Augmentation of *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) for the control of California red scale *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae) on citrus

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Abstract

Aphytis lingnanensis was reared and tested in South Africa in the early 2000s for augmentation against red scale on citrus and was found to be ineffective. *Aphytis melinus* is now commercially available and it is important that the efficacy of augmentation thereof on red scale is determined locally. Field trials, fitness assessments and molecular identification on *A. melinus* from two insectaries were done. Field trials was done in seven, five and six pairs of comparable release and control orchards across the Eastern and Western Cape during the seasons of 2019/2020, 2020/2021 and 2021/2022 respectively. Red scale infestation was monitored and a sample of 20 infested fruit from each orchard was randomly collected every four weeks. *Aphytis* spp. responsible for parasitism were identified and the percentage parasitism recorded. Results of this study of field trials suggest that the augmentation of *A. melinus* did not significantly increase the level of parasitism above that of the untreated control. Five repetitions with six replicates of flight and longevity tests were performed with wasps from each insectary. Wasps in the longevity test from two insectaries were kept at 23 °C and 65% RH with honey. Flight tests were performed in tubes of 16 by 30 cm, with a light above a clear, sticky ceiling at 23 °C and 65% RH. On average in five replicates, 65%, 33% and 17% *A. melinus* wasps were alive on day one, five and 10 respectively. The overall sex ratio was 1.58 for females to males, but 1.05, 2.19 and 2.66 for non-flyers, non-crawlers, crawlers, and flyers respectively. In flight tests for both insectaries combined, only 36.97% of wasps could initiate flight in 24 h while 56.96% remained on the tube floor, and 6.05% attempted to crawl upwards. No significant differences in flight performance were recorded between the two insectaries. Wasps from the local insectary lived significantly longer during the longevity tests but were shorter in transit than wasps from the overseas insectary. COI genes were sequenced and compared against Genbank sequences using BLAST. Molecular identifications did not confirm morphological identifications for all species, indicating unexpected genetic complexity.

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List of abbreviations

%	percentage
µL	micro litre
°C	degree(s) Celsius
ANOVA	Analysis of variance
BLASTn	basic local alignment search tool nucleotide
bp	base pair
C	Crawler
cm	centimetre
CO1	cytochrome c oxidase subunit 1
DNA	deoxyribonucleic acid
EC	emulsifiable concentrate
EDTA	ethylenediaminetetraacetic acid
et al.	*et. alia* (and others)
EW	oil emulsion in water
F	Flyer
g/ Kg	grams per kilogram
h	hour(s)
Ha	hectare(s)
ID	identification
IPM	integrated pest management
L	litre
L-NFNC	live non-flyer non-crawler
m	metre
MAFFT	multiple alignment using fast Fourier transform
mg/ L	milligrams per litre
ml	millilitre
ML	maximum likelihood
ml/ L	millilitres per litre
mm	millimetre
mM	millimolar
Molecular ID	molecular identification
Morph. ID	morphological identification
NaCl	sodium chloride

NCBI	National Centre of Biotechnology Information
NFNC	Non-flyer non-crawler
nt	nucleotide
OB	occlusion bodies
PCR	polymerase chain reaction
pH	potential of hydrogen
R1	repeated measures
RH	relative humidity
SC	suspension concentrate
SDS	sodium dodecyl sulphate
SE	standard error
SL	soluble liquid
SP	soluble powder
s	seconds
TNES	a recipe containing Tris, NaCl, EDTA and SDS
WG	water dispersible granule
WP	wettable powder

Chapter 1: Introduction and literature review

1.1. Introduction

Aonidiella aurantii Maskel (Hemiptera: Diaspididae) is an important pest of citrus in South Africa. To combat this pest, parasitoids in the genus *Aphytis* are regarded as the primary biological control agents worldwide (Grout & Richards, 1989b; Bedford, 1998; Bedford & Cilliers, 1994; Samways, 1986a; Forster et al., 1995; Quednau & Annecke, 1963).

Aphytis lingnanensis Compere (Hymenoptera: Aphelinidae) was mass reared in South Africa by various insectaries for augmentation against red scale on citrus in the early 2000s. However, unpublished research by Daneel and Ware (2000) concluded that there was no evidence that augmentation of *A. lingnanensis* influenced the population dynamics of red scale. They argued that as farmers had paid for beneficial insects, they were reluctant to apply disruptive chemicals, which played a big part in allowing other, naturally occurring beneficial insects such *A. africanus* Quednau to contribute to the suppression and control of red scale and thus the augmentation of *A. lingnanensis* indirectly resulted in control of red scale. Added to this, trials by Moore & Richards (2002, unpublished data) in the Sundays River Valley (Eastern Cape Province) demonstrated that, despite not releasing any *A. lingnanensis* during the trial season, *A. lingnanensis* had established in all orchards. Although only nominal releases of *A. lingnanensis* were made during the following season of the trial, *A. lingnanensis* numbers surpassed those of *A. melinus*, leading to the conclusion that *A. lingnanensis* simply displaced *A. melinus* and *A. africanus* without increasing the overall level of parasitism. However, further research by Moore and Richards (2002, unpubished data) over five consecutive seasons showed that, if *Aphytis* spp. was the dominant group of parasitoid species over *Comperiella bifasciata* Howard (Hymenoptera: Encyrtidae), then *Aphytis* spp. would leave less live red scale on fruit at harvest. These findings confirm those of Atkinson (1983) and Samways (1986b).

Aphytis melinus is now commercially available for augmentation in South Africa. Wasps are reared by an overseas insectary and imported into South Africa. A local insectary in South Africa, has also started rearing *A. melinus*. Consequently, it is important that the efficacy of augmentation of this species on red scale is determined locally, as it is already established in South Africa, well-suited to these environments, and is considered the primary biological control agent for red scale elsewhere (Forster et al., 1995; Pekas, 2010; Garcerá et al., 2013; Rill et al., 2008). Murdoch et al. (1985) have shown that successful biological control by *A. melinus* on red scale was the most convincing out of six generally agreed successful classical biological control cases.

Although various economically important species of *Aphytis*, such as *A. lingnanensis*, *A. melinus* and *A. chrysomphali* Mercet, are established in South African citrus orchards along with the native *A. africanus*, the augmentation of *A. melinus* has never been tested in South Africa. The outcome of this research will guide future red scale control programmes on whether to adopt augmentation programmes or, rather, to pursue the conservation of established *Aphytis* populations.

1.2. Red scale

1.2.1. Classification and taxonomy

The history of red scale provides a typical case study of costly setbacks due to incomplete taxonomic and biographic knowledge of a pest species (Kennett et al., 1999; Compere, 1961; Nel, 1933; McKenzie, 1937). Red scale originated from the tropics and subtropics of Southeast Asia (McKenzie, 1937) and became a citrus pest when it was introduced to other parts of the world where it had no natural enemies (Compere, 1961). Many parasitoids of the closely related *Aonidiella* spp. are not effective against red scale. The inability to distinguish red scale from closely related yellow scale (*Aonidiella citrina*) was the main reason why early attempts at biological control on red scale failed after the introduction of promising parasitoid species into the USA. This principle was supported when scale taxonomist, Howard McKenzie, discovered that red scale could be separated from yellow scale by microscopic morphological differences (Beardsley & Gonzalez, 1975; Rosen & DeBach, 1979).

Class: Insecta
Order: Hemiptera
Suborder: Sternorhyncha
Superfamily: Coccoidea
Family: Diaspididae
Tribe: Aspidiotini
Genus: *Aonidiella*
Species: *aurantii* (Maskell, 1878).

1.2.2. Distribution and host range

Diaspididae (armored scales) constitute one of the most successful groups of plant-feeding arthropods and include some of the most damaging and stubborn pests of perennial crops (Beardsley & Gonzalez, 1975). Red scale apparently originated in Southeast Asia and has since spread to all citrus-growing countries except for Japan, Bermuda, Kuwait and Columbia (CABI, 2019). Environmentally, low temperatures are the most limiting factor determining its distribution and abundance (Abdelrahman, 1973).

Red scale has become the most problematic pest in citrus-producing countries worldwide, and in South Africa, it has infested all the above-ground parts of citrus trees (Bedford, 1998; Ebeling, 1959; Forster et al., 1995; Grout & Richards, 1989a). The literature available on this pest is extensive, with most of the research having been done in California, USA, hence the name California red scale (Bedford, 1998). As early as 1895, C.P. Lounsbury reported that red scale occurred on more than 200 different plant species in South Africa and was a serious pest on citrus in the Cape Colony (Bedford, 1998; Annecke & Moran, 1982). Red scale occurs on a variety of host plants – various trees, creepers, vines, and agricultural crops (Quayle, 1938; Munro & Fouche, 1936) – across the world but is mainly known for its association with citrus (Bedford, 1998). Red scale occurs on various host plants across the world but is mainly known for its association on citrus (Bedford, 1998). According to Quayle (1938) a list of host plants is the following: *Acacia* species, alfalfa, aloe, apple, ash, asparagus, aspidistra, athel, avocado, banana, bay sweet, bauhinia, box elder, box wood, breadfruit, bur marigold, camphor tree, carob, carnation, castor bean, China berry, citrus, cottonwood, coconut, coprosma, elder berry, English ivy, elm, elaegnus, Eucaluptus, euonymus, gif, fuchsia, golden rod, grape, blackberry, hibiscus, holly, horseweed, jasmine, ligustrum, lauristinus, locust, mango, mistletoe, mulberry, nightshade, oak, oleander, olive, palm, passion vine, pear, pine, pistachio, podocarpus, poplar, poinsettia, potato, privet, quince, ragweed, rose, sago palm, Scotch broom, sesbania, squash, sumac, sycamore, tamarix, tea, walnut, watermelon, willow and yucca and numerous weeds. In South Africa red scale has been noted on 201 host plants that include fruit trees, cultivated shrubs and indigenous plants (Munro & Fouche, 1936).

1.2.3. Damage and economic importance

Red scale inhabits and feeds on all above-ground plant parts of citrus, that is, wood, leaves, twigs and fruit (Ebeling, 1959; Bedford, 1998; Grout & Richards, 1992; Quayle, 1938). However, they prefer the softer and exterior plant parts – green twigs, leaves and fruit – for reproduction and growth (Carroll & Luck, 1984; Murdoch et al., 1989). Citrus cultivars vary in susceptibility to infestation, where lemon (*Citrus limon* Osbeck) is the most susceptible, followed by grapefruit (*Citrus paradisi* Macfadyen), sweet orange (*Citrus sinensis* Osbeck) and lastly, various Mandarin types (e.g. *Citrus reticulata* Blanco, *C. unshiu* Marcovitch) (Hare et al., 1990; Bedford, 1998; Habib et al., 1972).

Red scale males and females feed by deeply penetrating plant tissue with their rostrum (Fig. 1.1) during the instar stages of the life cycle (Forster et al., 1995; Beardsley & Gonzalez, 1975).

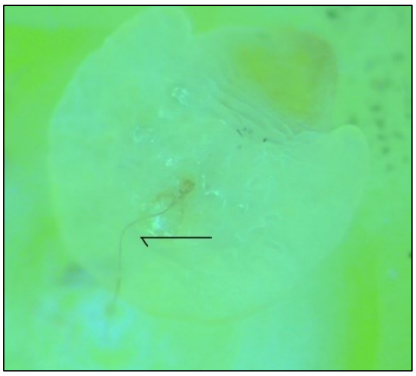


Figure 1.1. An inverted third instar red scale female revealing the rostrum.

A cross-section of the rostrum shows that the two mandibular stylets form the outer lateral structure of the fascicle, surrounding the interlocking maxillary stylets in between to complete the bundle. The faces of the maxillary stylets are so formed that the juxtaposition enables two channels. The larger dorsal channel is used for plant sap intake, and a smaller ventral channel for saliva delivery (Beardsley & Gonzalez, 1975). The stylet path penetrates the cell and feeding mostly takes place in the parenchyma tissue (Washington & Walker, 1990). Washington & Walker (1990) found that saliva excreted as part of the feeding process spreads via intercellular spaces, where it causes an outflow of nutrients from cells by means of a tactical chemical reaction, in line with the hypothesis proposed by Miles (1987) for mirids and coreids. In this way, feeding instars can reach and affect target cells without directly piercing them. This phenomenon explains the symptom that led to the term *toxic saliva*, coined by previous researchers as the cause of dieback in areas adjacent to scale bodies, (Bedford, 1998).

Feeding effects are mostly associated with depressions, discolouration and other malformation of host tissue, so reducing the quantity and quality of citrus crops (Beardsley & Gonzalez, 1975; Bedford, 1998). Heavy infestations can lead to severe tree damage that includes twig and branch dieback, leaf chlorosis, defoliation, and ultimately, tree death. Longitudinal cracking of bark on the main trunk can also occur, leading to permanent problems in tree structural development (Ebeling, 1959; Badary & Abd-Rabou, 2010; Grout, 2012a; Quayle, 1938).

Even light infestations of red scale on fruit lead to a fruit cull, due to the unacceptable appearance of affected fruit rind. Some export destinations such as Japan and South Korea regard red scale as a phytosanitary pest and will not accept a single scale individual on any fruit (Bedford, 1998; DALRRD, 2005). Severe infestations of red scale lead to pits forming on fruit at the early stages of development, which are still evident at maturation, rendering the fruit unsuitable for export markets. Another effect of heavy infestations on yield is that quantity might be affected by reduced flowering, premature fruit drop and diminished final pack-out volumes. Crop quantity and quality of the following season can also be adversely affected by current season presence of serious infestations of the pest

(Bedford, 1998; Grout, 2012a; Annecke & Moran, 1982). Furthermore, dead wood and twigs that result from infestation dieback are responsible for fruit scarring and blemishes, and they facilitate post-harvest fruit decay pathogens (Bedford, 1998).

It is difficult to quantify crop and quality losses caused by red scale because of the great variation across the citrus industry. Personal communications with technical field personnel support statements of export cull being between 0% and 60% in some cases, and the loss of young trees in newly planted orchards.

1.2.4. Biology and morphology

The adult female is the most prominent life stage of red scale; the scale covering is flat, circular, about 2 mm in diameter and dull-yellow or red brown in colour (Fig. 1.2). It is a mainly sedentary insect with reduced appendages and stylet-like mouth parts (rostrum) that penetrate the host plant (Annecke & Moran, 1982). Red scale is ovoviviparous and parthenogenesis is non-existent (Tashiro & Chambers, 1967).

Figure 1.2. Citrus fruit infested with red scale.

Red scale adults exhibit sexual dimorphism. Males and females can be separated from the first moult. A ventral view of the first exuvia of the female appears shiny and polished, while that of the male is dull (Bedford, 1998). From the second instar onwards, males and females can be distinguished from each other morphologically when the male cover elongates (Fig. 1.3) and develops eyes (Forster et al., 1995). Adult males have wings and are mobile but are weak flyers, fragile and short lived. Females are sedentary from the crawler stage onwards (Fig. 1.4) and have no wings or legs (Beardsley & Gonzalez, 1975; Forster et al., 1995).

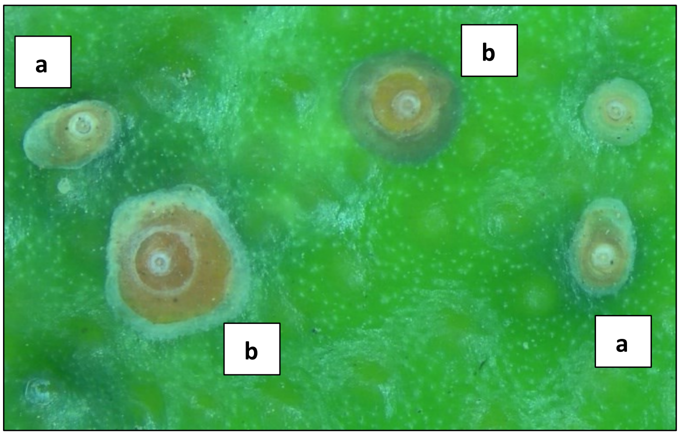


Figure 1.3. Male (a) and female (b) scales on fruit.

In most citrus-producing areas of South Africa, red scale has four to six generations on orange trees and five to seven on lemon trees per year (Grout et al., 1989b). Generations tend to overlap each other during the season and it is usual to find all scale growth stages (Fig. 1.4) in any representative population sample (Annecke & Moran, 1982; Grout et al., 1989b).

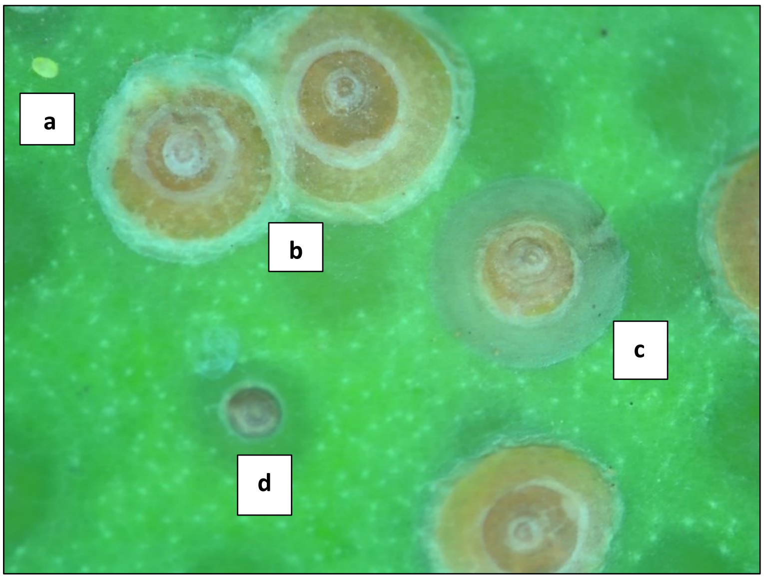


Figure 1.4. Various life stages of red scale on an infested citrus fruit; crawler (a), gravid female (b); third instar virgin female (c) and first moult (d).

The immobile stages of red scale can be separated into two distinct stages, namely the instar and moult stages (Fig. 1.5). Feeding takes place during the instar stages when the scale inserts its feeding tube (rostrum) into the plant. The moult stages are regarded as dormant, and no feeding occurs. Males undergo only one moult stage while females undergo two (Forster et al., 1995; Bedford, 1998; Quayle, 1938). During instar stages, the edge of the scale body under the scale cover is vertically rounded and the scale body is loose from the scale cover. The colour of the scale body is typically lemon-yellow. The moulting stages are defined by the orange body colouration and the fused edge where the scale body and scale cover join and are inseparable (Forster et al., 1995; Tashiro & Beavers, 1968).

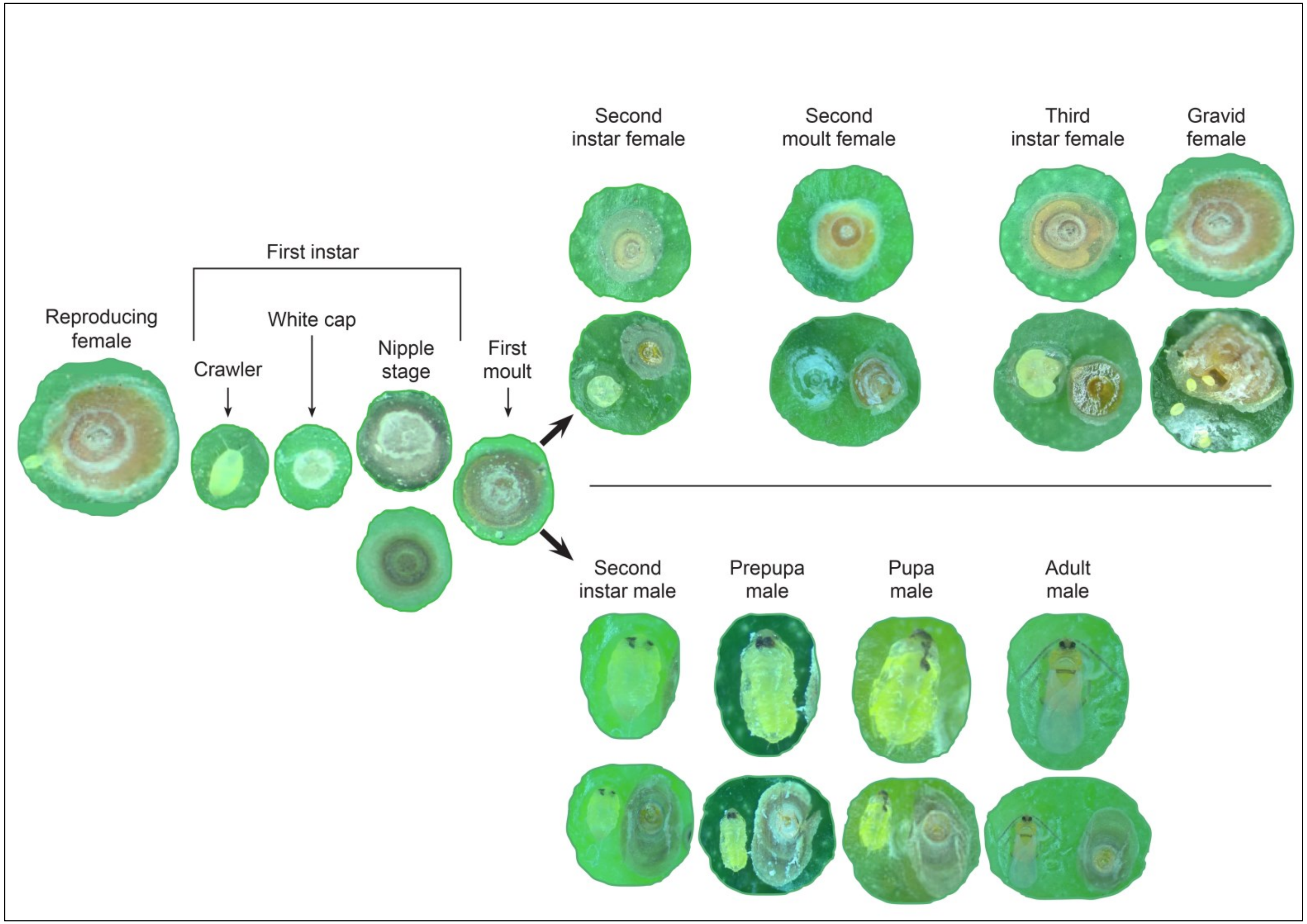


Figure 1.5. Life cycle of red scale.

