6	0.15 ± 0.86	2-4
A. caulescens	140 (7)	57	40.71	412	2.94 ± 0.46	2-21
A. coddii	100 (5)	4	4.00	15	0.15 ± 0.54	2-6
A. inapertus	320 (16)	170	53.12	1954	6.11 ± 0.30	2-37
A. praecox	400 (20)	131	32.75	894	2.23 ± 0.27	1-26

Table A2: Number of plant sampled per *Agapanthus* subspecies showing the intensity of the egg infestation.

Species	Subspecies	Total No. of plants sampled (clumps)	No. of plants with eggs	Percentage of infested plants (%)	Total No. of eggs found	Mean number of eggs (± S.E.)	Min and Max No. of eggs per egg batch
A. caulescens	*angustifolius*	60 (3)	7	11.67	56	0.93 ± 0.37	3-13
A. caulescens	*caulescens*	60 (3)	38	63.33	265	4.42 ± 0.64	2-21
A. caulescens	*gracilis*	20 (1)	12	60.00	91	4.55 ± 1.13	4-19
A. inapertus	*hollandii*	60 (3)	12	20.00	64	1.07 ± 0.30	3-9
A. inapertus	*inapertus*	80 (4)	68	85.00	948	11.85 ± 1.05	2-37
A. inapertus	*intermedius*	60 (3)	5	8.33	21	0.35 ± 0.16	2-7
A. inapertus	*pendulus*	120 (6)	85	70.83	921	7.67 ± 0.72	2-34
A. praecox	*minimus*	140 (7)	10	7.14	53	0.38 ± 0.13	2-13
A. praecox	*orientalis*	140 (7)	73	52.14	480	3.43 ± 0.34	2-17
A. praecox	*praecox*	120 (6)	48	40.00	361	3.01 ± 0.44	1-26

Table A3: The leaf length and width of each of the species of *Agapanthus*.

Species	Total No. of leaves assessed	Mean No. of leaves per plant	No. of leaves per plant (Min-Max)	Mean Length of leaves ± S.E. (cm)	Leaf length Range (cm)	Mean Width of leaves ± S.E. (cm)	Leaf Width Range (cm)
A. campanulatus	239	5.97 ± 0.19	4-9	54.42 ± 1.30	49.6-60.4	2.58 ± 0.08	2.2-3.0
A. caulescens	968	6.91 ± 0.12	4-12	40.44 ± 2.19	6.5-53.7	3.58 ± 0.13	2.3-5.1
A. coddii	905	9.05 ± 0.11	7-12	49.39 ± 1.47	42.4-54.3	4.56 ± 0.26	3.5-5.8
A. inapertus	2974	9.29 ± 0.14	5-17	44.18 ± 2.31	14.4-74.2	3.34 ± 0.19	0.9-6.5
A. praecox	3996	9.99 ± 0.15	5-19	40.42 ± 2.35	3.2-84.4	3.41 ± 0.16	0.3-8.1

Table A4: The leaf length and width of each of the species of *Agapanthus*.

Species	Subspecies	Total No. of leaves assessed	Mean No. of leaves per plant	No. of leaves per plant (Min-Max)	Mean Length of leaves ± S.E. (cm)	Leaf length Range (cm)	Mean Width of leaves ± S.E. (cm)	Leaf Width Range (cm)
A. caulescens	*angustifolius*	421	7.02 ± 0.12	5-9	40.18 ± 5.06	6.5-53.7	2.91 ± 0.13	2.3-3.4
A. caulescens	*caulescens*	369	6.15 ± 0.15	4-10	41.32 ± 1.70	31.8-48.3	3.75 ± 0.04	3.5-3.9
A. caulescens	*gracilis*	178	8.90 ± 0.31	7-12	39.81 ± 4.25	12.4-52.9	4.08 ± 0.28	2.7-5.1
A. inapertus	*hollandii*	392	6.53 ± 0.12	5-8	40.16 ± 3.89	14.4-52.6	2.51 ± 0.23	0.9-3.2
A. inapertus	*inapertus*	765	9.56 ± 0.13	7-12	62.5 ± 2.91	48.6-74.2	3.24 ± 0.25	1.8-4.7
A. inapertus	*intermedius*	560	9.33 ± 0.28	6-15	41.27 ± 3.68	24.3-53.2	4.41 ± 0.51	2.5-6.5
A. inapertus	*pendulus*	1257	10.47 ± 0.72	7-17	32.79 ± 1.01	28.5-36.3	3.21 ± 0.16	2.1-3.6
A. praecox	*minimus*	1013	7.24 ± 0.15	5-11	26.17 ± 1.46	3.2-35.3	2.27 ± 0.15	0.3-3.0
A. praecox	*orientalis*	1612	11.51 ± 0.20	8-17	55.8 ± 4.40	16.5-84.4	3.69 ± 0.14	2.5-4.5
A. praecox	*praecox*	1371	11.42 ± 0.25	7-19	40.39 ± 3.55	9.6-69.6	4.48 ± 0.33	2.4-8.1