1.2.4.1. Adult male and virgin female behaviour

The developmental timeline of the two sexes is closely correlated, as shown in Fig. 1.5. In any given cohort, the adult male emerges at the same time as the adult female attains sexual maturity (Beardsley & Gonzalez, 1975). Flying males locate receptive females largely in response to female sex pheromones. Males do not live long, and under laboratory conditions, mortality starts two hours from emergence, where 50% die within the six-to seven-hour period, and none survive 14 hours after emergence (Beardsley & Gonzalez, 1975; Tashiro & Beavers, 1968). The process of finding a receptive female is dependent on female pheromones (Beardsley & Gonzalez, 1975); in dispersal tests, marked males were found up to 189 m downwind and up to 92 m upwind from release points on traps loaded with female pheromones (Rice & Moreno, 1970). Furthermore, although yellow cards attracted the most red scale males, all colours were equally attractive when female pheromone was added to the cards (Rice & Moreno, 1969).

When the third instar virgin female is ready to mate (Fig. 1.6) the pygidium extends beyond the lateral lobes of the thorax towards the edge of the scale cover and sometimes even slightly beyond the edges of the scale cover (Tashiro & Moffitt, 1968; Forster et al., 1995; Beardsley & Gonzalez, 1975). Fertilisation by the male scale takes place during the early phases of this stage.

The female scale body and the surrounding grey covering grows remarkably during this stage. The grey scale cover again develops past the edge of the scale body to facilitate accommodation for the soft, loosened, and growing scale body. The silhouette of the scale body is mostly visible through the almost transparent grey scale cover (Fig. 1.6 a) before the body and covering fuses together to end the third instar female stage (Bedford, 1998; Quayle, 1938). Parasitism by *Aphytis* spp. can also occur during the third instar until fertilisation takes place, which ends this scale growth stage and starts with the gravid female stage, when the scale is no longer susceptible to *Aphytis* spp. parasitism (Quayle, 1938; Forster et al., 1995).

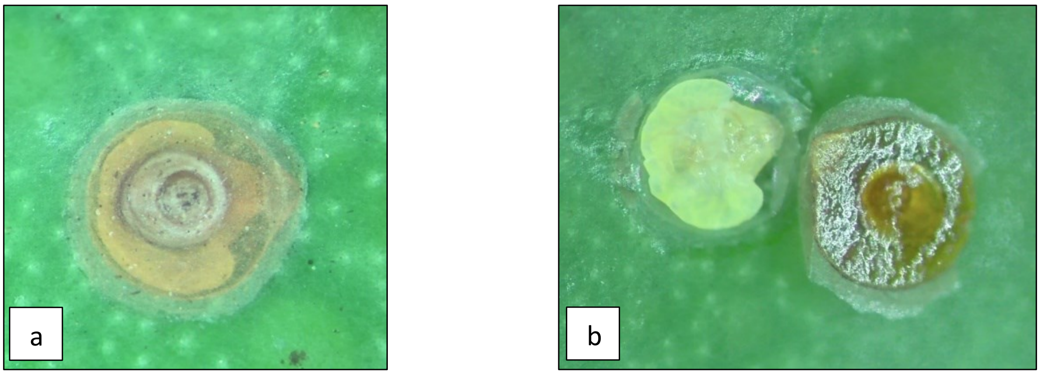


Figure 1.6. A third instar virgin red scale female intact on a fruit (a) and inverted (b). Note the extended pygidium outwards to the rind of the scale cover.

Third instar females continue to grow but will not develop into mature females until mated. The length of the developmental period of the third instar female is thus determined by when mating take place (Forster et al., 1995; Beardsley & Gonzalez, 1975). Virgin females are attractive to males as soon as the grey margin is formed, which is about 23 days after settling. If mating does not occur, virgin females may remain attractive to males up to a maximum of 107 days. Males are ready for mating as soon as they emerge from their scale cover. Individual males were able to fertilise up to 30 females and, on average 11.9, in laboratory tests. Mating males position themselves on the side of the scale cover, facing towards the centre of the female scale. The aedeagus is inserted under the edge of the scale cover by thrusting it forward and downward (Beardsley & Gonzalez, 1975).

1.2.4.2. Gravid female

Within one day after mating, permanent retraction of the pygidium starts. Three days after mating the body shape is typical reniform and the lateral thoracic lobe extends past the pygidium (Beardsley & Gonzalez, 1975; Forster et al., 1995). At this stage, the body of the scale is sealed inside the cover and feeding gradually ceases. The female body now looks similar to the moult stages and will start to produce crawlers (Fig. 1.7) in approximately 12 days (Forster et al., 1995). Production of crawlers ranged from 102 to 479 per female, of which 90% emerged during the first 10 weeks (Tashiro & Beavers, 1968).

Figure 1.7. Red scale crawlers emerging from a gravid female (a) and crawlers revealed from the same gravid female only now inverted (b).

Red scale overwinters as virgin and gravid females; the latter will produce male and female progeny in spring. Males maturing from this generation will then fertilise existing virgin females (Abdelrahman, 1973).

1.2.4.3. First instar

The first instar is made up of the crawler, white cap and nipple stages (Beardsley & Gonzalez, 1975; Forster et al., 1995).

1.2.4.3.1. Crawler stage

The crawlers may stay under the covering of the gravid female for a few hours to a day or two after hatching (Quayle, 1938). After emerging from the female scale's cover, larvae crawl out (Fig. 1.8) into the surrounding environment to settle permanently on a suitable location.

Figure 1.8. Red scale crawlers.

Crawler dispersal by means of wind mainly happens during the morning, probably to shield them from high temperatures during mid-day and afternoon (Willard, 1973). Crawlers will also settle on older branches and leaves, but they are primarily phototactic and inclined to move outwards to settle on scaffold branches, new growth, and fruit. They settle on the upper surfaces of leaves near main veins. Crawlers favour depressions in the oil glands as sites for settling; however, older fruit might often be completely covered and encrusted by scale coverings of all sizes (Bedford, 1998).

1.2.4.3.2. White cap stage

Immediately after settling, the first instar scale positions its legs and antennae under the body. Within hours, a white, cottony secretion covers the body surface and extends down the sides of the crawler to become fixed (Fig. 1.9).

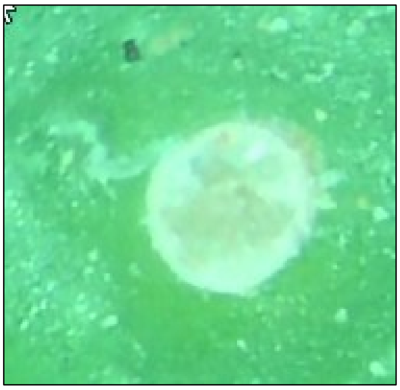


Figure 1.9. The white cap stage of red scale.

The appearance becomes circular, and the insect revolves to mould the covering with a thin waxy layering that is secreted by the pygidium. The cumulative covering forms a flat top and vertical sides from which the name “white cap” is derived. White caps are clearly visible when a new generation of females starts reproducing. Once the crawlers settle, they remain fixed in that location for the rest of their life, except for the winged male that emerges from its covering later (Bedford, 1998; Quayle, 1938).

1.2.4.3.3. Nipple stage

As the body of the scale continues to develop, a circular, ring-like ridge is formed on the previously flat top of the white cap stage (Fig. 1.10).

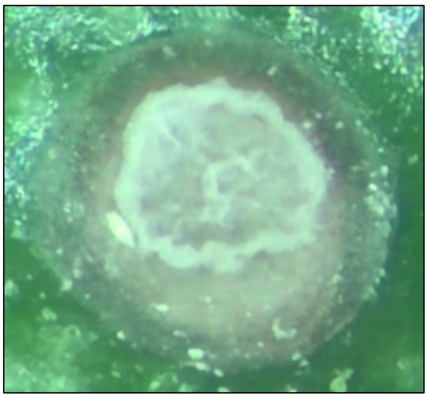


Figure 1.10. The nipple stage of red scale.

This is now the "nipple stage," which is the last phase of the first instar. Towards the end of the first instar, the young scale prepares to moult. The rostrum is detached and the scale body is sealed off inside the scale cover (Bedford, 1998; Forster et al., 1995; Quayle, 1938). The first instar is the only stage when the scale does not have any orange pigmentation (Forster et al., 1995).

1.2.4.3.4. First moult

After the first instar, the first cast skin becomes part of the scale covering to form the first moult (Fig. 1.11).

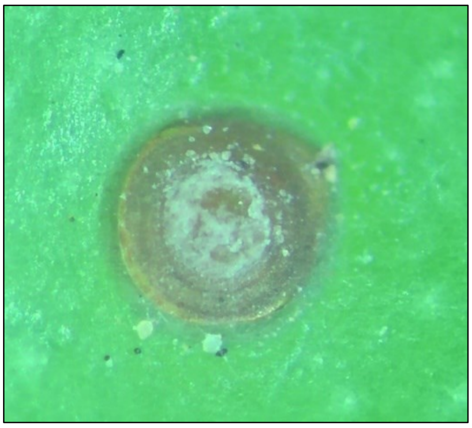


Figure 1.11. The first moult stage of red scale.

During the moulting process, the body is swollen and watery, while the construction is hard, brittle, and joined onto the scale covering (Bedford, 1998; Quayle, 1938). The insect is dormant, and no feeding takes place during the moulting stage. The body shape is slightly convex when lifted and is sealed off inside the cover. Generally, red scale stays in the first moult for about four days. When the feeding tube again extends into the plant surface, first moults begin to transform into second instars (Forster et al., 1995).

Males and females can be separated by body shape during the next life stage, which is the second instar stage, when they develop further. Females remain circular, while males develop an elongated body shape (Forster et al., 1995; Bedford, 1998).

1.2.4.4. Female life stages

Female red scales complete a further three well-defined life stages, namely second instar, second moult and third instar (Forster et al., 1995).

1.2.4.4.1. Second instar female

All second instars can be distinguished by the orange moult ring, which is the same diameter as the total width of the first moult. The orange ring is the cast skin from the first moult that has become a part of the scale cover (Fig. 1.12). The grey skirt surrounding the moult ring consists of wax and protein, which is produced during the second instar and secreted through the pygidium (Forster et al., 1995).

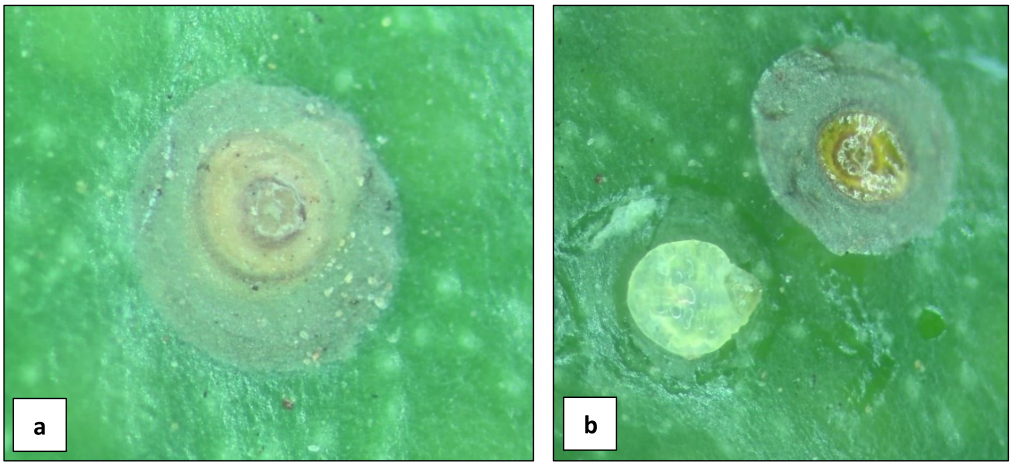


Figure 1.12. The second instar of red scale, intact beneath scale cover (a) and the scale cover inverted (b).

The development of the grey skirt, as an extension of the scale cover that widens with instar age, is necessary to accommodate the increased size of the scale body. During immature stages, the scale body is loose from the scale cover and only becomes firmly attached again during the beginning of the second moult (Quayle, 1938). *Aphytis* spp. prefer to parasitise the instars over moulting stages; the scale body of the instars is softer and is free from the scale cover, enabling the *Aphytis* spp. female to lay eggs on top of and, or below the scale body under the scale cover. The second instar can already be parasitised by most *Aphytis* spp. (Bedford, 1998; Forster et al., 1995; Opp & Luck, 1986).

1.2.4.4.2. Second moult female

When the second cast skin is incorporated into the scale cover, the second moulting stage is initiated (Quayle, 1938). Parasitism by *Aphytis* spp. does not occur during the moulting stages of scale, probably because of the harder scale cover. The absence of the soft grey waxy rim around the scale cover edge and the fused scale body and cover make egg-laying unfavourable for *Aphytis* spp. females during a moulting stage (Forster et al., 1995) (Fig. 1.13). The actual moulting process lasts for about two to seven days (Quayle, 1938).

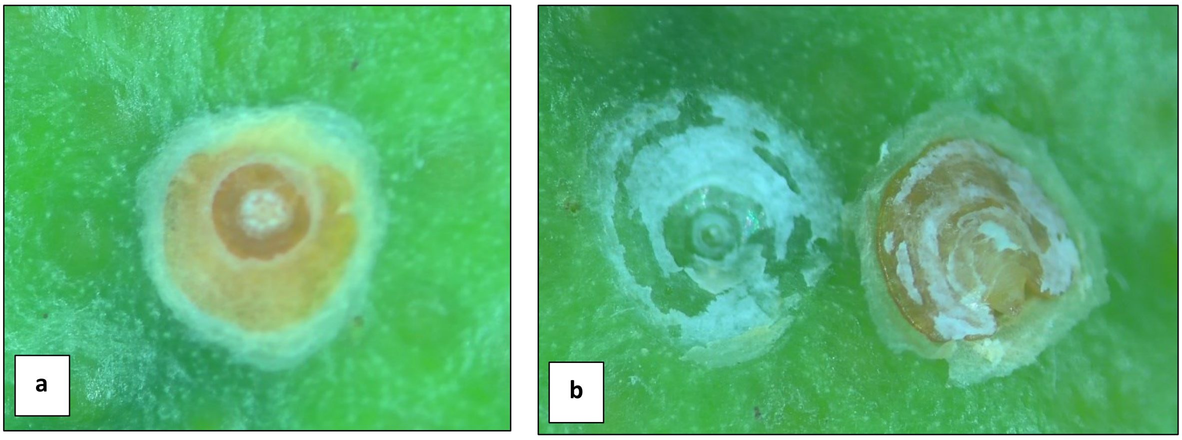


Figure 1.13. View of the second moult stage of red scale intact (dorsal) (a) and inverted (ventral) (b).

1.2.4.4.3. Third instar and adult female

The female grows during the third instar (Fig. 1.14), also known as the grey adult stage, when the female is ready to mate (Bedford, 1998). During this stage, the grey skirt is expanded when the protein-wax-like material is excreted by the pygidium to enlarge the scale cover. This is accomplished when the loose, soft scale body rotates first to the one side and then to the other with the feeding tube probably still inserted in the host plant tissue (Annecke & Moran, 1982).

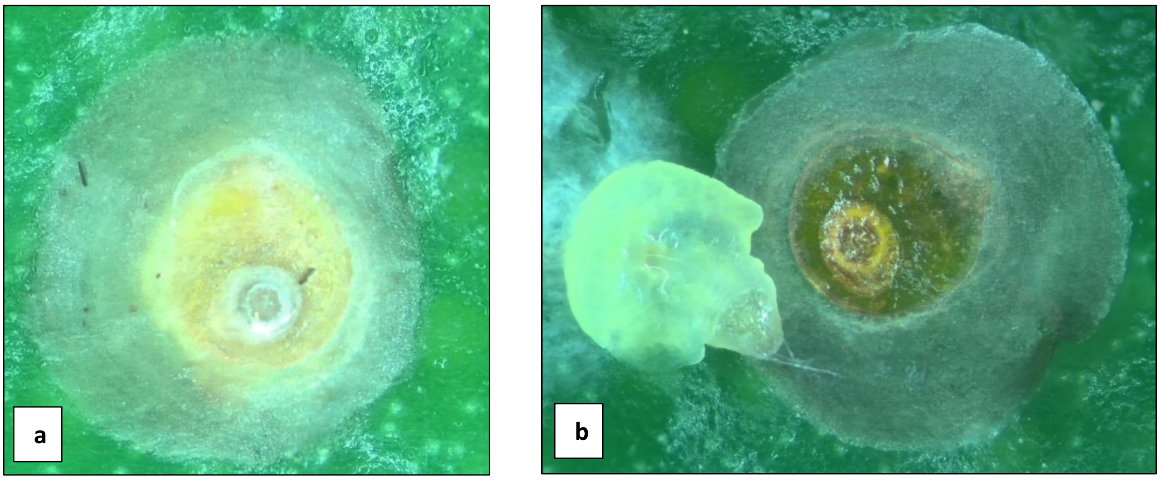


Figure 1.14. The third instar red scale female (a), expanding the grey skirt with the pygidium (b).

1.2.4.5. Male life stages

After the first instar and moult, male scales complete three additional definite juvenile stages, namely the second instar, prepupa and pupa (Forster et al., 1995).

1.2.4.5.1. Second instar male

Male scales elongate during the second instar and develop eyes. The second instar male is distinguishable from the prepupa and pupa by the conspicuous V-shaped, light brown pygidium (Fig. 1.15).

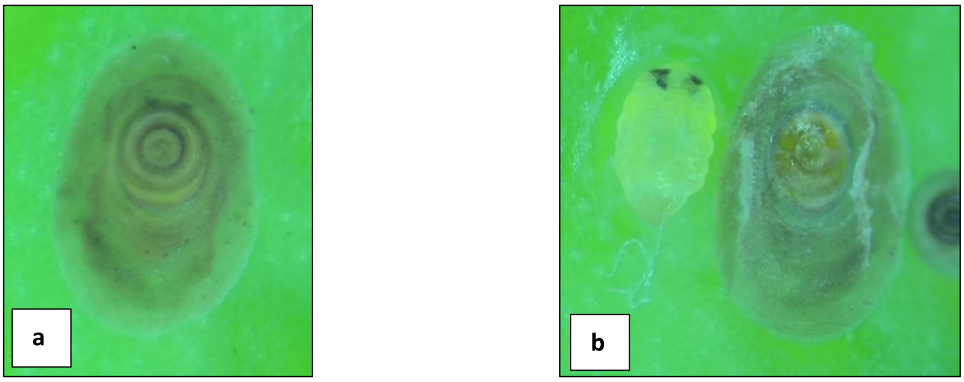


Figure 1.15. The second instar male stage intact beneath the scale cover (a) and inverted (b).

The eventual size of the male scale covering is 1 mm to 1.3 mm long, 0.5 mm to 0.65 mm broad, and is reddish-brown in colour. Second instar males begin to pupate after approximately five days (Bedford, 1998; Forster et al., 1995). The second instar male is often parasitized by *A. africanus* (Bedford, 1968).

1.2.4.5.2. Prepupal male

The second instar stage is followed by a prepupal stage during which the scale develops short wing pads (Bedford, 1998). Slight flattening of the pygidium area and loss of the brown colouration takes place (Fig. 1.16). The prepupal stage is quite short and, within about 36 hours, the prepupal male develops into a male pupa (Forster et al., 1995).

Figure 1.16. The prepupal stage of males.

1.2.4.5.3. Pupa male

The male pupa shows more developed and clearly defined appendages as well as distinct, pointed genitalia (Fig. 1.17). Adult males emerge about three days after pupation (Bedford, 1998; Forster et al., 1995).

Figure 1.17. The male pupa.

1.2.4.5.4. Adult male

The adult male is a weak, two-winged insect that emerges from the pupal scale covering after about three days (Forster et al., 1995). Long feathery antennae and a dark dorsal band across the thorax are characteristic of the adult male scale (Fig. 1.18) (Bedford, 1998; Forster et al., 1995).

Figure 1.18. An exposed adult male just before eclosing.

Male emergence is triggered by diminishing light, thus male flights peak just before sunset in the colder months when temperature drops after sunset. In the summer months, when temperatures do not drop after sunset, male flights peak just after sunset (Tashiro & Beavers, 1968; Rice & Moreno, 1970).

1.2.5. Ecology

1.2.5.1. Biotic factors

1.2.5.1.1. Host varieties

Different citrus varieties vary greatly in their susceptibility to infestation by red scale (Habib et al., 1972; Hare et al., 1990; Salama & Mostafa, 1984; Cameron et al., 1969). Physical factors include the density of oil glands on leaves and fruits as well as bark suitability (Hare et al., 1990; Habib et al., 1972). Red scale infestation decreases with an increase in oil glands per mm^2 on leaves and fruit. Mandarins are the most resistant, sweet orange are intermediate, and lemon and grapefruit are the most susceptible (Habib et al., 1972). Some components of the essential oil found in the oil glands correlated with susceptibility to red scale infestation when lemon and sweet orange were compared. More susceptible cultivars showed a higher hydrocarbon content and a reduced level in less susceptible hosts, like sweet orange. Alcoholic terpene content correlated negatively with red scale suitability (Salama & Mostafa, 1984).

Bark, as a substrate for growth and development of red scale, also exhibits variation amongst cultivars. Lemon and grapefruit have been shown to be least resistant to red scale infestation, while sweet orange were intermediate, and mandarins the most resistant to red scale (Hare et al., 1990).

1.2.5.1.2. Interaction with natural enemies

Natural enemies of red scale occur worldwide, and each citrus area will have a different constitution of parasitoid and predator species that prey on red scale. Each beneficial insect that attacks red scale has different temperature and humidity restrictions and requirements of certain host species and the specific life stage thereof (Forster et al., 1995).

Ectoparasitoids, of which *Aphytis* spp. is the predominant group, comprise the most effective part of the biological control complex of red scale (Forster et al., 1995; Bedford & Cilliers, 1994; Bedford, 1998; Quayle, 1938; Ben-Dov & Rosen, 1969; Rosen & DeBach, 1979). *Aphytis* spp. affect red scale population dynamics mainly in two ways: namely, parasitism (Fig. 1.19) and predatory host feeding (Fig. 1.20 and 1.21).

Figure 1.19. *Aphytis melinus* pupa visible through the transparent scale covering of a second instar female red scale (a), and then clearly visible after the scale cover has been removed (b). Note the exuviae (top left, b) which is the only remains of the soft scale body.

Typically, only 25% parasitism has been found at any given time, with populations in equilibrium at low levels (Rosen & DeBach, 1979; Ben-Dov & Rosen, 1969; DeBach, 1969). Parasitism is not the only way that red scale is killed by *Aphytis* spp. Predatory host feeding and stinging without feeding by *Aphytis* spp. kills as many or even more hosts than parasitism (Forster et al., 1995; Rosen & DeBach, 1979; DeBach & Sundby, 1963; DeBach & White, 1960; Cebolla et al., 2017). This category of mortality is perplexing and field counts reveal only a small portion of it. It is difficult to record host feeding and stinging apart from unknown death causes as scale bodies dry up soon after dying, which compromises the establishment of the cause of death (Fig. 1.21).

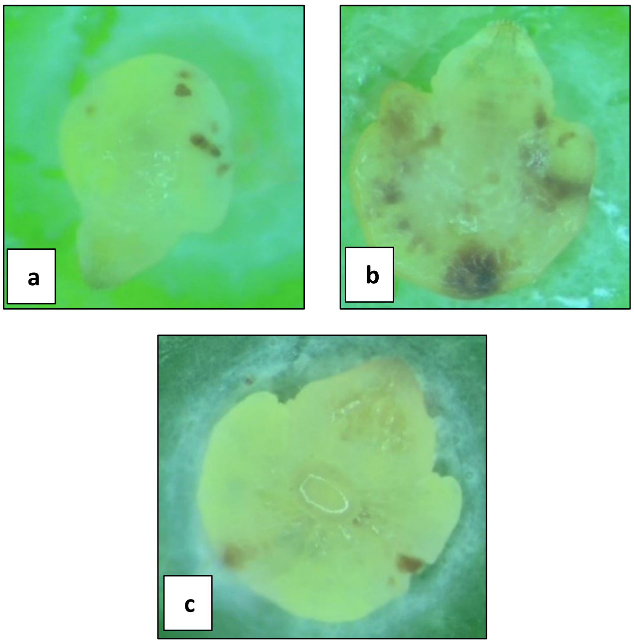


Figure 1.20. Mutilation marks caused by host feeding of *Aphytis* spp. visible on the bodies of a second (a) and a third instar red scale female (b & c). Note the developing second instar *Aphytis* sp. larva (c).

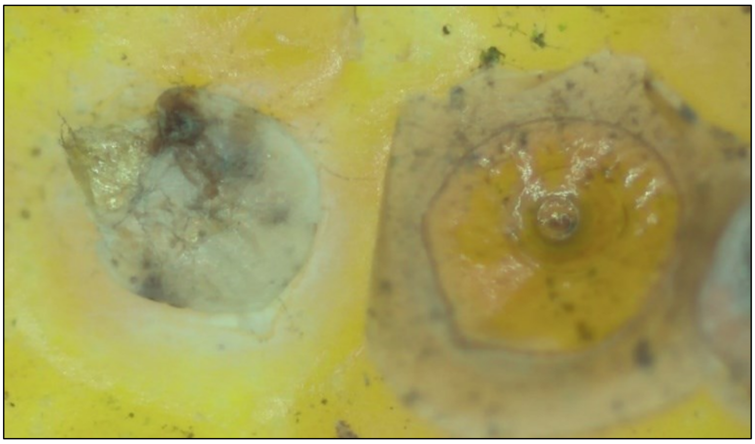


Figure 1.21. Host feeding by *Aphytis* spp. causes red scale to die and consequent quick desiccation of the soft body.

We therefore know that a relatively low percentage of parasitisation by *Aphytis* spp. can be associated by a greater summation of *Aphytis*-caused mortality as explained by Fig. 1.22 (DeBach, 1969).

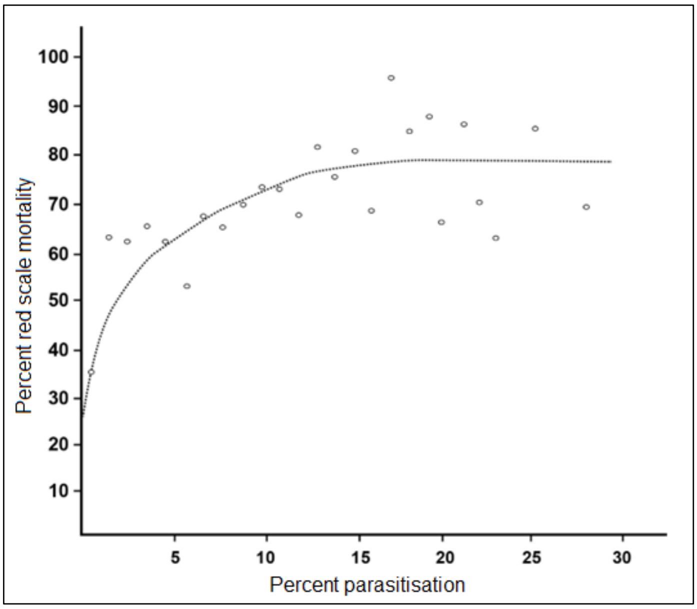


Figure 1.22. Relation between percent parasitisation by *A. melinus* and field mortality of red scale (DeBach, 1969).

A stable equilibrium between *A. melinus* and red scale has been recorded under natural conditions where neither the host nor the parasitoid is eradicated. Bark on tree trunks as a substrate on which red scale enjoy refuge is confirmed as a mechanism for host population stability as it was found that parasitism on those interior plant parts was significantly lower than on exterior parts like twigs, leaves, and fruit. It was subsequently shown that the quality of the scale as hosts played a more significant role than the position of the scale, when infested fruit positioned inside and outside the tree canopy were tested (Murdoch et al., 1989; Walde et al., 1989; Murdoch et al., 1985; Reeve & Murdoch, 1986; Murdoch et al., 2006).

Comperiella bifasciata is an endoparasitoid (Figs. 1.23 & 1.24) but also kills red scale by host feeding and occasional probing. It attacks almost all red scale life stages but prefers the third instar (Martinez-Ferrer et al., 2003; Flanders, 1945). However, *C. bifasciata* is regarded of lesser importance than *Aphytis* spp. in terms of impacting on red scale populations (Forster et al., 1995; Martinez-Ferrer et al., 2003).

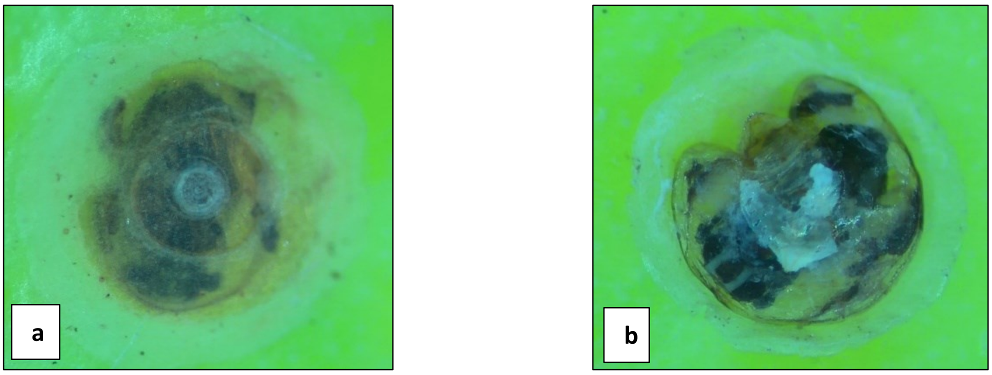


Figure 1.23. The pupa of a *C. bifasciata* female wasp inside a third instar gravid red scale female intact on a fruit (a) and the ventral view of same red scale female inverted (b).

Comperiella bifasciata was noted as a strong competitor to *A. africanus* when red scale density was high, but as soon as biological control took effect during later seasons, and scale density subsequently dropped to low levels, *C. bifasciata* became scarce (Bedford, 1998). Where *A. lingnanensis* and *C. bifasciata* were found to parasitise red scale as a set, high parasitism percentages realised, but *A. lingnanensis* contributed twice as much towards parasitism than *C. bifasciata* (Smith, 1978).

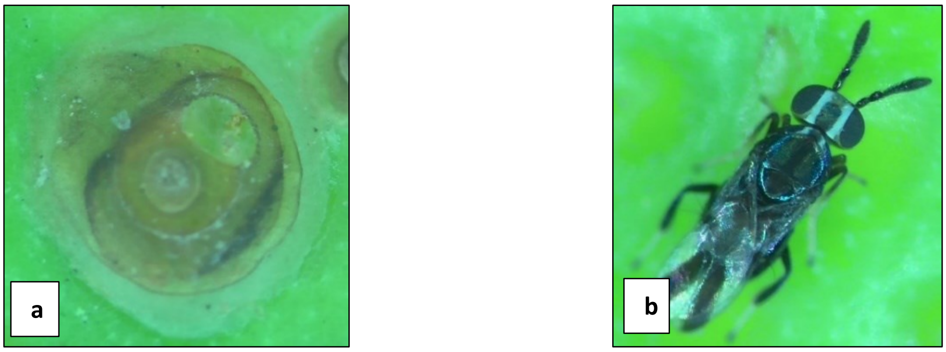


Figure 1.24. A third instar red scale female that has been parasitised by *C. bifasciata*, showing the typical faecal crescent with the exit hole on the top through the hard scale integument (a); an adult *C. bifasciata* (b).

Another wasp from the Aphelinidae family, *Habrolepsis rouxi* Compere has been noted as an endoparasitoid, but of lesser importance. Research by Omer-Cooper and Whitehead (1950) in the Eastern Cape indicated it was too rare to play a role in the population dynamic of red scale.

Chilocorus nigritis Fabricius (Coleoptera: Coccinellidae) is a common predator of red scale and is an effective colonist, native to India (Pervez & Omkar, 2003). The addition of *C. nigritis* to the biological control complex of red scale in South Africa has been very useful. It attacks all life stages of red scale and feeds voraciously, and has proved to dramatically impact high scale densities to very low numbers in the Lowveld area of South Africa (Samways, 1984).

Predators like *Chilocorus distigma* Fabricius and *Rhyzobius lophanthae* Blaisdell (Coleoptera: Coccinellidae) often prey alongside *A. africanus* in high host densities. At low host densities, these predators tend to withdraw (Bedford, 1998). Other predators, like Chrysophidae and Coniopterygidae, among others found in citrus orchards, are probably not important in terms of having noticeable impacts on red scale ecology (Hesse, 1947; Bedford, 1998).

1.2.5.1.3. Hyperparasitism

Comperiella bifasciata was often under attack by the hyperparasitoid *Marietta javensis* which, in turn, affected its ability to parasitise red scale (Samways, 1985).

1.2.5.1.4. Pathogens of red scale

Numerous pathogens have been recorded from citrus pests in South Africa (Moore, 2002). Etomopathogenic fungi known to attack red scale include *Sphaerostilbe aurantiicola* Petch., (*Nectria coccophila* Berkerly and Broom), *Podonectria* sp. Petch., *Fusarium coccinellum* Kalch. and *F. lateritium* Nees (Catling, 1971; Bedford, 1968; Searle, 1964; Luttrell, 1944; Doidge, 1937). *Sphaerostilbe auranticola*, also known as the red-headed fungus, was found to frequently impact red scale populations but did not lead to significant effects in field trials (Bedford, 1968; Bedford, 1954).

1.2.5.2. Abiotic factors

1.2.5.2.1. Crop nutrition

Availability of adequate nitrogen, phosphorous and potassium increased the resistance of citrus seedlings to infestation by red scale (Salama et al., 1972). Studies done at Letaba Estates found that red scale females started to reproduce 5.21 days (SE ±0.77; $P = 0.001$) earlier on seedlings grown in a low nitrogen regime, which is significant. Low calcium nutrient levels led to a notable increase in the period of one crawler life stage to the next crawler stage of development (Steyn, 1951). These results suggest that citrus grown under nutritional programmes with high nitrogen, phosphorous, and potassium levels but low calcium levels, might reduce the number of red scale generations per season.

1.2.5.2.2. Temperature

The life cycle duration of red scale is greatly influenced by temperature (Kennett & Hoffmann, 1985; Willard, 1968; Beardsley & Gonzalez, 1975; Atkinson, 1983; Yu & Luck, 1988; Bimboni, 1970). Low temperature is detrimental to red scale and seems to be the most important limiting factor determining species distribution and abundance. The growing stages and the egg maturation stage proved to be the most tolerant of extreme temperatures. The gravid female, moulting stages, prepupal and pupal stages of males, in declining order, are less tolerant (Abdelrahman, 1973). Although crawlers are sensitive to low humidity, they nevertheless survived for 5.7 hours at 35 °C and 25% RH (Willard, 1973), which suggests that they can overcome the elements during wind dispersal.

Yu and Luck (1988) found the lower developmental threshold for red scale females to be 11.5 °C, which is similar to the 11.6 °C previously reported (Willard, 1972; Bimboni, 1970). However, the lower and upper developmental threshold generally used for male and female red scale is 11.7 °C (Kennett & Hoffmann, 1985; Morse et al., 1985) and 37.8 °C (Morse et al., 1985). Physiological time lines can thus be drawn to eliminate periods unsuitable for growth ruled by temperature by incorporating these thresholds into a degree day model (Grout & Richards, 1989b; Kennett & Hoffmann, 1985). The average number of degrees amassed between the set of thresholds can be calculated using a formula developed by Higley et al. (1986), which incorporates daily minimum and maximum temperatures based on the sine wave method of Allen (1976).

Development duration for most stages decreased with increasing temperature up to 30 °C, as shown in Table 1.1. Above 30 °C, duration increased except for first instars, first moults and differentiated second male and female instars. Thus, as temperature increases, the time during which a second and third instar scale was available for parasitism by *A. melinus* diminished, except at 33 °C, when the third instar period increased slightly again. The size of non-growing stages, second moult and prepupa, became smaller as temperature increased, and high temperature reduced the size of second and third instars. Higher temperatures thus reduced both the period during which scales were sensitive to *A. melinus* attack as well as the suitable instar sizes at which female progeny could be produced (Table 1.1) (Yu & Luck, 1988).

Table 1.1. The development times for red scale at different constant temperatures (Yu & Luck, 1988).

Growth stage	Mean developmental time (days) (±SE)					
	Temperature (60% RH; photoperiod 16 light: 8 dark)					
	17 °C	**20 °C**	**25 °C**	**26.7 °C**	**30 °C**	**33 °C**
First instar (±SE)	19.09 (1.09)	13.94 (1.08)	8.28 (0.71)	6.19 (0.60)	5.42 (0.64)	5.19 (0.35)
First moult	12.63 (0.35)	10.18 (0.55)	4.82 (0.42)	3.94 (0.35)	3.76 (0.34)	3.51 (0.37)
Second instar (before sex differentiation)	9.94 (0.90)	7.11 (0.56)	3.75 (0.36)	3.38 (0.35)	2.10 (0.43)	3.51 (0.37)
Total development	41.66	31.23	16.65	13.51	11.28	15.09
Female						
Second instar	3.06 (0.86)	2.36 (0.39)	1.92 (0.51)	1.68 (0.29)	1.59 (0.37)	1.48 (0.40)
Second moult	13.70 (0.50)	13.07 (0.64)	6.92 (0.41)	5.53 (0.3)	4.34 (0.44)	5.19 (0.38)
Second instar	12.56 (0.84)	10.02 (0.85)	7.82 (0.65)	6.9 (0.45)	6.4 (0.33)	7.4 (0.35)
Matured female	34.97 (0.88)	24.86 (0.46)	15.04 (0.67)	12.35 (0.39)	11.16 (0.4)	12.46 (0.78)
Total female development	105.95 (1.36)	81.54 (0.74)	48.55 (1.22)	39.97 (0.96)	34.77 (0.71)	41.62 (1.22)
Male						
Second instar	10.72 (0.73)	9.58 (0.68)	5.91 (0.49)	4.82 (0.37)	4.00 (0.36)	3.41 (0.60)
Prepupa	2.81 (0.61)	2.41 (0.61)	1.56 (0.44)	1.35 (0.43)	1.15 (0.31)	1.48 (0.61)
Pupa	9.23 (0.87)	7.16 (0.84)	3.47 (0.51)	2.87 (0.39)	2.44 (0.33)	2.87 (0.40)
Total male development	64.42 (1.21)	50.38 (2.10)	27.79 (0.87)	22.55 (0.66)	18.87 (0.46)	22.85 (0.73)

1.2.5.2.3. Wind

Wind plays a part in the dispersal, dislodging and mortality of red scale crawlers (Beardsley & Gonzalez, 1975; Willard, 1974; Willard, 1968). Wind can carry crawlers, adult males, and mature reproductive females up to 312 m from an infested lemon orchard. Numbers of established red scale on potted lemons trees that served as traps decreased significantly from the source to 19.5 m away and then more slowly from 19.5 m to 312 m away. Gravid females could not re-establish after dislodgement and dispersal because of broken mouth parts, but they still could produce a mean of

2.55 (± 0.234) crawlers in laboratory trials after being repositioned and so still play a part in the dispersal of red scale (Willard, 1974; Willard, 1976; Willard, 1968).

1.2.5.2.4. Dust

Hulley (1962) found that dust particles irritated scale crawlers and ultimately led to intensified infestations, which can most likely be explained because crawling distance and wandering time was reduced by the dust; and consequently, exposure time to elements such as wind dislodgement was limited. The direct effects of dust on natural enemies, which are even more important, are more fully explained in "Dust" as an abiotic factor.

1.2.6. Management

1.2.6.1. Monitoring

The main objective of monitoring red scale is to determine its presence and phenology, something which, traditionally, was only possible by means of physical orchard inspections (Moreno, 1983) and the use of cumbersome and costly traps baited with mature virgin female scales (Shaw et al., 1971; Roelofs et al., 1978). The red scale sex pheromone was discovered by Tashiro and Chambers (1967), making it possible to deploy this species-specific pheromone impregnated in rubber septa onto sticky card traps to attract and catch adult red scale males. Pheromone-baited traps can be used commercially to detect the presence of red scale infestations over large regions, replacing costly in-person field assessments (Moreno, 1983). These traps can also be used as a research tool to study pest phenology (Grout et al., 1989; Samways, 1988; Wessels & Meyer, 1982; Grout & Richards, 1989b) and to aid in orchard scouting where farm workers do not survey high tree tops and scaffold branches located in darker shaded tree canopy areas. Other useful applications of pheromone traps are to indicate different levels of current red scale infestations amongst various orchards, to help in the timing of *A. melinus* releases in the coastal areas of California (Phillips, 1987), and timing of insecticide applications (Grout & Richards, 1991; Walker et al., 1990). In South Africa there is no evident correlation between the maximum number in red scale male catches and fruit infestation at harvest, in all probability due to the variable effect of *Aphytis* spp. Hence predicting expected red scale infestation levels based on high trap counts during the season and formulating decision support thresholds is not possible (Grout & Richards, 1991).

1.2.6.2. Chemical control

Classical control by means of insecticide applications is still the mainstay of control in South Africa (Grout, 2012a; Moore et al., 2012; Moore & Richards, 2002, unpublished data). Imidacloprid and pyriproxyfen, both remedies against red scale, were the two most common residues detected among all insecticides in residue tests done during the last ten years. Spirotetramat ranked tenth and might have increased after the use of the product multiplied when cheaper, generic formulations appeared on the market (Fourie, 2020). Horticultural mineral oil is used mainly in addition to pyriproxyfen and spirotetramat. Winter sprays with horticultural mineral oil after harvest are effective, but its use has been neglected during the last three decades (Grout, 2020). Imidacloprid, which was for many years the pillar of red scale control in South Africa, has shown to increase false codling moth, *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae) populations (Grout & Richards, 1989a; Grout, 2000; Grout, 2012a; Moore et al., 2012) and decrease *A. melinus* and *C. bifasciata* incidence in treated orchards (Grafton-Cardwell et al., 2008; Moore et al., 2012). Red scale resistance to imidacloprid has developed in the northern regions of South African citrus production areas (Grout et al., 2016). Pyriproxyfen is used extensively as a remedy against red scale across South Africa, but its effect on predatory beetles and on certain parasitoids like *Coccidexonoides perminutus* Timberlake (Hymenoptera: Encytidae) (Grout, 2000; Grout & Richards, 1989a) is an important disadvantage in terms of integrated pest management (IPM) compatibility. Spirotetramat applications against red scale did not significantly affect *A. melinus* and is therefore an integrated pest management compatible insecticide (Garcerá et al., 2013).

The shortcomings of chemical control highlight the need to develop additional control measures for red scale as a key citrus pest. The development of a biological control option as a single input or as a contribution to integrated control in *Aphytis* spp. against red scale would aid to achieve this end.

1.2.6.3. Mating disruption

Mating disruption is a behavioural-modification tactic used for pest control. It is founded on the deployment of synthetic sex pheromones formulated to be systematically dispersed in the air to prevent mating of the target pest (Suckling, 2000). The idea of applying relevant sex pheromones within a crop to orchestrate eventual reduced reproduction of insect pests started in the early 1970s. Sex pheromones are some of the most potent sensory stimuli known, triggering special effects like the timeous and accurate behavioural reactions they can elicit from the target organism. Furthermore the promise that the use of this environmentally friendly natural substance against various insect pests, including red scale, could also reduce the load of conventional insecticide usage, was attractive (Miller & Gut, 2015; Hefetz et al., 1988; Curtis et al., 1985; Franco et al., 2022). In addition to mating disruption as a prospect, other semiochemical-based methods currently used against insect pests include attract-and-kill approaches, and mass trapping (Welter et al., 2005).

After the isolation of the red scale pheromone was pioneered by Roelofs et al. (1978) and Tashiro and Chambers (1967), Hefetz et al. (1988) were the first to suggest and test the use of the red scale sex pheromone in orchards as an application towards achieving mating disruption. Although Hefetz et al. (1988) found that the overall population decreased after treatment, they did not conclude that mating disruption could be a direct control method against red scale infestations but suggested that higher dosages and better pheromone dispersal, potentially through other devices, could achieve that end. Recent research in South Africa, Portugal, Spain, and California has shown that male trap catches are affected by the deployment of mating disruption devices in orchards (i.e., their ability to locate the female pheromone), and that overall red scale fruit and branch infestation was reduced. But the level of control was not adequate to reckon mating disruption as a single control method constituting a full control strategy (Sousa et al., 2008; Grout et al., 2016; Vacas et al., 2015; Leonard, 2019; Vacas et al., 2009). The current method of disruption used against red scale can possibly be classified as non-competitive disruption as opposed to competitive disruption where the targeted pest is lured to eventually find the pheromone dispenser as described by Miller and Gut (2015). As red scale males are very weak flyers and short lived (Beardsley & Gonzalez, 1975), the inherent potential to attract and kill them is limited. Research in this field is still limited. A possible reason for the lack of sufficient control can be that adult males, whose emergence is physically very close to and coincides with the appearance of the sedentary mature virgin females, often do not require vulnerable flights to mate (Vacas et al., 2009; Leonard, 2019; Vacas et al., 2010).

1.2.6.4. Biological control

Biological control of many insects is regarded as the result of a stable interaction between a host and one or more of its natural enemies as a natural enemy complex where the resultant pest damage is less damaging and more acceptable (Hassell & Comins, 1978; Huffaker & Messenger, 1964; Eilenberg et al., 2001). A natural enemy complex can be such effective biological control that George Compere stated that no insect is a pest of sufficient gravity in its native environment to justify artificial control measures, as nature always provides a means of control through parasitic or predacious insects (Compere, 1961).

Biological control is constituted by the orchestration of either some, or a combination of classical (introduction), conservation and, or augmentative biological control (Heimpel & Mills, 2017; Van Lenteren, 2012a). Of these mentioned, classical biological control is, by a great margin, the most effective approach (Rosen & DeBach, 1979; Van Lenteren, 2012a).

In classical biological control, the progeny of the initially released agents effect control on the subsequent generations of the target pest. The importation of such exotic arthropods is thus inoculated into an environment with the aim of establishing (Elzen & King, 1999; Eilenberg et al., 2001). A textbook example of classical biological control, the Vedalia beetle, *Novius cardinalis* Mulsant (Coleoptera: Coccinellidae), was introduced from Australia to California to control the cottony cushion scale, *Icerya purchasi* Maskell (Homoptera: Margarodidae), which was an introduced species that was devastating the citrus industry in Southern California (Doutt, 1958) (Table 1.2). This project was the first outstanding success in classical biological control and attracted much interest in this method of pest control (Kennett et al., 1999). Also, the introduction of *A. lingnanensis* into California from China in 1947 against the California red scale has been the turning point against this serious pest as an effective biological control agent (Rosen & DeBach, 1979). Although not many other introductions of natural enemies have been as successful as the case with *N. cardinalis* and *A. lingnanensis*, the number of successful schemes in citrus far surpasses that of any other crop (Kennett et al., 1999; Rosen & DeBach, 1979).

Table 1.2. Adapted table from "Types of classical biological control programmes based on the history of the pest and natural enemy association and the geographical origins of the pest and natural enemy with some examples (Eilenberg et al., 2001; Barrows & Hooker, 1981; Doutt, 1958; Compere, 1961; Van Lenteren & Tommasini, 2003)".

Origin of pest	Origin of biological control agent	Recommended terminology	Practical examples
Exotic	Exotic (same as pest)	Classical biological control	*N. cardinalis* on *I. purchasi*
Native	Exotic	New association classical biological control	*Pediobius foveolatus* on *Epilachna varivestis*
Exotic	Exotic (different from pest)	New association classical biological control	*A. melinus* on *A. aurantii*
Native (Europe)	Native (Europe)	Classical biological control	*Chrysoperla carnea* on native aphid species
Exotic	Native	New association classical biological control	*Diglyphus isaea* on *Lyriomyza spp.*

With conservation biological control, naturally occurring beneficial arthropods, such as natural enemies or pathogens of pests, are conserved and often enhanced to play an improved role in attacking pests (Heimpel & Mills, 2017; Eilenberg et al., 2001). Although it is a tactic that the specific agricultural field contains a healthy and diverse community of plants and arthropods for biological control to contribute meaningful to pest management, it is not a guarantee of favourable outcomes (Jonsson et al., 2017; Tscharntke et al., 2016). Agriculture is a drastic modification of a natural ecosystem towards a remarkably simple agro-ecosystem with a reduced biodiversity. This is achieved by means of mostly chemical and mechanical weed control leading to monoculture where, in most cases, only the cultivated crop plant species remains. Such a disrupted and eventually simplified ecosystem inflicts ecological stresses on beneficial arthropods in higher trophic levels on which conservation agriculture pivot (Michaud, 2018). In practice, the use of selective rather than broad-spectrum pesticides, alternating strip treatments with approved insecticides, cultivation of cover crops, and the use of certain crop varieties would help to maintain conservation agriculture (Rill et al., 2008; Grout, 2000; Grout, 2012b; Bedford, 1968; Moore & Richards, 2002, unpublished data; Garcerá et al., 2013; Michaud, 2018; DeBach & Landi, 1961).

Augmentative biological control entails the deployment of artificially mass-reared beneficial arthropods in inundative or inoculative quantities and fashions to agro-ecosystems with the aim of controlling certain agricultural pests (Heimpel & Mills, 2017; Gontijo & Carvalho, 2020; Michaud, 2018; Elzen & King, 1999; Eilenberg et al., 2001). With inundative augmentative biological control, success depends on the immediate agents inundatively released and not on their progeny to achieve control (Elzen & King, 1999; Van Lenteren, 2012a). The beneficial arthropod that is supplemented might already prevail in the relevant environment but lacks sufficient numbers. With inoculative augmentation it is expected from the released biological control agent to multiply in the new environment and then its progeny is to suppress or control the target pest organism, but not to a permanent extent (Eilenberg et al., 2001).

When exotic beneficial arthropods are to be augmented, they should first be subjected to the relevant environmental risk assessment of the relevant authority (Gontijo & Carvalho, 2020; Van Lenteren, 2012b). The idea of augmentative biological control was first promoted by Speyer in 1926 after he realised that the parasitoid, *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae), could provide satisfactory control of whiteflies *Trialeurodes vapararriorum* Westwood (Hemiptera: Aleyrodidae) in greenhouses in Britain (Speyer, 1927). Many challenges still exist to improve the efficacy of augmentative biological control. The main challenges with augmentative biological control are insecticide selectivity and resistance, public policies and perceptions, and quality and fitness of mass-reared insects (Van Lenteren, 2012b; Bellows & Morse, 1993; Vasquez & Morse, 2012).

South Africa is one of the few countries where the indigenous natural enemies of native scale insects were able to attack the introduced red scale of citrus, making it an ideal environment to pursue conservation biological control (Bedford & Grobler, 1981; Compere, 1961). Searle (1964) listed four parasitoid and 31 predator species, all of which are natural inhabitants to South Africa, as enemies of red scale.

1.2.6.4.1. Parasitoids

Aphytis spp. are the most important genus of all the important parasitoids and constitutes the largest part of the biological control complex on red scale worldwide and in South Africa (Forster et al., 1995; Bedford, 1968; Bedford & Grobler, 1981; Pekas, 2010). Among these, the parasitoid *A. africanus* is of superior importance and is effective in controlling red scale in most South African citrus-producing areas, even keeping population levels low, provided that the criteria for IPM are adhered to (Grout & Moore, 2015; Grout, 2012a; Bedford, 1998). Another indigenous parasitoid, *Habrolepsis rouxi*, contributes to control and was mostly found in low-lying areas with higher humidities (Compere, 1961; Annecke & Mynhardt, 1970; Catling, 1971). A third parasitoid, *Encarsia lounsburyi* Berlese and Paoli, that was probably accidentally introduced from neighbouring Mozambique, is quite scarce and can be regarded as unimportant (Bedford, 1998).

Aphytis chrysomphali must have been introduced with citrus material and was at some stage considered to be the main parasitoid of red scale in South Africa; it was later displaced by *A. africanus* and is now found only in certain limited localities (Bedford, 1998; Quednau, 1964b; Quednau, 1964a). *Aphytis lingnanensis*, *A. melinus* and *A. coheni* DeBach were introduced to South Africa after 1962 with the aim of acquiring better and faster biological control of red scale (Quednau & Annecke, 1963; Annecke, 1969). Initially it seemed that these three species did not establish well because of competitive displacement (DeBach & Sundby, 1963) in favour of the indigenous *A. africanus* (Annecke, 1969), notwithstanding the fact the *A. africanus* laid fewer eggs in laboratory trials and does not lay more than one egg per host, unlike all the imported species (Annecke & Moran, 1982; Quednau & Annecke, 1963; Quednau, 1964b). The introduced *A. melinus* is now permanently established in all the citrus-producing areas of South Africa (Bedford, 1998). *Aphytis lingnanensis* also established well in South Africa. This species was previously referred to as *A. lingnanensis-coheni* DeBach (Prinsloo, 1984) and, according to Bedford (1998), Paul DeBach originally identified these adult specimens as *A. lingnanensis,* but with some reservations.

Ulleytt unsuccessfully introduced the parasitoid, *C. bifasciata,* in 1942 and then reintroduced it in 1966 (Annecke, 1969). This parasitoid established and spread to all the citrus-producing parts of South Africa within 10 years (Bedford & Grobler, 1981).

1.2.6.4.2. Predators

The second predator introduced after *Rhyzobius lophanthae* was *Chilocorus cacti* Linnaeus. This predator, although well-established, specialises as a feeder at very high host densities due to its strong feeding habits and therefore does not play an important role in IPM in South Africa today because of its absence when pest levels are low (Bedford & Grobler, 1981).

Chilocorus nigritis was unintentionally introduced into South Africa. This predator has recently proved to be valuable in IPM systems to control red scale and has been regarded by Samways (1984) as important as it is able to survive on low host populations and attacks all life-stages of red scale. It is mostly found in the northern citrus-producing areas of South Africa.

1.2.6.4.3. Cultural practices and environmental manipulation

Common practices in orchards that can be altered to serve as measures that exclude or reduce pest damage and environmental manipulations to facilitate biological control have been practised successfully for many years (Van der Bosch, 1971; Van der Bosch & Telford, 1964; DeBach, 1974; Rosen & DeBach, 1979; Grafton-Cardwell et al., 2014): tactical pruning, dust reduction, improvement of overall tree health, using resistant varieties, timing of harvest, fertilisation and the omission of broad spectrum pesticides are all ways to improve orchard environments towards biological control (Grafton-Cardwell et al., 2014).

There are several further practices that can limit red scale infestation. Planting red scale-free trees is an important measure to prevent scale infestation early in the life of the tree. It is also critical that tree skirts do not allow ants to move from the orchard floor to the tree canopy when stem treatments, such as ant barriers, are used for ant control, by creating bridges that circumvent the ant barrier (Grout, 2012a; DeBach et al., 1951a). Support for biological control agents is also valuable: artificially providing sugar in orchards as well as the natural or arranged presence of nectar-providing vegetation has been proven to enhance the performance of *A. melinus* and other parasitoid wasps significantly (Tena et al., 2015; Pekas, 2010; Lenaerts et al., 2016; Tena et al., 2013).

1.3. *Aphytis melinus*

This species of the genus *Aphytis* comprises small, yellow, or greyish wasps, generally not exceeding 1 mm in length. *Aphytis* spp. develop exclusively as primary ectoparasites of armoured scale insects (Hemiptera: Diaspididae) and are recognised as the most effective and important natural enemies of armoured scale insects (Rosen & DeBach, 1979; Samways, 1988; Grafton-Cardwell & Stewart-Leslie, 1998; Bedford, 1998).

With the introduction of *A. lingnanensis* from China to California, red scale was well suppressed in the coastal areas, but not in the inland areas. During 1957, *A. melinus* was introduced to California and rapidly displaced *A. lingnanensis* (Luck & Podoler, 1985; Rosen & DeBach, 1979). According to Luck and Podoler (1985) *A. melinus* has competitive advantages over *A. lingnanensis*. Although both species attack mostly the same host stage (third instar females), *A. melinus* parasitises scale at a smaller size threshold, lays more eggs per visit to a host, and produces more daughters at smaller scale sizes. *Aphytis melinus* is more tolerant of extreme temperatures and is also more vagile. *Aphytis lingnanensis* also cannot utilize its fecundity advantage in female progeny when it competes against *A. melinus* because the latter seize red scale growth stages before it reaches the size range preferred by *A. lingnanensis.* Furthermore, *A. melinus* feeds extensively on hosts it does not parasitize, and host mortality is greater than the parasitism rate, which brings the added benefit of a predator-like approach to parasite augmentation (Murdoch et al. 1985). Consequently, *A. melinus* is regarded as the primary biological control agent of red scale in California, proving to be more effective than *A. lingnanensis* (Forster et al., 1995; Moreno & Luck, 1992).

1.3.1. Classification and taxonomy

Despite the economic importance of *Aphytis* spp., systematic knowledge of this large genus has remained rather enigmatic. Identification and separation of the species of *Aphytis* is difficult, mainly because of their minute size. Other factors that add to the challenging task is the few reliable distinguishing characteristics, the possibility of sibling species, and that a substantial portion of *Aphytis* spp. is uniparental (thelytokous). Even so, it is of critical importance, that when biological control agents are being sought, that species identification are done precisely (Rosen & DeBach, 1979).

Class: Insecta
Order: Hymenoptera
Suborder: Apocrita
Superfamily: Chalcidoidea
Family: Aphelinidae
Genus: *Aphytis*
Species: *Melinus* (Debach, 1959).

1.3.2. Distribution and host range

The geographical distribution of any given species of *Aphytis* is eventually limited by climatic factors (Rosen & DeBach, 1979). *Aphytis melinus,* initially mainly from India, is now distributed in all citrus-producing countries worldwide and is firmly established in all citrus-producing areas of Southern Africa (Bedford, 1998). The host range of *Aphytis* spp. consists mainly of the Diaspididae family of insects (Rosen & DeBach, 1979).

1.3.3. Economic importance

The species of the genus *Aphytis* are now generally recognised as the most effective natural enemies of armoured scales and are recognised in all citrus-producing countries (Rosen & DeBach, 1979; Forster et al., 1995; Bedford & Cilliers, 1994; Pekas, 2010).

1.3.4. Biology and morphology

Aphytis spp. exhibit complete metamorphosis and their development entails an egg, larval, prepupa, pupa and adult stage as can be seen in Fig. 1.25 (Rosen et al., 1978; DeBach et al., 1978).

Most known species of *Aphytis,* like *A. melinus, A. africanus* and *A. lingnanensis*, are biparental and reproduce sexually. However, approximately 25% of all species whose sexuality is known are uniparental, like *A. chrysomphali*. The biparental species presents arrhenotokous reproduction, where unfertilised eggs develop into males and fertilised eggs develop into females. Unmated females produce males only, while mated females produce both males and females. Although males are common in such species, females normally constitute 60% to 70% of the population. The uniparental species of *Aphytis* exhibit thelytokous parthenogenesis. Females produce female progeny without being fertilised by males. Males are, however, still often produced in this these species, but at a rate of one to five percent (Rosen & DeBach, 1979).

Unless interrupted by diapause, the development of *Aphytis* spp. is relatively short (Rosen & DeBach, 1979). *Aphytis melinus* completed its entire development from oviposition to adult emergence in 24.83, 16.96 and 13.25 days at 20 °C, 25 °C and 26 °C, respectively, as in Table 1.3 (Yu & Luck, 1988). Egg incubation and the prepupal period is relatively short, whereas pupal development takes about one-third of the total period (Rosen & DeBach, 1979) (Table 1.3 and Fig. 1.26).

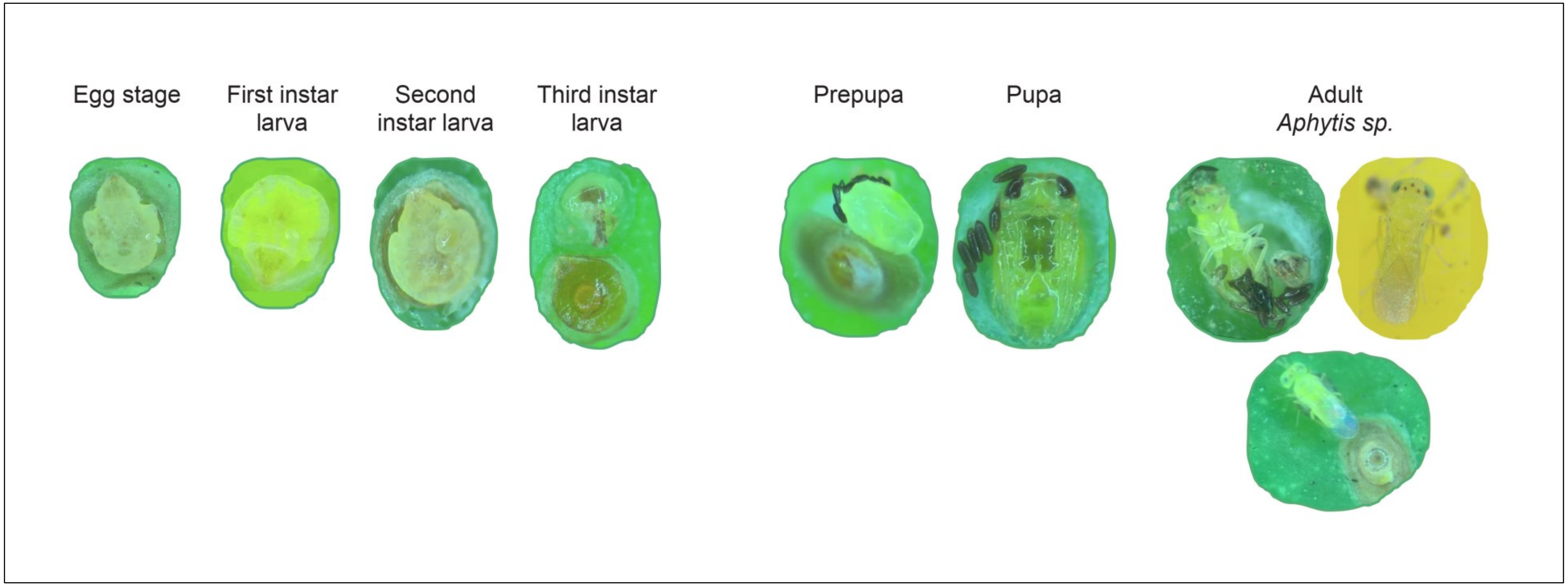


Figure 1.25. Life cycle of *Aphytis* spp.

Table 1.3. Duration of development of *A. melinus* under six constant temperatures (Yu & Luck, 1988).

Stage	**Developmental time in days (SE)**						**Time spent in life stage as a percentage**
	Temperature (°C)						
	17 °C	**20 °C**	**25 °C**	**26.7 °C**	**30 °C**	**33 °C**	
Egg	6.23 (0.12)	4.93 (0.15)	3.00 (0.00)	2.00 (0.00)	1.94 (0.04)	2.00 (0.00)	18%
Larvae	10.85 (0.71)	8.54 (0.26)	6.04 (0.21)	5.19 (0.1)	4.06 (0.1)	4.20 (0.10)	36%
Prepupa	2.77 (0.23)	1.73 (0.12)	1.36 (0.1)	0.96 (0.04)	1.08 (0.05)	0.83 (0.10)	8%
Colourless pupa	6.23 (0.2)	4.6 (0.16)	3.08 (0.08)	2.43 (0.06)	2.09 (0.05)	2.13 (0.09)	18%
Red-eyed pupa	6.85 (0.27)	3.4 (0.13)	2.36 (0.1)	1.64 (0.06)	1.47 (0.08)	1.46 (0.10)	13%
Green-eyed pupa	1.69 (0.17)	1.6 (0.13)	1.12 (0.07)	1.03 (0.04)	0.69 (0.08)	0.92 (0.10)	7%
Total	34.62	24.83	16.96	13.25	11.33	11.54	100%

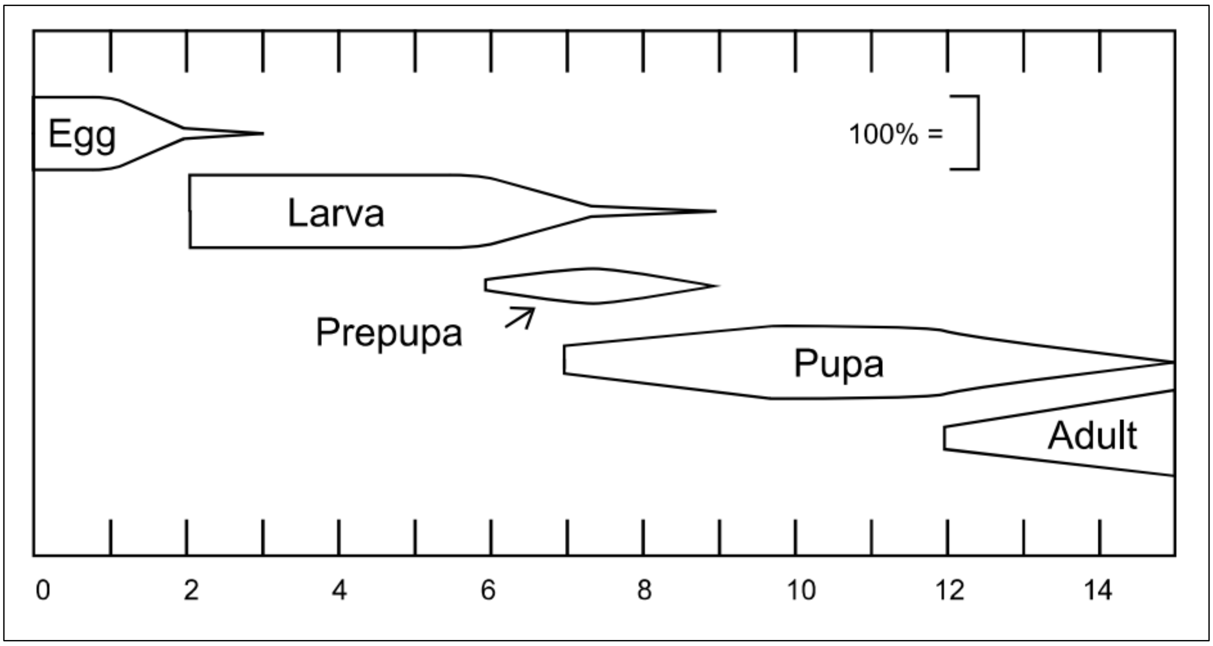


Figure 1.26. Days after oviposition of *A. holoxanthus* DeBach: duration at 27 °C (Rosen & DeBach, 1979).

Abdelrahman (1974a) observed that the developmental period of *A. melinus* from egg to emergence of adult that was laid the same day in different host scales on one lemon varied significantly. Emergence occurred at 12 to 15 days at 30 °C, 15 to 18 days at 25 °C and from 25 to 32 days at 20 °C. However, eggs laid in the same scale always developed in synchronisation and emerged within minutes from each other.

Most *Aphytis* spp. are multivoltine, develop throughout the year and have two or three generations to one of their hosts. However, diapause is known to occur in the larval stage, mostly in species that develop on hosts that overwinter as eggs (Rosen & DeBach, 1979).

1.3.4.1. *Aphytis* egg

Aphytis eggs are teardrop-shaped and semi-transparent (Forster et al., 1995). *Aphytis* mostly deposit the eggs on the dorsal or ventral aspect of the scale body under the covering when scales are in the instar stages. Eggs adhere to the scale body (Fig. 1.27) with an adhesive pad (Rosen & DeBach, 1979; Rosen et al., 1978).

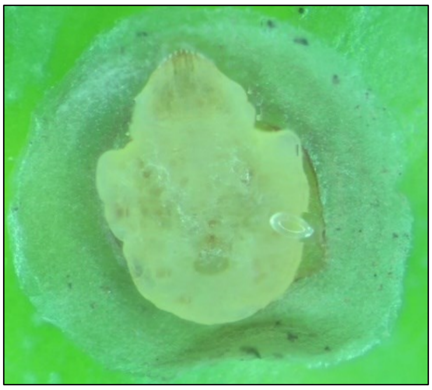


Figure 1.27. *Aphytis* egg deposited on the ventral side of a virgin third instar red scale female.

The duration of the egg stage constitutes 18% of the life cycle, with development increased as temperature increased to six, three and two days at 17 °C, 25 °C and 26.7 °C, respectively (Yu & Luck, 1988).

1.3.4.2. *Aphytis* larva

The larval stage takes up 36% of the total life cycle, that is, 10.85, 6.04 and 5.19 days at 17 °C, 25 °C and 26.7 °C, respectively (Yu & Luck, 1988). Three instars exist in Aphelinidae and can be separated by the different sizes and shapes of their mandibles, and the number of open spiracles (Rosen & DeBach, 1979; Rosen et al., 1978). Individuals in this stage vary in size and can be recognised by their elongated sac appearance (Fig. 1.28) with body segments (Forster et al., 1995).

Figure 1.28. An early third instar *Aphytis* sp. larva feeding on a second instar male red scale (a), a second instar *Aphytis* sp. larva feeding on a third instar red scale female (b) and two third instar *Aphytis* sp. larvae that fed from one host scale (c).

The newly hatched first instar larvae of *Aphytis* are ovoid in shape and fully developed after three days. Body segmentation is not clearly visible during this development stage. In this instar, *Aphytis* has four pairs of spiracles, one pair of which is in the mesothoracic segment and one in each of the first three abdominal segments (Rosen & DeBach, 1979).

The second instar is reached five to six days after oviposition and lasts for two to three days. Nine days after oviposition, the larva enters the third and last instar, which lasts for five to six days. As the *Aphytis* larva grows and develops, the host becomes depleted and gradually shrinks, becoming creased in appearance (Rosen & DeBach, 1979). During larval feeding, gut material becomes visible through the segmented body (Fig. 1.29) and changes colour from golden yellow to brown, as the larva matures (Forster et al., 1995).

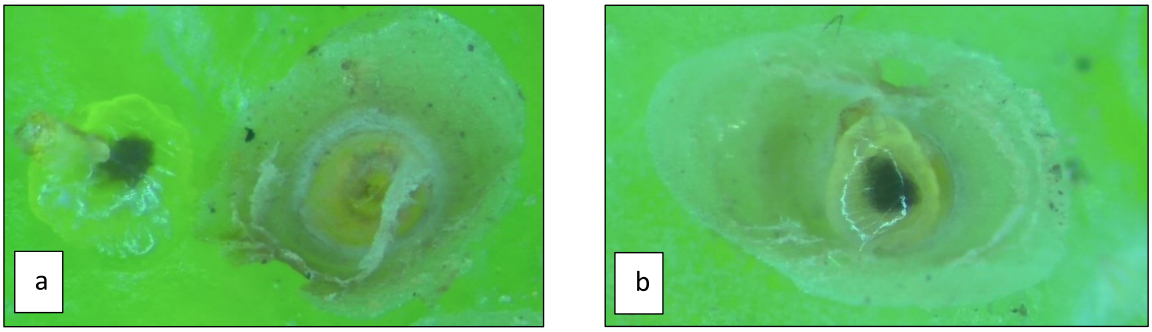


Figure 1.29. A third instar *Aphytis* sp., with the gut material still visible inside the larval body (a, b).

On the 17th, 9th and 7th days after oviposition at 17 °C, 25 °C and 26.7 °C, respectively, the third instar larva transforms into the prepupal stage and all feeding stops (Yu & Luck, 1988).

1.3.4.3. *Aphytis* prepupa

The *Aphytis* larva then turns on its back with its ventral aspect towards the scale covering and excretes between seven and 15 meconial pellets (Rosen & DeBach, 1979) (Fig. 1.30). This stage therefore lacks the colouration of the gut, due to the absence of gut material (Forster et al., 1995). The larva enters a resting period in the prepupal stage, lasting about two days, during which rapid development occurs and organs gradually start to gain pigmentation. Pigmentation of pupal organs systematically progresses, starting first with the inner margins of the eyes, then the bases and tips of the antennal cases, the tips of the mouthparts, legs, and wing cases, and eventually, the point of the abdomen. Pigment spreads gradually from the top to the base of each organ (Rosen et al., 1978).

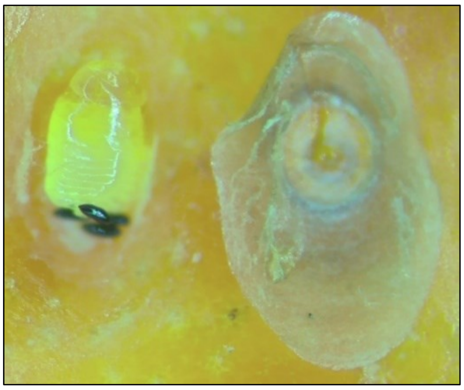


Figure 1.30. *Aphytis* sp. prepupa from beneath the cover of a male red scale, with the recent excreted meconial pellets.

As with other Hymenoptera, pupation of *Aphytis* spp. is exarate and normally begins about 14 days after oviposition and lasts for about six to seven days when partial pigmentation has been reached during the prepupal stage.

1.3.4.4. *Aphytis* pupa

As pupae mature, the eyes gradually change from colourless to red and then to green (Fig. 1.31), providing an indication of the developmental stage of the pupae (Rosen & DeBach, 1979; Forster et al., 1995; Yu & Luck, 1988; Wang et al., 2021). The colourless pupal stage comprises 18% of the life cycle and varies from 6.23, 3.08 and 2.43 days at 17 °C, 25 °C and 26.7 °C, respectively. The red- and green-eyed pupal stages are shorter than the colourless stage and make up only 13% and 7% of the life cycle, respectively (Yu & Luck, 1988).

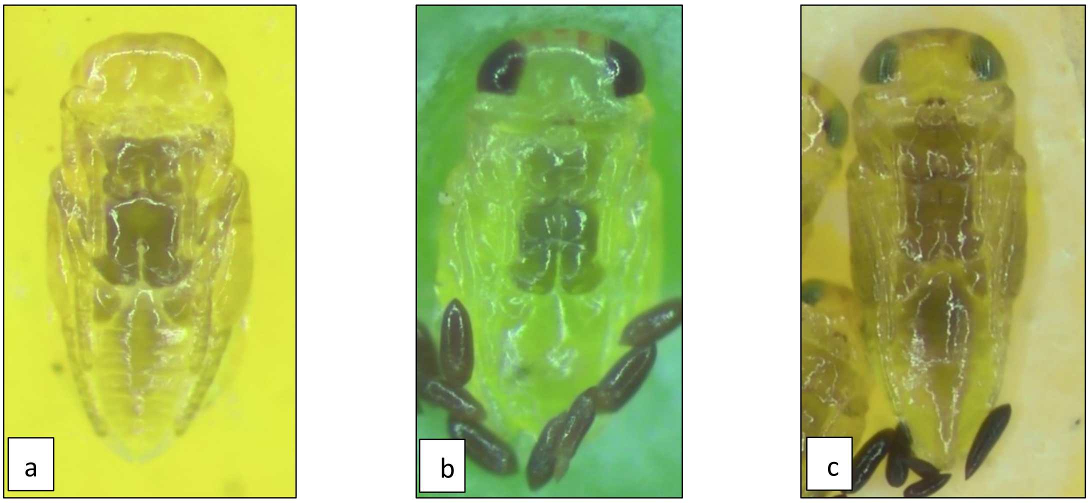


Figure 1.31. *Aphytis* spp. in various stages of pupal development: colourless-eyed pupal stage (a), red-eyed pupal stage (b) and the green-eyed pupal stage (c).

Males and females can be distinguished from each other at the pupal stage for the first time during this development stage: female pupae have a pair of minute rectangular plates present ventrally near the tip of the abdomen (Rosen & DeBach, 1979; Rosen et al., 1978). Adult *Aphytis* emerge about one day after the eyes of the pupa have turned green, a feature that is retained in the adult (Forster et al., 1995; Abdelrahman, 1974b).

1.3.4.5. *Aphytis* adult

Eclosion happens beneath the scale covering of the host and the pupal skin is broken into three parts (Fig. 1.32). The cephalic and thoracic exuviae are usually recognisable after emergence, whereas the abdominal exuvium is thinner and more fragile. Like the pupa, the emerging wasp lies on its dorsum while facing the scale cover with its ventral aspect and mouth parts. Emergence usually starts by biting an exit hole through the scale covering or by simply pushing the cover upwards to exit between the scale covering and the fruit surface. If more than one wasp has developed in the same host, they will always exit through the same hole. Even for a prolonged period after emergence, the characteristic meconium exuviae and the exit hole, if present, are tell-tale signs that a dead armoured scale insect was parasitized by *Aphytis* (Rosen & DeBach, 1979; Forster et al., 1995).

Figure 1.32. Eclosion of an adult *Aphytis* sp. wasp from the pupal skin covering. The red scale exuvia and meconial pellets can be seen on the right and bottom, respectively.

After emergence from the host scale, the adult *Aphytis* rests for a while and cleans itself. Active movement of an adult in the presence of a host population usually entails running. Short-distance flight mostly occurs on warm sunny days. Longer distance dispersal is assumed to happen on warmer, sunny days and is aided by light wind. Wasps will cling to plant surfaces and take refuge during strong winds and storms (Rosen & DeBach, 1979).

Sexual behaviour occurs without delay in the absence of a premating period in *Aphytis* spp. Males and females are sexually receptive immediately after eclosion; females will mate during any time of the day or night with conspecific males. The *Aphytis* female is monogamous and mates only once in her life, storing sufficient sperm in her spermatheca to fertilise all her eggs. Such females will refuse subsequent mating. By contrast, males are polygamous and are capable of mating in rapid succession with several females soon after eclosion (Rosen & DeBach, 1979).

Aphytis adults mostly emerge in proximity to their next potential host, owing to the clustered presence of red scale on plants. Host location itself encompasses a random search of the host plant surface. The female parasitoid moves quickly over the host plant area, while touching the surface rapidly with her antennae. Short flights are sometimes accomplished. Scale selection entails a ritual of exploration and investigation of potential hosts, until a specific criterion is met. After host selection, oviposition takes place when the hard scale cover is penetrated at the margin (Rosen & DeBach, 1979) (Fig. 1.33). *Aphytis melinus* and *A. lingnanensis* quite often oviposit two or three eggs on one host, while *A. africanus* always only oviposits one egg per host scale (Annecke & Moran, 1982).

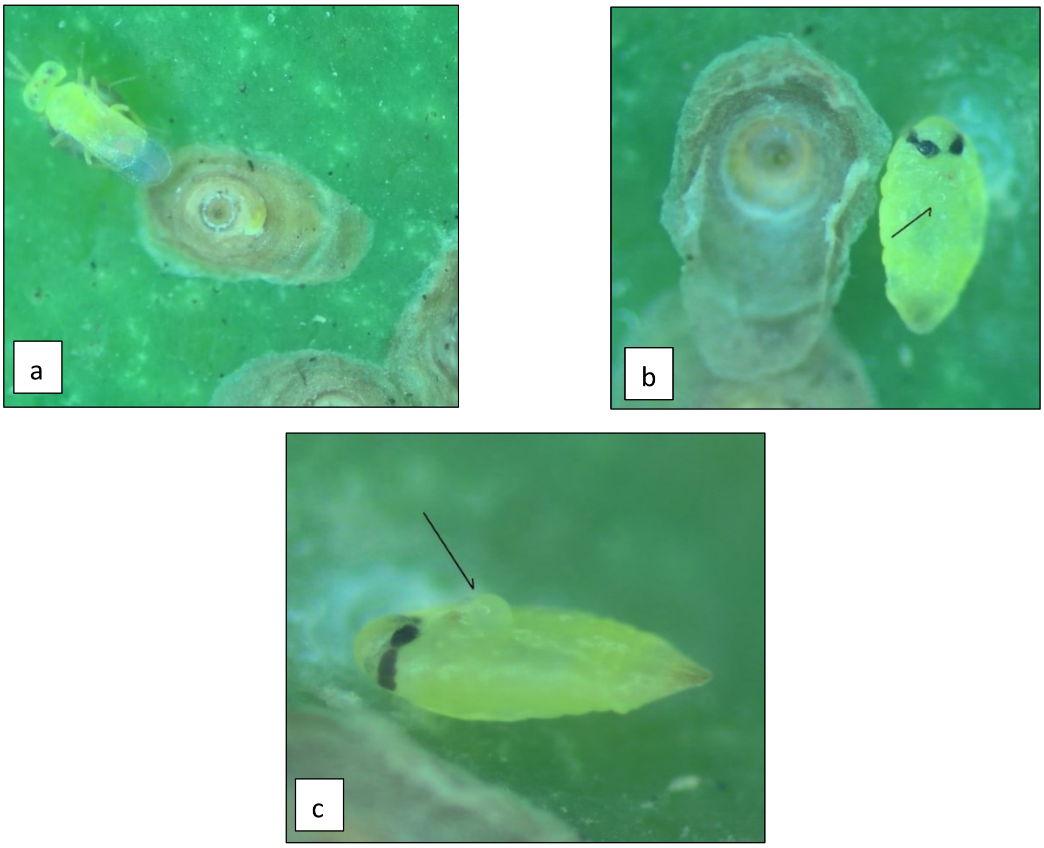


Figure 1.33. Female *Aphytis* sp. ovipositioning in a second instar red scale male (a). Note the freshly laid egg on the dorsal part of the male scale from a dorsal and lateral perspective (b & c respectively).

1.3.5. Ecology

1.3.5.1. Biotic factors

1.3.5.1.1. Host scale

Host scales affect the fecundity of *Aphytis* in several ways. The suitability of a host to most given *Aphytis* spp. determines how attractive the *Aphytis* spp. find the host. Fecundity, in turn, is determined by suitability (Rosen & Debach, 1977) with *Aphytis* having higher fecundity on preferred hosts than on less preferred hosts (Rosen & DeBach, 1979). For example, *Aphytis lingnanensis* produced more than double the number of female progeny on oleander scale, *Aspidiotis nerii* Bouché (Hemiptera: Diaspididae), as hosts than on red scale (DeBach & White, 1960).

The host development stage plays an important role, as red scale is susceptible to attack by *A. melinus* only at certain stages, unlike oleander scale. Only second instar males and females, and third instar females are vulnerable to attack by *Aphytis* (Abdelrahman, 1974b; Rosen & DeBach, 1979; Forster et al., 1995; Annecke & Moran, 1982). Only 20% of the total life cycle of red scale is available for parasitism, whereas, for example, up to 90% of the life cycle of oleander scale is susceptible. This limited window of opportunity in the time line of the red scale life cycle makes effective biological control of the pest more difficult (DeBach, 1969). The moulting stages of red scale are not suitable for parasitism by *Aphytis* spp. because of the hard integument that unifies the soft body and stronger cover (Baker, 1976; Forster et al., 1995).

The specific host growth stage size plays a role in determining the sex ratio and fecundity *per se* of the *Aphytis*. *Aphytis melinus* lays male eggs in small scales and female eggs in larger ones (Opp & Luck, 1986), so *Aphytis* progeny is inclined to be more males on smaller scales; furthermore, females developed in smaller host scales are smaller and have a lower rate of development and number of ovarian eggs. The exact relationship, however, between female size and potential fecundity differs between *Aphytis* spp. (Opp & Luck, 1986).

1.3.5.1.2. Natural enemies of *Aphytis* spp.

Hyperparasitoids may have significant potential to disrupt biological control in natural and agricultural systems (Tougeron & Tena, 2019). *Marietta javensis* Motshulsky (Hymenoptera: Aphelinidae) is an ectoparasite of *Aphytis* spp. and well established in South Africa (Fig. 1.34) (Annecke & Moran, 1982; Hafez, 1988; Hayat, 1974; Annecke & Insley, 1972).

Figure 1.34. *Marietta javensis* adult.

This hyperparasitoid oviposits on the pupa of the primary parasitoid of the host scale (Fig. 1.35). No host feeding takes place (Kfir & Rosen, 1981).

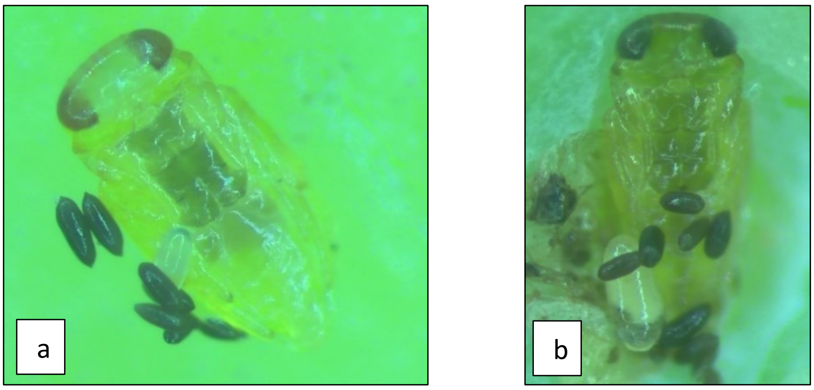


Figure 1.35. *Aphytis melinus* pupae with hyperparasitoid larvae in early development stages.

Very few records exist of natural enemies having a substantial effect on *Aphytis* populations other than the adverse effect of ants on adult *Aphytis* (Rosen & DeBach, 1979). However, Annecke and Moran (1982) state that all red scale parasitoids, except *A. africanus,* are subjected to attack by *M. javensis*. One larva matures on one host and feeds externally (Fig. 1.35) from which a pupa develops (Figs. 1.36 and 1.37) (Annecke & Moran, 1982).

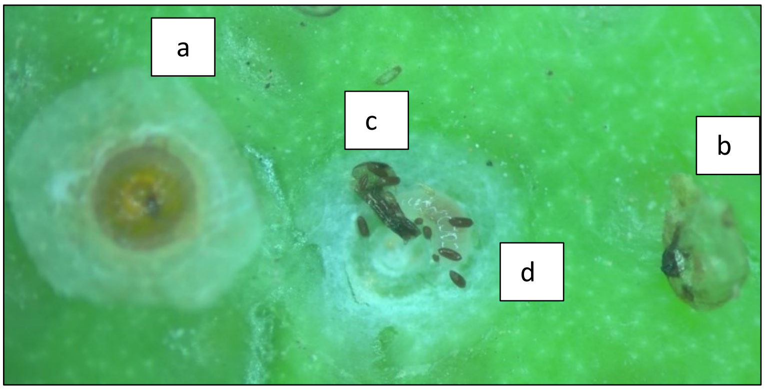


Figure 1.36. *Aphytis* sp., parasitised by *M. javensis*. Note the inverted third instar host scale cover (a), host scale body remains (b), *Aphytis* sp. remains (c) and the *M. javensis* larva (d).

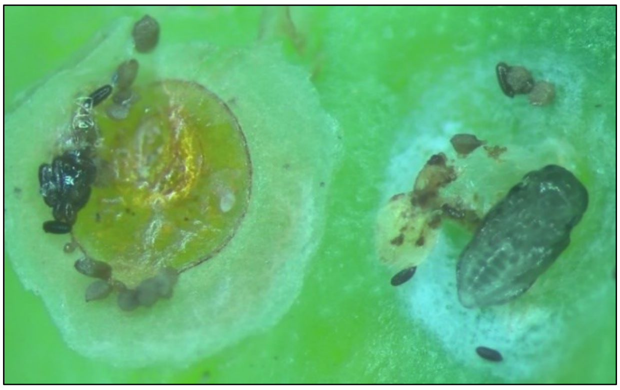


Figure 1.37. *Marietta javensis* pupa from an inverted third instar red scale. Note the two distinct types of meconia and the remains of the *Aphytis* sp. pupa on the scale cover. The exuvium of the red scale lies on the immediate left of the *M. javensis*. pupa.

1.3.5.1.3. Ants

Ants intentionally and directly visit Hemipteran insect pests such as soft scales, aphids, whiteflies and mealybugs because of the honeydew they produce (Samways et al., 1998; Bedford, 1968; Buckley, 1987; Bartlett, 1961; Itioka & Inoue, 1996; Samways et al., 1982). According to Samways et al. (1982), of the 123 ant species that have been recorded in citrus orchards in South Africa to date, only 44 foraged in trees and only 25 attended to honeydew-producing insects. However, only four species have attained pest status and are listed in order of importance: the pugnacious ant, *Anoplolepsis custodiens* Smith (Hymenoptera: Formicinae), the brown house ant, *Pheidole megacephale* Fabricius, the black pugnacious ant, *A. steingroeveri* Forel, and the Argentine ant, *Linepithema humile* Mayr.

Ant attendance on honeydew-producing hemipteran insect pests is mainly to harvest honeydew, thus protecting the host against parasitoids, predators and even some entomopathogenic fungi. Observations record that ants assist with transport of insects and parental care of some hemipteran species to enhance brood production, so confirming their mutualistic, symbiotic relationship. Red scale does not produce honeydew and it is not, therefore, directly attended by ants. However, increased ant activity disrupts the parasitoids and predators of red scale, and it has been established that certain ant species cause coincident infestations of red scale, among other non-honeydew producing insect pests (Rosen & DeBach, 1979; DeBach et al., 1951; Steyn, 1954b; Compere, 1961; Annecke, 1958; Bedford, 1968; Steyn, 1954a; Pekas et al., 2011; Samways et al., 1982; Samways, 1983; Samways et al., 1998; Flanders, 1945). It has also been demonstrated that ants compete with parasitoids, including *Aphytis* spp., for honeydew as a source of carbohydrates produced by non-*Aphytis* hosts (Calabuig et al., 2015; Tena et al., 2013) and also prey on *Aphytis* wasps (Heimpel, Rosenheim & Mangel, 1997). The constant disruption and interference of host examination and oviposition leads to dramatically reduced efficacy in host parasitism (Rosen & DeBach, 1979; Martinez-Ferrer et al., 2003).

Valencia trees treated with special ant-limiting bands had significantly lower ant (*P. megacephala*) and red scale infestations than untreated trees (Steyn, 1954a). Similar observations were made by Itioka and Inoue (1996), where the infestation by mealybug (*Pseudococcus citriculus* Green) in a Satsuma orchard decreased by 94% when the attendant ants (*Lasius niger* Linnaeus) were excluded. Moreno et al. (1987) found that in the absence of ants, populations of honeydew-producing citrus mealybug (*Planococcus citri* Risso), woolly whitefly (*Aleurothrixus floccosus* Maskell) and non-honeydew-producing red scale were effectively reduced by their respective natural enemies. Even in the absence of honeydew-producing scale, the reduction of parasitism of armoured scale by *Aphytis* spp. due to ant disturbance can be significant, and the effect of ants on *Aphytis* is rated next to weather extremes and detrimental pesticides (Heimpel, Rosenheim & Mangel, 1997; DeBach, Fleschner, et al., 1951b; Rosen & DeBach, 1979). Consequently control of ants is recommended in order to facilitate the biological control of red scale (Rosen & DeBach, 1979; DeBach, Dietrick, et al., 1951; Buckley, 1987; Bartlett, 1961).

1.3.5.1.4. Host varieties

In more ways than one, host plants may influence the fecundity of *Aphytis* spp. The efficacy of *Aphytis* spp. in smaller trees is much lower than in large mature trees (Rosen & DeBach, 1979), and the size of scale insects (and most probably the nutrition for their parasitoids) is affected by their host plant. When given a choice, *A. lingnanensis* parasitised more red scale on Sago palm than on Yucca (Smith, 1957). *Aphytis chrysomphali* preferred red scale on various infested fruit in the following declining order: citron melon, grapefruit, oranges and potato tubers, with no parasitism on the latter (Smith, 1957).

As mentioned earlier, different citrus varieties present great variation in their susceptibility to infestation by red scale (Habib et al., 1972; Hare et al., 1990; Salama & Mostafa, 1984) and consequently their primary biological control agent, *Aphytis* spp. *Aphytis melinus* wasps reared from scales on leaves of lemon produced nearly double the proportion of female offspring than those from scales reared on leaves of grapefruit, orange, or mandarin (Hare & Luck, 2018).

1.3.5.1.5. Competition

Competition in *Aphytis* can be allocated into interspecific and intraspecific categories. Intraspecific competition entails super-parasitism, cannibalism, interference, and discrimination. When individuals that operate in a population exert characteristics like these mentioned, a feedback mechanism is created to maintain population balance, preventing the specific population from increasing and decreasing excessively. These threats are only a problem under artificial circumstances when rearing of *Aphytis* spp. takes place and optimal output is needed (Rosen & DeBach, 1979). Very few cases have been recorded on *Aphytis* spp. which can discriminate between parasitised and unparasitised scales and then avoid oviposition in parasitised scales (Van Lenteren pers. comm. to Rosen and DeBach, 1978).

Competition between supernumerary larvae of the same species leads to a reduction in the survival rate in the pupal stages. When there are three larvae per host scale, in the case of *A. lingnanensis,* 40% die, 34% of *A. fisheri,* but only 6% of *A. melinus*. This compares to a 100% survival rate when only one *Aphytis* larva is present per host scale (Rosen & DeBach, 1979). Interspecific competition between larvae produces a greater effect than intraspecific competition, and the density of adult females also has a substantial effect on *Aphytis* fecundity. When four adult *Aphytis* were kept under comparable conditions per scale-infested lemon fruit, the progeny was 31.8 per female for *A. lingnanensis*, 25.9 for *A. fisheri* and 24.1 for *A. melinus*. When adult *Aphytis* numbers were increased to 40 per fruit, the progeny per female was 7 for *A. lingnanensis*, 7.1 for *A. fisheri* and 6.5 for *A. melinus* (DeBach & Sundby, 1963).

Research in interspecific competition has made significant contributions to basic ecology theory, and the theory and practice of biological control. Competition between two or more ecological homologues mostly involves closely related *Aphytis* spp. in competition with each other (Rosen & DeBach, 1979). An example of interspecific competition occurred where two homologues competed: *A. melinus* replaced *A. lingnanensis* in Californian citrus-producing areas during the 1960s after the introduction of the former in 1956 to 1957. *Aphytis melinus* can use a smaller scale size threshold to produce female offspring, and thus pre-empts red scale individuals before they grow to a size that is favourable for *A. lingnanensis* (Luck & Podoler, 1985). In another study in South Africa, *A. melinus* was displaced by *A. lingnanensis* when the latter was augmented. When the augmentation was halted, the displacement simply reversed and no overall increase in red scale parasitism was noted while *A. lingnanensis* dominated (Moore & Richards, 2002, unpublished data). This finding does not agree with findings in other studies which is difficult to explain. The result is competitive displacement or exclusion of one species by another. Competition between non-homologues normally involves a species of *Aphytis* competing with one or more endoparasitic species for the same host and does not result in displacement but rather in complementary effects which increase the overall biological control result (Rosen & DeBach, 1979).

1.3.5.2. Abiotic factors

1.3.5.2.1. Temperature and humidity

Temperature extremes are the most important natural cause of mortality and other adverse consequences for *Aphytis* spp. (Rosen & DeBach, 1979; DeBach & Sundby, 1963). In general, heat was more critical than cold for the survival of red scale natural enemies. The pupal stage of *A. melinus* is the most tolerant of cold, followed in levels of increasing sensitivity by the adult, the egg, and finally the larval stage, which is the most sensitive. The pupa was less sensitive to heat, while eggs and larvae showed intermediate sensitivity and adults were the most vulnerable to heat, making the pupal stages of *Aphytis* spp. the most tolerant to temperature extremes. Low winter temperatures cause greater red scale mortality than *Aphytis* spp., with the reverse being true in hot summer months. However, DeBach et al. (1955) found consistent evidence from field data that populations of *Aphytis* spp. were depleted out of proportion to its red scale host during the winter and, in some areas, at other times of the year. Laboratory tests confirm that low temperatures are the main reason for *Aphytis* mortality. The threshold for development of *A. melinus* is 11 °C (Abdelrahman, 1974a) and it appears that stages of *Aphytis* spp. are slightly less tolerant of cold and heat extremes than the stages at which red scale can be parasitised, with the exception of one instance: cold susceptibility of the host pre-pupal and pupal stages (males) (Abdelrahman, 1973). High temperatures (32.2 °C) dramatically lowered life expectancy of adult *Aphytis* spp. as well as the survival of immature stages (Rosen & DeBach, 1979; DeBach et al., 1955). Apart from direct effects, high temperatures reduce the size and duration of the scale second and third instars, which in turn, leads to decreased *A. melinus* female progeny and a shorter period in which scale hosts are susceptible for parasitism (Yu & Luck, 1988).

The fertility of *A. melinus* was not impacted as much by extreme temperatures. In a study conducted where batches of mated females were subjected to LD_{50} cycles for low and high temperature, through peaks of between -1.1 °C and 38.7 °C, the sex ratio of the progeny was normal (2 : 1; female : male) (Abdelrahman, 1973). These results differ significantly from the findings of Kfir and Luck (1979) and DeBach et al. (1955), whose trials entailed pre-ovipositional exposure to the same extreme high and low temperatures, but maintained at pre-determined periods instead of high-low cycles. Abdelrahman (1973) explained these differences by describing that sudden changes in temperature as opposed to gradual change could cause this response. Abdelrahman's (1973) work is more applicable to this study because, in nature, temperature fluctuates regularly in a 24-hour cycle. Trials with extreme constant temperatures involve sudden and potentially traumatic changes in thermal conditions, of which the effects on insects are unknown (Messenger & Flitters, 1959). Thus, data obtained from situations under constant temperature may be significantly different from those obtained from more natural, fluctuating temperatures that are characteristic of natural habitats (Bursell, 1964).

The influence of temperature on the development of *Aphytis* spp. is an important aspect of their regulatory ability (Rosen & DeBach, 1979). The rate of development for many species (*A. lingnanensis*, *A. coheni*, *A. africanus*, *A. melinus*, *A. fisheri* and *A. holoxanthus*) was the same, taking

12 to 13 days at a constant temperature of 26.6 °C to complete the life cycle from egg to the adult stage (Rao & DeBach, 1969). According to Kfir and Luck (1984) the duration of development of *A. melinus*, *A. lingnanensis* and *A. chrysomphali* was progressively shorter at higher temperatures, (Table 1.4). *Aphytis chrysomphali*, *A. lingnanensis* and *A. melinus* developed from oviposition to adult in 17.70, 20.33 and 19.69 days respectively at 21 °C. At 32 °C, the species in the same order developed from oviposition to adult in 11.28, 10.43 and 11.03 days, respectively.

Table 1.4. Duration of development of *A. chrysomphali*, *A. lingnanensis* and *A. melinus* from oviposition to adult emergence at five constant temperatures with red scale as host (Kfir & Luck, 1984).

Temperature (°C)	Number of parasitoids		Duration of development in days (Mean SE)
	Female	Male	
A. chrysomphali			
21	43	-	17.70 (0.13)
24	45	-	14.11 (0.13)
27	19	-	12.89 (0.18)
29	29	-	11.28 (0.10)
32	-	-	-
A. lingnanensis			
21	104	103	20.33 (0.06)
24	10	15	15.72 (0.17)
27	29	14	13.13 (0.11)
29	119	110	11.95 (0.12)
32	21	21	10.43 (0.11)
A. melinus			
21	13	21	19.69 (0.25)
24	16	11	15.49 (0.12)
27	9	10	13.62 (0.20)
29	40	35	11.90 (0.11)
32	18	13	11.03 (0.19)

The effect of humidity on *Aphytis* has been much less marked than that of temperature, although the desiccating effect of low humidity negatively affects *Aphytis* spp. Immature stages of *Aphytis* are less exposed than adults when sheltered against the effect of low humidity and still intact under the host cover where the unique microclimate protects them from drying out (Rosen & DeBach, 1979). Kfir and

Luck (1984) subjected adult *A. melinus* to temperatures 21 °C, 27 °C and 32 °C and each at 10%, 40% and 70% relative humidity respectively. Adults survived longer (4.24 vs. 2.36 and <1 day respectively) at a lower temperature (21 °C) at 10% RH than at a higher temperature (27 °C and 32 °C) and the same RH. The longest life duration was recorded at 21 °C and 70% RH.

1.3.5.2.2. Light

The effect of light on *Aphytis* spp. in the field has not been studied, but it plays a key role in flight initiation and searching. Laboratory tests showed that *A. lingnanensis* females were positively phototactic and that adult *A. melinus* can be trapped on yellow red scale pheromone traps as opposed to white ones (Rosen & DeBach, 1979). *Aphytis lingnanensis* responded to light intensity in laboratory studies, showing that they are positively phototactic (DeBach & White, 1960).

1.3.5.2.3. Insecticides

The adverse effect of chemical insecticides is extensive in relation to various active ingredients applied to orchards. The different reactions of pests are often instantly drastic and have long-term effects, which are often overlooked (Rosen & DeBach, 1979; Grout, 2012b). The use of less broad-spectrum pesticides, which interfere with the effect of predators and parasitoids in a biointensive IPM programme, improved biological control success (Rill et al., 2008).

Various pesticides have been evaluated with pyrethroids, carbamates and organophosphates, in declining order, shown to be the ones most toxic to *A. melinus*. Beneficial insects like *Aphytis* spp. are more sensitive to insecticides like carbamates and organophosphates aimed at scale control than the target scale itself (Bellows & Morse, 1993). Red scale populations increased between 40- and 200-fold in inland and coastal orchards under biological control in treated plots when sprayed with DDT, toxaphene, endrin, dieldrin, parathion and carbaryl (Debach, 1965).

Resistance to organophosphates by red scale became evident in 1975 in the northern areas and nine years later in the southern parts of South Africa, and in 1995 in the San Joaquin Valley of California (Nel et al., 1979; Georgala, 1975; Grafton-Cardwell & Vehrs, 1995). Field populations of *A. africanus*, *A. lingnanensis A. melinus* also showed development of resistance to organophosphate applications (Schoonees & Giliomee, 1982; Rosenheim & Hoy, 1986; Javier et al., 1991). Organophosphates caused significant sub-lethal effects on the longevity and progeny production rates of *A. melinus* and *A. lingnanensis* (Rosenheim & Hoy, 1988; Javier et al., 1991). Chlorpyrifos also moved the progeny sex ratio away from the strong female-biased characteristic of this species (Hoffmann & Kennett, 1985). Horticultural mineral oil as a standalone application has been proven safe for use in IPM programmes where *Aphytis* spp. are important parasitoids (Rosen, 1967), but DeBach and Bartlett (1951) found that horticultural mineral oil applications might upset *Aphytis* spp. activity for up to three to five years, while concentrations of 1 to 2% killed 45.1% of juvenile *Aphytis* spp. This unsuspected result might be explained in that an increase in dead scale also means death of the immature *Aphytis* spp. stages as the mortality rate of red scale and immature *Aphytis* spp. were closely related (Davies & McLaren, 1977). Furthermore, the mere mechanical blast of applying remedies to citrus trees also has a negative effect on beneficial insects (Beirne, 1970). However, the addition of horticultural mineral oil with organophosphate insecticides reduced the toxicity of the organophosphate to *Aphytis* spp. and other beneficial insects (Schoonees & Giliomee, 1982; Searle, 1964; Wiese, 1962).

1.3.5.2.4. Dust

More severe red scale infestations have often been observed on trees adjacent to dusty roads (Compere, 1961; Rosen & DeBach, 1979), where dust particles precipitate on all host plant surfaces. The most widely accepted explanation for this outcome is that dust particles interfere with the well-being of natural enemies (DeBach, 1951). If the dust particles are very fine and deposited regularly, the effect can be the same as deleterious chemicals to beneficial arthropods (Rosen & DeBach, 1979). The specific mode of action, as currently understood, is that particles eventually causes desiccation by wearing away the integument of the intersegmental membranes of beneficial arthropods (Bartlett, 1951). *Aphytis* spp. adults that have been exposed to dust have spent considerable time intensively grooming themselves, demonstrating their sensitivity towards dust (Rosen & DeBach, 1979).

1.4. Objective of the study

The overall objective of this study was to determine if the augmentation of insectary-reared *A. melinus* could be a useful contribution to IPM programmes against red scale for citrus growers in Southern Africa. This was made up of field trials where mass-reared *A. melinus* wasps were released and the level of parasitism was evaluated on a regular basis.

Quality assessments in terms of sex ratio, flight tests and longevity assessments of reared *A. melinus* were performed with the aim of comparing insects that were reared locally versus imported ones. The potential effect of logistical timelines was also investigated with longevity comparisons to determine whether transit days play a significant part in the quality of reared *A. melinus*.

Molecular and morphological identification methods were utilised to identify and compare *Aphytis* spp. present in trial and other orchards.

Chapter 2: Field trials to evaluate the augmentation of *Aphytis melinus* (Hymenoptera: Aphelinidae) in controlling *Aonidiella aurantii* (Hemiptera: Diaspididae).

2.1. Introduction

The emphasis in recent years to reduce chemical insecticide applications in pest control programmes has been driven by the prospect of higher revenue from retailers for producers who deliver fruit with no or very low, residues to the target markets. Concomitantly, however, tolerance for pest presence and the accompanying fruit damage are at an all-time low, owing to severe competition between these same high-end supermarkets, especially in the European Union. This demand, among other factors, has led to a situation where growers have resorted to the augmentation of beneficial insects, which has subsequently reached an unprecedented high level in pest control programmes (Moore, 2023). However, mandatory calendar-bound spray applications of disruptive remedies against citrus black spot, *Phyllosticta citricarpa* McAlpine (Botryoshaeriales: Phyllostictaceae), (Grout, 2012b) and the exaggerated fear, especially, of citrus thrips, *Scirtothrips aurantii* Faure (Thysanoptera: Thripidae), fruit damage, means that IPM programmes have never been in such an unhealthy state (Moore, 2023). Along with other beneficial insects, *A. melinus* is currently being sold to many growers for augmentation within an IPM programme. Although *A. melinus* was introduced, and has been successfully established for more than 50 years in South Africa, it is still outcompeted by the indigenous species, *A. africanus*, in most citrus-producing areas (Bedford & Grobler, 1981; Bedford & Cilliers, 1994; Daneel & Ware, 2000; Debach, 1959).

It is therefore important that the contribution of augmented *A. melinus* towards red scale control is evaluated in South Africa by conducting field trials.

2.2. Materials and methods

2.2.1. Orchard selection

Pairs of orchards similar in cultivar, age, and tree size, and with comparable levels of red scale infestation were selected in the Eastern (Kirkwood and Hankey) and Western Cape (Citrusdal). The trial areas in orchards were about 1 ha in surface area and each pair was separated by at least 150 m in order to prevent released wasps moving to control sites as shown by Zappalà et al.(2012) (Table 2.1).

Table 2.1. Pairs of trial orchards used for *A. melinus* release and as untreated controls.

Treatment	Farm, orchard, (area), year planted	Season			Variety (cultivar)	Region
		2019/2020	2020/2021	2021/2022		
Release	Hexfarm 1350 T (1 ha) 2007	X	X	X	Mandarin (Nadorcott)	Citrusdal
Control	Hexfarm 1350 B (3.08ha) 2007	X	X	X	Mandarin (Nadorcott)	Citrusdal
Release	Hexfarm 1371 (1.40 ha) 2014	X	X	X	Lemon (Eureka)	Citrusdal
Control	Hexfarm 1272 (2.70 ha) 2013	X	X	X	Lemon (Eureka)	Citrusdal
Release	Modderfontein 401 (1.45 ha) 2008	X	X	X	Lemon (Eureka)	Citrusdal
Control	Modderfontein 405 (1.81 ha) 2008	X	X	X	Lemon (Eureka)	Citrusdal
Release	Mandaryn T (1.04 ha) 1997	X	X	X	Valencia (Midknight)	Hankey
Control	Mandaryn B (1.04 ha) 1997	X	X	X	Valencia (Midknight)	Hankey
Release	Normandale 33 (1 ha) 2011	X		X	Lemon (Eureka)	Kirkwood
Control	Step Aside 26 (1.10 ha) 2011	X		X	Lemon (Eureka)	Kirkwood
Release	Sunad 6 (1.41 ha) 2012	X			Lemon (Eureka)	Kirkwood
Control	Sunad 61 (8.92 ha) 2011	X			Lemon (Eureka)	Kirkwood
Release	Sunad 60 T (1.13 ha) 2009	X			Valencia (Midknight)	Kirkwood
Control	Sunad 60 C (2.22 ha) 2009	X			Valencia (Midknight)	Kirkwood

Release	Step Aside 23 T (1.13 ha) 2009		X	X	Valencia (Midknight)	Kirkwood
Control	Step Aside 32 C (2.22 ha) 2009		X	X	Valencia (Midknight)	Kirkwood

2.2.2. Monitoring scale using fruit infestation assessments

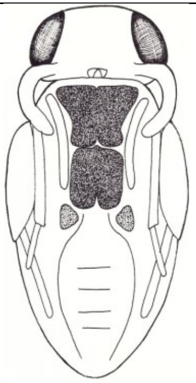
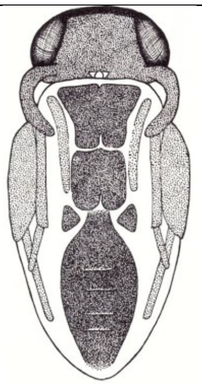
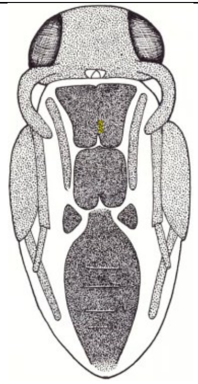
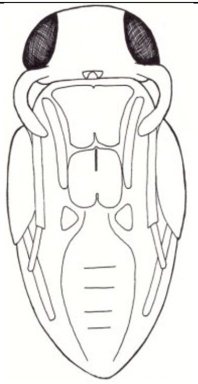
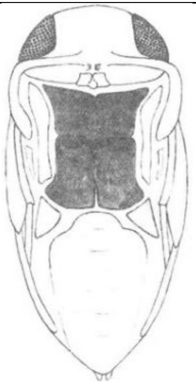
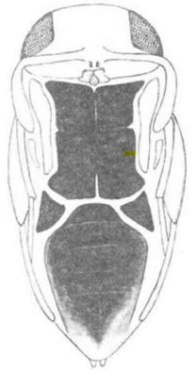
Fruit were inspected and red scale infestation levels were determined in all the trial orchards, in order to assess the infestation levels. Ten fruit on 10 marked trees within an unsprayed section in the middle of each orchard were examined in every orchard every fortnight, each year from January onwards. If one or more red scales were found on fruit, fruit were considered as infested.

2.2.3. Microscopic assessments of red scale

A sample of 20 well-infested fruit were randomly picked from each orchard within the 35 m wide unsprayed section every four weeks from January onwards. An assessment was carried out on up to 20 red scales per fruit when present. All stages of males and females in the second instar and older were microscopically examined to determine the percent of healthy, parasitised, and dead scale.

Red scale death was recorded as mutilated when fresh signs of recent host feeding, and abandoned probing were found. Unidentified *Aphytis* sp. was recorded when red scale parasitism was confirmed by the presence of any parasitoid life stage (eggs, larvae, or pre-pupae) or tell-tale signs of red scale parasitism evident by the existence parasitoid meconia, pupal exuvium, or exit holes in red scale coverings, but without the evidence required to provide species-level identification. *Aphytis* spp. pupae found in the late colourless-, red- and green-eyed stages were morphologically identified to the specific *Aphytis* sp., based on the identification key developed by (Prinsloo, 1984; DeBach, 1959; Wang et al., 2021; Quednau, 1964a; Bedford, 1998) (Table 2.2). Other parasitoids that were found, of which *C. bifasciata* was the only species, were identified according to the key provided by Forster et al. (1995). Predation was relatively negligible and was consequently not specifically considered in this study. Sampling stopped when the crop was harvested.

Table 2.2. Key used to identify *Aphytis* spp. pupae during the green and red-eyed stage

A. melinus	***A. africanus***	***A. lingnanensis***	***A. chrysomphali***
(Prinsloo, 1984)			
			
(Debach, 1959)			
			
Author's pictures based on above keys as well as Bedford, (1998), Quednau (1964a) and Wang et al. (2021).			
			

2.2.4. Monitoring of *Aphytis* spp. adults using yellow sticky trap cards

Four yellow sticky trap cards (Horiver®; 100 mm × 257 mm) were hung in each orchard. Traps were hung on the eastern side of the tree at a height of 1.8 m and plant material was regularly cleared around each trap. Each trap was replaced weekly and the *Aphytis* spp. wasps caught were counted. During the first (2019/2020) season, traps were baited with the red scale sex pheromone, (3S, 6R)-3-methyl-6-isopropenyl-9-decen-1-yl & (3S, 6S)-3-methyl-6-isopropenyl-9-decen-1-yl acetate, impregnated in a rubber septum supplied by Scentry Biologicals Inc. Traps without lures were used during the second (2020/2021) and third (2021/2022) seasons to avoid over-accumulation of male red scales on the cards, which could affect the accurate counting of the *Aphytis* spp. wasps. (Sorribas & Garcia-Marí, 2010; Moreno et al., 1984). Unfortunately trap cards of the 2020/2021 season from orchards Step Aside 23 and 32 were lost and therefore omitted.

2.2.5. Pesticide application records

Pesticides were applied in most orchards during the season to control common citrus pests (Tables 2.3 to 2.15). The produce of the orchards Modderfontein 401 and 405, as well as Hexfarm 1371 and 1272, is grown according to organic certified guidelines, hence no pesticides were used apart from one horticultural mineral oil application to orchard Modderfontein 405 in August 2021 (Table 2.12). In the middle of the rest of the orchards, a 35 m-wide section was left unsprayed immediately after releases were made.

2.2.5.1. The 2019/2020 season

Table 2.3. Pesticides applied in Hexfarm 1350 T and B orchards during the 2019/2020 season.

Date	Trade name	Active ingredient	Concentration	Dose/ 100 L water	Application volume/ ha (L)	Manufacturer/supplier
14-Sep-19	Eco BB	*Beauveria bassiana* R444	2 × 10^9 spores/ g	15 g	4000	Andermatt Madumbi
	Buprofezin 500 WG	Buprofezin	500 g/ kg	30 g		Villa Crop Protection
	Scalex 100 EC	Pyriproxyfen	100 g/ L	30 ml		Villa Crop Protection
	Orosorb	Orange oil	50 g/ L	50 ml		Oro Agri
		Borax	10 g/ L			
18-Oct-19	Delegate 250 WG	Spinetoram	250 g/ kg	10 g	3000	Corteva AgriSciences
26-Nov-19	Mirror	Kaolin	1000 g/ kg	1000 g	600	Metson World
27-Dec-19	Mirror	Kaolin	1000 g/ kg	1000 g	600	Metson World
30-Jan-20	Mirror	Kaolin	1000 g/ kg	1000 g	600	Metson World
15-Apr-20	Microthiol	Sulphur	800 g/ kg	150 g	3000	Total South Africa

Table 2.4. Pesticides applied in Mandaryn T and B orchards during the 2019/2020 season.

Date	Trade name	Active ingredient	Concentration	Dose/ 100 L water	Application volume/ ha (L)	Manufacturer/supplier
31-Jul-19	Aliette 800 WG	Fosetyl-Al	800 g/ L	200 g	2500	Bayer CropScience
04-Sep-19	Dursban 750 WG	Chlorpyrifos	750 g/ kg	64 g	6000	Dursban 750 WG
	Scalex 100 EC	Pyriproxyfen	100 g/ L	30 ml		Villa Crop Protection
	Charge	Polyether-polymethylsiloxane-copolymer	1000 g/ L	3 ml		Villa Crop Protection
02-Oct-19	Tenderbuff	Acetic acid and ammonia	500 g/ L	50 ml	5000	Rolfes Agri
	Methomyl 900 SP	Methomyl	900 g/ kg	50 g		Villa Crop Protection
	Pro Gibb 40 WG	Gibberellic acid	400 g/ kg	2.5 g		Valent BioSciences
	Buprofezin 500 WG	Buprofezin	500 g/ kg	30 g		Villa Crop Protection
	Charge	Polyether-polymethylsiloxane-copolymer	1000 g/ L	5 ml		Villa Crop Protection
	Scalex 100 EC	Pyriproxyfen	100 g/ L	30 ml		Villa Crop Protection
17-Oct-19	Unizeb 750 WG	Mancozeb	750 g/ kg	200 g	5000	Villa Crop Protection
	Dicarzol 500 SP	Formetanate	500 g/ kg	25 g		Klub M5
	Charge	Polyether-polymethylsiloxane-copolymer	1000 g/ L	5 ml		Villa Crop Protection
	Feeder	Sucrose, fructose, and glucose	650 g/ L	100 ml		KBS
04-Nov-19	Agrimec Gold 84 SC	Abamectin	84 g/ L	4.3 ml	5000	Syngenta
	Unizeb 750 WG	Mancozeb	750 g/ kg	200 g		Villa Crop Protection
	Cryptogran SC	Cryptophlebia leucotreta granulovirus	5×10^{10} OB / ml	10 ml		River BioScience
	Methomyl 900 SP	Methomyl	900 g/ kg	25 g		Villa Crop Protection
	Cipron	Mineral oil (medium-heavy)	846 g/ L	400 ml		H & R Sout Africa Sales
19-Nov-19	Tenderbuff	Acetic acid and ammonia	500 g/ L	50 ml	5000	Rolfes Agri
	Feeder	Sucrose, fructose, and glucose	650 g/ L	100 ml		KBS

	Dicarzol 500 SP	Formetanate	500 g/ kg	25 g		Klub M5
	Charge	Polyether-polymethylsiloxane-copolymer	1000 g/ L	3 ml		Villa Crop Protection
27-Nov-19	Aqua Right 7	Acetic acid	381 g/ L	100 ml	5000	Rolfes Agri
		Alkyl polyglucoside	86 g/ L			
	Delegate 250 WG	Spinetoram	250 g/ kg	20 g		Corteva AgriSciences
	Blackout SL	Dipotassium phosphate	297 g/ L	100 ml		River BioSciences
	Charge	Polyether-polymethylsiloxane-copolymer	1000 g/ L	3 ml		Villa Crop Protection
	Feeder	Sucrose, fructose, and glucose	650 g/ L	100 ml		KBS
	Eco BB	*Beauveria bassiana* R444	2×10^9 spores/ g	15 g		Andermatt Madumbi
19-Dec-19	Eco BB	*Beauveria bassiana* R444	2×10^9 spores/ g	15 g	5000	Andermatt Madumbi
	Delegate 250 WG	Spinetoram	250 g/ kg	12 g		Corteva AgriSciences
	Aqua Right 7	Acetic acid and ammonia	381 g/ L	100 ml		Rolfes Agri
		Alkyl polyglucoside	86g/ L			
		Alkyl polyglucoside	86 g/ L			
	Feeder	Sucrose, fructose, and glucose	650 g/ L	100 ml		KBS
15-Jan-20	Tenderbuff	Acetic acid and ammonia	500 g/ L	50 ml	5000	Rolfes Agri
	Blackout SL	Dipotassium phosphate	297 g/ L	100 ml		River BioSciences
	Agrimec Gold 84 SC	Abamectin	84 g/ L	4.3 ml		Syngenta
	Cipron	Mineral oil (medium-heavy)	846 g/ L	400 ml		H & R South Africa Sales

Table 2.5. Pesticides applied in Normandale 33 and Step Aside 26 orchards during the 2019/2020 season.

Date	Trade name	Active ingredient	Concentration	Dose/ 100 L water	Application volume/ ha (L)	Manufacturer/supplier
24-Sep-19	Commodobuff	Acetic acid and ammonia	660 g/ L	50 ml	3000	Villa Crop Protection
	Profenfos 500 EC	Profenofos	500 g/ L	100 ml	8000	Villa Crop Protection
	RB 1	Dipotassium phosphate	297 g/ L	100 ml		River BioSciences
	Break Thru S 240	Polyether-polymethylsiloxane-copolymer	1000 g/ L	3 ml		Evonik Degussa
14-Oct-19	Commodobuff	Acetic acid and ammonia	660 g/ L	50 ml	3000	Villa Crop Protection
	Klartan 240 EW	Tau-fluvalinate	240 g/ L	30 ml		Adama
24-Oct-19	Biomectin 18 EC	Abamectin	18 g/ L	20 ml	8000	Villa Crop Protection
	Benomyl 500 WP	Benomyl	500 g/ kg	50 g		Villa Crop Protection
	Pennfluid	Mancozeb	420 g/ L	150 ml		Total/ Cerexagri SA
	Scalex 100 EC	Pyriproxyfen	100 g/ L	30 ml		Villa Crop Protection
	Citrole 100	Medium mineral oil	810 g/ L	200 ml		Total South Africa
14-Nov-19	Commodobuff	Acetic acid and ammonia	660 g/ L	50 ml	2000	Villa Crop Protection
	Dicarzol 500 SP	Formetanate	500 g/ kg	25 g		Klub M5
	White sugar	Sugar	1000 g/ kg	200 g		Tongaat Huletts
19-Dec-19	Biomectin 18 EC	Abamectin	18 g/ L	20 ml	8000	Villa Crop Protection
	Citrole 100	Medium mineral oil	810 g/ L	300 ml		Total South Africa
	Scalex 100 EC	Pyriproxyfen	100 g/ L	30 ml		Villa Crop Protection
	Nordox	Copper oxide	860 g/ kg	90 g		Ascendis BioSciences
	Cerato 250 EC	Pyraclostrobin	250 g/ L	10 ml		Villa Crop Protection
16-Dec-19	Delegate 250 WG	Spinetoram	250 g/ L	10 g	2000	Corteva AgriScience
	Commodobuff	Acetic acid and ammonia	660 g/ L	25 ml		Villa Crop Protection
	Break Thru S 240	Polyether-polymethylsiloxane-copolymer	1000 g/ L	2 ml		Evonik Degussa

Table 2.6. Pesticides applied in Sunad 6 and 61 orchards during the 2019/2020 season.

Date	Trade name	Active ingredient	Concentration	Dose/ 100 L water	Application volume/ ha (L)	Manufacturer/supplier
23-Sep-19	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
07-Oct-19	Entrust WP	Spinosad	800 g/ kg	9 g	2000	Corteva AgriScience
21-Oct-19	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
11-Nov-19	Entrust WP	Spinosad	800 g/ kg	9 g	2000	Corteva AgriScience
18-Nov-19	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
02-Dec-19	Entrust WP	Spinosad	800 g/ kg	9 g	2000	Corteva AgriScience
13-Jan-20	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
10-Feb-20	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
10-Mar-20	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience

Table 2.7. Pesticides applied in Sunad 60 T and C orchards during the 2019/2020 season.

Date	Trade name	Active ingredient	Concentration	Dose/ 100 L water	Application volume/ ha (L)	Manufacturer/supplier
23-Sep-19	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
07-Oct-19	Entrust WP	Spinosad	800 g/ kg	9 g	2000	Corteva AgriScience
21-Oct-19	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
11-Nov-19	Entrust WP	Spinosad	800 g/ kg	9 g	2000	Corteva AgriScience
18-Nov-19	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
02-Dec-19	Entrust WP	Spinosad	800 g/ kg	9 g	2000	Corteva AgriScience
13-Jan-20	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
10-Feb-20	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
10-Mar-20	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
23-Sep-19	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
07-Oct-19	Entrust WP	Spinosad	800 g/ kg	9 g	2000	Corteva AgriScience
21-Oct-19	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
11-Nov-19	Entrust WP	Spinosad	800 g/ kg	9 g	2000	Corteva AgriScience
18-Nov-19	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience
02-Dec-19	Entrust WP	Spinosad	800 g/ kg	9 g	2000	Corteva AgriScience
13-Jan-20	Coprox Super	Copper oxychloride	850 g/ kg	200 g	8000	Arysta LifeScience

2.2.5.2. The 2020/2021 season

Table 2.8. List of pesticides applied in Hexfarm 1350 T and B orchards during the 2020/2021 season.

Date	Trade name	Active ingredient	Concentration	Dose/ 100 L water	Application volume/ ha (L)	Manufacturer/supplier
24-Sep-20	Helicovir	Helicoverpa armigera nucleopolydrovirus	5×10^9 OB/ ml	12 ml	3000	River BioScience
20-Oct-20	Aqua Right 7	Acetic acid and ammonia	381 g/ L	100 ml	2000	Rolfes Agri
		Alkyl polyglucoside	86g/ L			
	Delegate 250 WG	Spinetoram	250 g/ Kg	10 g		Corteva AgriScience
05-Nov-20	Scalex 100 EC	Pyriproxyfen	100 g/ L	30 ml	6000	Villa Crop Protection
	Biomectin 18 EC	Abamectin	18 g/ L	20 ml		Villa Crop Protection
	Orosorb	Orange oil	50 g/ L	100 ml		Oro Agri
		Borax	10 g/ L			
10-Dec-20	Deccoshield	Calcium carbonate	55%	2000 ml	1000	Citrashine
		Calcium metasilicate	5%			
		Calcium	24%			
19-Feb-21	Tivoli 240 SC	Spirotetramat	240 g/ L	20 ml	6000	Villa Crop Protection
	Orosorb	Orange oil	50 g/ L	200 ml		Oro Agri
		Borax	10 g/ L	100 ml		

Table 2.9. List of pesticides applied in Mandaryn T and B orchards during the 2020/2021 season.

Date	Trade name	Active ingredient	Concentration	Dose/ 100 L water	Application volume/ ha (L)	Manufacturer/supplier
31-Aug-20	Buprofezin 500 WG	Buprofesin	500 g/ kg	30 g	6000	Villa Crop Protection
	Nemesis 100 EC	Pyriproxyfen	100 g/ L	30 ml		Sumitomo
	Supawett	Polyether-polymethylsiloxane-copolymer	1000 g/ L	5 ml		Rolfes Agri
25-Sep-20	Savage 360 SC	Chlorphenapyr	360 g/ L	30 ml	2000	Villa Crop Protection
	Feeder	Sucrose, fructose, and glucose	650 g/ L	100 ml		KBS
22-Oct-20	Tenderbuff	Acetic acid and ammonia	500 g/ L	50 ml	6000	Rolfes Agri
	Unizeb 800 WP	Mancozeb	800 g/ kg	200 g		Villa Crop Protection
	Methomex 900 WP	Methomyl	900 g/ kg	100 g		Adama
	Supawett	Polyether-polymethylsiloxane-copolymer	1000 g/ L	5 ml		Rolfes Agri
10-Nov-20	Feeder	Sucrose, fructose, and glucose	650 g/ L	100 ml	2000	KBS
	Dicarzol 500 SP	Formetanate	500 g/ kg	25 g		Klub M5
	Unizeb 800 WP	Mancozeb	800 g/ kg	200 g		Villa Crop Protection
25-Nov-20	Tenderbuff	Acetic acid and ammonia	500 g/ L	50 ml	6000	Rolfes Agri
	Unizeb 800 WP	Mancozeb	800 g/ Kg	200 g		Villa Crop Protection
	Nemesis 100 EC	Pyriproxyfen	100 g/ L	30 ml		Sumitomo
	Biomectin 18 EC	Abamectin	18 g/ L	20 ml		Villa Crop Protection
	Benomyl 500 WP	Benomyl	500 g/ kg	50 g		Villa Crop Protection
	Methomex 900 WP	Methomyl	900 g/ kg	100 g		Adama
18-Dec-20	Aqua Right 5	Acetic acid	820 g/ L	50 ml	2000	Rolfes Agri
		Alcohol etoxylate	56 g/ L			
	Delegate 250 WG	Spinetoram	250 g/ L	10 g		Corteva AgriScience
	Feeder	Sucrose, fructose, and glucose	650 g/ L	100 ml		KBS

	Supawett	Polyether-polymethylsiloxane-copolymer	1000 g/ L	5 ml		Rolfes Agri
08-Jan-21	Tenderbuff	Acetic acid and ammonia	500 g/ L	50 ml	6000	Rolfes Agri
	Blackout SL	Dipotassium phosphate	297 g/ L	100 ml		River BioSciences
	Cryptex SC	Cryptophlebia leucotreta granulovirus	2x10^{10} OB/ ml	3.3 ml		River BioSciences

Table 2.10. List of pesticides applied in Step Aside 23 and 32 orchards during the 2020/2021 season.

Date	Trade name	Active ingredient	Concentration	Dosage rate/ 100 L water	Application volume/ ha (L)	Manufacturer
02-Nov-20	Cerato 250 EC	Pyraclostrobin	250 g/ L	10 ml	7000	Villa Crop Protection
	RB1	Dipotassium phosphate	297 g/ L	100 ml		River BioScience
	Citrole 100	Medium mineral oil	810 g/ L	300 ml		Total
26-Nov-20	Cerato 250 EC	Pyraclostrobin	250 g/ L	10 ml	7000	Villa Crop Protection
	RB1	Dipotassium phosphate	297 g/ L	100 ml		River BioScience
	Citrole 100	Medium mineral oil	810 g/ L	300 ml		Total
	Cryptogran SC	Cryptophlebia leucotreta granulovirus	5×10^{10} OB / ml	10 ml		River BioScience
	Biomectin 18 EC	Abamectin	18 g/ L	20 ml		Villa Crop Protection
	Commodobuff	Acetic acid and ammonia	660 g/ L	50 ml		Villa Crop Protection
08-Dec-20	Commodobuff	Acetic acid and ammonia	660 g/ L	75 ml	1500	Villa Crop Protection
	Dicarzol 500 SP	Formetanate	500 g/ kg	25 g		Avima
	Sugar	Sugar	1000 g/ kg	200 g		Huletts
14-Jan-21	Citrole 100	Medium mineral oil	810 g/ L	300 ml	7000	Total
	Biomectin 18 EC	Abamectin	18 g/ L	20 ml		Villa Crop Protection
	RB1	Dipotassium phosphate	297 g/ L	100 ml		River BioScience
20-Feb-21	Commodobuff	Acetic acid and ammonia	660 g/ L	50 ml	7000	Villa Crop Protection
	Break Thru S 240	Polyether-polymethylsiloxane-copolymer	1000 g/ L	3 ml		Evonik Degussa
	RB1	Dipotassium phosphate	297 g/ L	100 ml		River BioScience
	Cryptogran SC	Cryptophlebia leucotreta granulovirus	5×10^{10} OB / ml	10 ml		River BioScience

2.2.5.3. The 2021/2022 season

Table 2.11. List of pesticides applied in Hexfarm 1350 T and B orchards during the 2021/2022 season.

Date	Trade name	Active ingredient	Concentration	Dose/ 100 L water	Application volume/ ha (L)	Manufacturer/supplier
26-Oct-21	Meothrin 200 EC	Fenpropathrin	200 g/ L	50 ml	2000	Philagro SA
11-Nov-21	Biomectin 18 EC	Abamectin	18 g/ L	20 ml	8000	Villa Crop Protection
	Scalex 100 EC	Pyriproxyfen	100 g/ L	30 ml		Villa Crop Protection
	Orosorb	Orange oil	50 g/ L	100 ml		Oro Agri
		Borax	10 g/ L			
10-Dec-20	Deccoshield	Calcium carbonate	55%	2000 ml	2000	Citrashine
		Calcium metasilicate	5%			
		Calcium	24%			
12-Jan-22	Tartar Emetic 995 SP	Tartar emetic	995 g/ kg	400 g	2000	Villa Crop Protection
	Sugar	Sugar	1000 g/ kg	400 g		Huletts
18-Jan-22	Deccoshield	Calcium carbonate	55%	2000 ml	1000	Citrashine
		Calcium metasilicate	5%			
		Calcium	24%			
21-Feb-22	Deccoshield	Calcium carbonate	55%	2000 ml	1000	Citrashine
		Calcium metasilicate	5%			
		Calcium	24%			

Table 2.12. List of pesticides applied in Modderfontein 401 and 405 orchards during the 2021/2022 season.

Date	Trade name	Active ingredient	Concentration	Dose/ 100 L water	Application volume/ ha (L)	Manufacturer/supplier
10-Aug-21	Citrole 100	Medium mineral oil	810 g/ L	1000 ml	6000	Total South Africa

Table 2.13. List of pesticides applied in Mandaryn T and B orchards during the 2021/2022 season.

Date	Trade name	Active ingredient	Concentration	Dose/ 100 L water	Application volume/ ha (L)	Manufacturer/Supplier
20-Sep-21	Savage 360 SC	Chlorphenapyr	360 g/ L	30 ml	2000	Villa Crop Protection
	Feeder	Sucrose, fructose, and glucose	650 g/ L	100 ml		KBS
06-Oct-21	Pherfect	Acetic acid and ammonia	550 g/ L	50 ml	6000	Inline Trading
	Buprofezin 500 WG	Buprofesin	500 g/ kg	30 g		Villa Crop Protection
	Devipan 1000 EC	Dichlorvos	1000 g/ L	100 ml		Adama
	Supawett	Polyether-polymethylsiloxane-copolymer	1000 g/ L	5 ml		Rolfes Agri
11-Oct-21	Pherfect	Acetic acid and ammonia	550 g/ L	50 ml	6000	Inline Trading
	Buprofezin 500 WG	Buprofesin	500 g/kg	30 g		Villa Crop Protection
	Bolldex SC	Helicoverpa armigera HearNPV	7.5x10^{12} OBS/ L	10 ml		Andermantt Madumbi
	Supawett	Polyether-polymethylsiloxane-copolymer	1000 g/ L	5 ml		Rolfes Agri
19-Oct-21	Tenderbuff	Acetic acid and ammonia	500 g/ L	50 ml	6000	Rolfes Agri
	Unizeb 800 WP	Mancozeb	800 g/ kg	200 g		Villa Crop Protection
	Methomex 900 WP	Methomyl	900 g/ kg	100 g		Adama

	Supawett	Polyether-polymethylsiloxane-copolymer	1000 g/ L	5 ml		Rolfes Agri
10-Nov-21	Pherfect	Acetic acid and ammonia	550 g/ L	50 ml	6000	Inline Trading
	Unizeb 800 WP	Mancozeb	800 g/ Kg	150 g		Villa Crop Protection
	Methomex 900 WP	Methomyl	900 g/ Kg	25 g		Adama
	Cerato 250 EC	Pyraclostrobin	250 g/ L	10 ml		Villa Crop Protection
	Cipron	Mineral oil (medium-heavy)	846 g/ L	300 ml		H & R Sout Africa Sales
11-Jan-22	Pherfect	Acetic acid and ammonia	550 g/ L	50 ml	6000	Inline Trading
	Devipan 1000 EC	Dichlorvos	1000 g/ L	100 ml		Adama
	Cerato 250 EC	Pyraclostrobin	250 g/ L	10 ml		Villa Crop Protection
	Blackout SL	Dipotassium phosphate	297 g/ L	100 ml		River BioSciences
	Scalex 100 EC	Pyriproxyfen	100 g/ L	30 ml		Villa Crop Protection
	Cipron	Mineral oil (medium-heavy)	846 g/ L	200 ml		H & R Sout Africa Sales
	Biomectin 18 EC	Abamectin	18 g/ L	20 ml		Villa Crop Protection
23-Feb-22	Pherfect	Acetic acid and ammonia	550 g/ L	50 ml	6000	Inline Trading
	Devipan 1000 EC	Dichlorvos	1000 g/ L	100 ml		Adama
	Cerato 250 EC	Pyraclostrobin	250 g/ L	10 ml		Villa Crop Protection
	Blackout SL	Dipotassium phosphate	297 g/ L	100 ml		River BioSciences
	Scalex 100 EC	Pyriproxyfen	100 g/ L	30 ml		Villa Crop Protection
	Cipron	Mineral oil (medium-heavy)	846 g/ L	250 ml		H & R Sout Africa Sales
	Biomectin 18 EC	Abamectin	18 g/ L	20 ml		Villa Crop Protection

Table 2.14. List of pesticides applied in Normandale 33 and Step Aside 26 orchards during the 2021/2022 season.

Date	Trade name	Active ingredient	Concentration	Dose/ 100 L water	Application volume/ ha (L)	Manufacturer/supplier
04-Oct-21	Commodobuff	Acetic acid and ammonia	660 g/ L	50 ml	3000	Villa Crop Protection
	Klartan 240 EW	Tau-fluvalinate	240 g/ L	30 ml		Adama
	Thuricide WP	*Bacillus thuringiensis* var. *kurstaki*	16 000 IU/ mg	25 g		Villa Crop Protection
25-Oct-21	Biomectin 18 EC	Abamectin	18 g/ L	20 ml	8000	Villa Crop Protection
	Unizeb 800 WP	Mancozeb	800 g/ L	150 g		Villa Crop Protection
	Bendazid 550 SC	Carbendasim	550 g/ L	55 ml		AECI
	Tivoli 240 SC	Spirotetramat	240 g/ L	10 ml		Villa Crop Protection
	Citrimist	Medium grade minderal oil	830 g/ L	300 ml		Protea chemicals
11-Nov-21	Commodobuff	Acetic acid and ammonia	660 g/ L	25 ml	2000	Villa Crop Protection
	Delegate 250 WG	Spinetoram	250 g/ L	12 g		Corteva AgriScience
	Break Thru S 240	Polyether-polymethylsiloxane-copolymer	1000 g/ L	3 ml		Evonik Degussa
24-Nov-21	Commodobuff	Acetic acid and ammonia	660 g/ L	50 ml	8000	Villa Crop Protection
	Biomectin 18 EC	Abamectin	18 g/ L	20 ml		Villa Crop Protection
	Citrimist	Medium grade minderal oil	830 g/ L	300 ml		Protea chemicals
	Cerato 250 EC	Pyraclostrobin	250 g/ L	10 ml		Villa Crop Protection
	RB 1	Dipotassium phosphate	297 g/ L	100 ml		River BioSciences
	Tivoli 240 SC	Spirotetramat	240 g/ L	30 ml		Villa Crop Protection
05-Jan-22	Commodobuff	Acetic acid and ammonia	660 g/ L	50 ml	8000	Villa Crop Protection
	Biomectin 18 EC	Abamectin	18 g/ L	20 ml		Villa Crop Protection
	Citrimist	Medium grade minderal oil	830 g/ L	300 ml		Protea chemicals
	Cerato 250 EC	Pyraclostrobin	250 g/ L	10 ml		Villa Crop Protection
	RB 1	Dipotassium phosphate	297 g/ L	100 ml		River BioSciences

Table 2.15. List of pesticides applied in Step Aside 23 and 32 orchards during the 2021/2022 season.

Date	Trade name	Active ingredient	Concentration	Dose/ 100 L water	Application volume/ ha (L)	Manufacturer/supplier
20-Oct-21	Commodobuff	Acetic acid and ammonia	660 g/ L	50 ml	8000	Villa Crop Protection
	Biomectin 18 EC	Abamectin	18 g/ L	20 ml		Villa Crop Protection
	Citrimist	Medium grade mineral oil	830 g/ L	300 ml		Protea chemicals
	Cerato 250 EC	Pyroclostobin	250 g/ L	10 ml		Villa Crop Protection
	RB 1	Dipotassium phosphate	297 g/ L	100 ml		River BioSciences
	Tivoli 240 SC	Spirotetramat	240 g/ L	10 ml		Villa Crop Protection
24-Nov-21	Commodobuff	Acetic acid and ammonia	660 g/ L	50 ml	8000	Villa Crop Protection
	Biomectin 18 EC	Abamectin	18 g/ L	20 ml		Villa Crop Protection
	Citrimist	Medium grade minderal oil	830 g/ L	300 ml		Protea chemicals
	Cerato 250 EC	Pyraclostrobin	250 g/ L	10 ml		Villa Crop Protection
	RB 1	Dipotassium phosphate	297 g/ L	100 ml		River BioSciences
	Tivoli 240 SC	Spirotetramat	240 g/ L	30 ml		Villa Crop Protection
	Cryptogran SC	Cryptophlebia leucotreta granulovirus	5 × 10^{10} OB / ml	10 ml		River BioScience
14-Dec-21	Delegate 250 WG	Spinetoram	250 g/ L	10 g	2000	Corteva AgriScience
	Commodobuff	Acetic acid and ammonia	660 g/ L	50 ml		Villa Crop Protection
	Break Thru S 240	Polyether-polymethylsiloxane-copolymer	1000 g/ L	3 ml		Evonik Degussa
05-Jan-22	Commodobuff	Acetic acid and ammonia	660 g/ L	50 ml	8000	Villa Crop Protection
	Biomectin 18 EC	Abamectin	18 g/ L	20 ml		Villa Crop Protection
	Citrimist	Medium grade minderal oil	830 g/ L	300 ml		Protea chemicals
	Cerato 250 EC	Pyraclostrobin	250 g/ L	10 ml		Villa Crop Protection
	RB 1	Dipotassium phosphate	297 g/ L	100 ml		River BioSciences

2.2.6. Temperature records

Temperature data were recorded at 30-minute intervals for each farm during the field trials with HOBO MX3201a loggers. See Figs. 2.1 to 2.12.

2.2.6.1. The 2019/2020 season

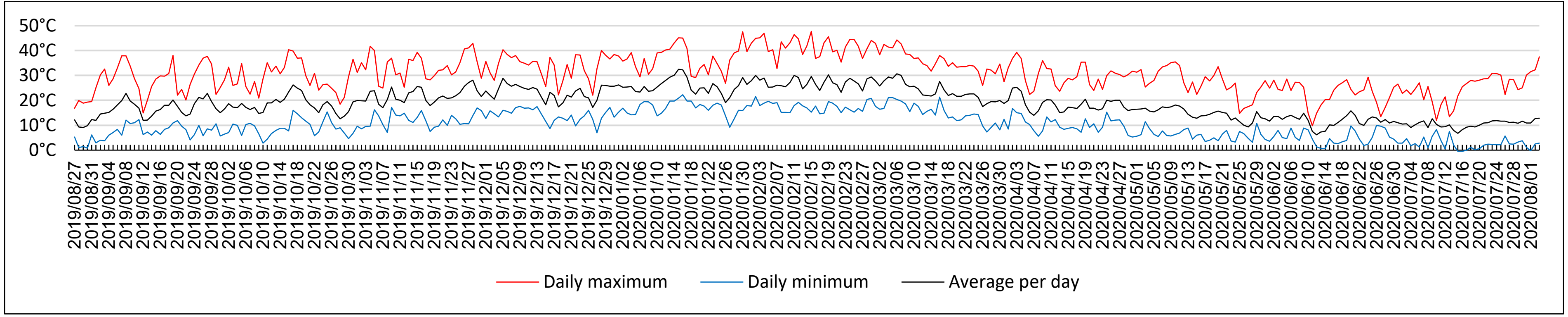


Figure 2.1. The daily maximum, minimum and average temperature during the 2019/2020 season for Hexfarm.

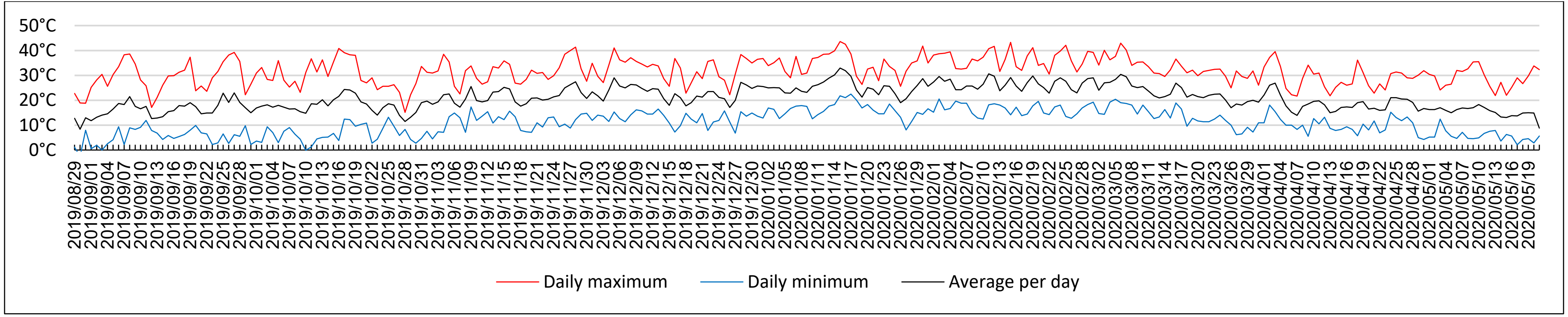


Figure 2.2. The daily maximum, minimum and average temperature during the 2019/2020 season for Modderfontein.

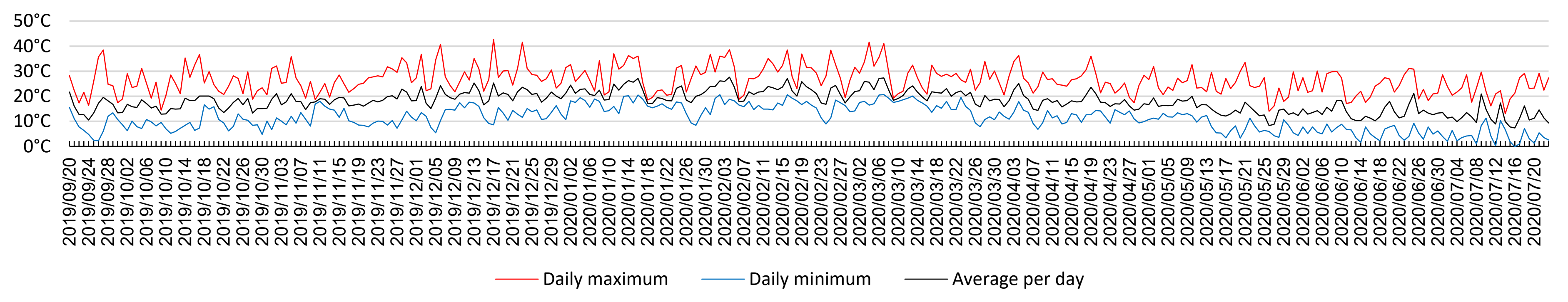


Figure 2.3. The daily maximum, minimum and average temperature during the 2019/2020 season for Mandaryn.

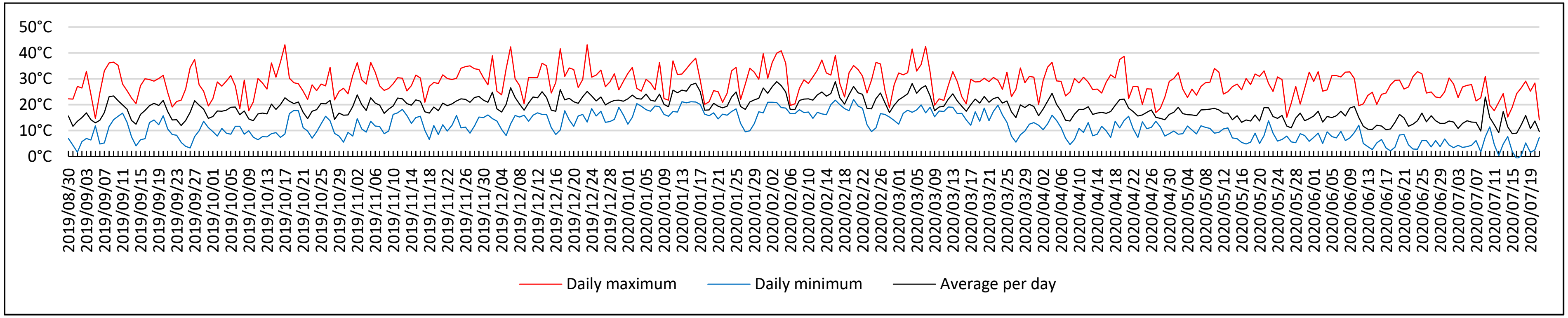


Figure 2.4. The daily maximum, minimum and average temperature during the 2019/2020 season for Sunad Farm, Step Aside and Normandale.

2.2.6.2. The 2020/2021 season

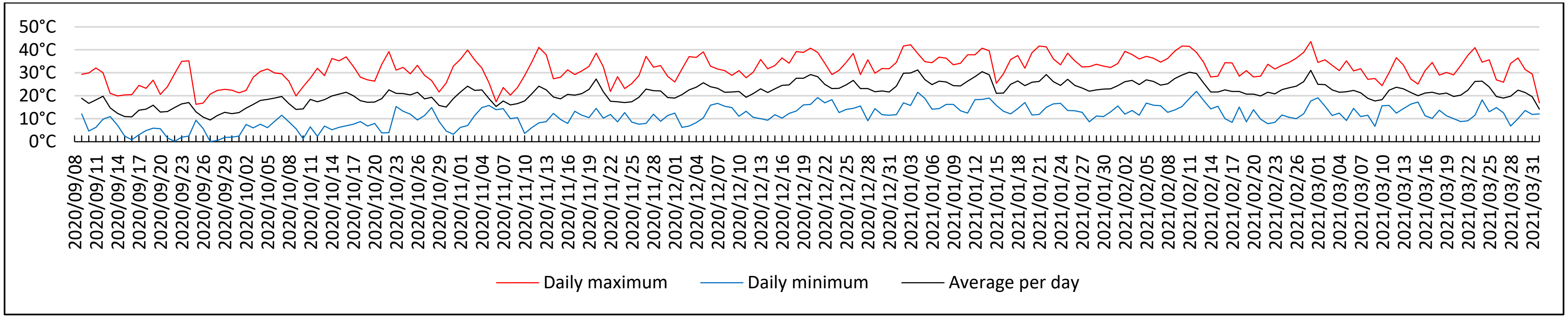


Figure 2.5. The daily maximum, minimum and average temperature during the 2020/2021 season for Hexfarm.

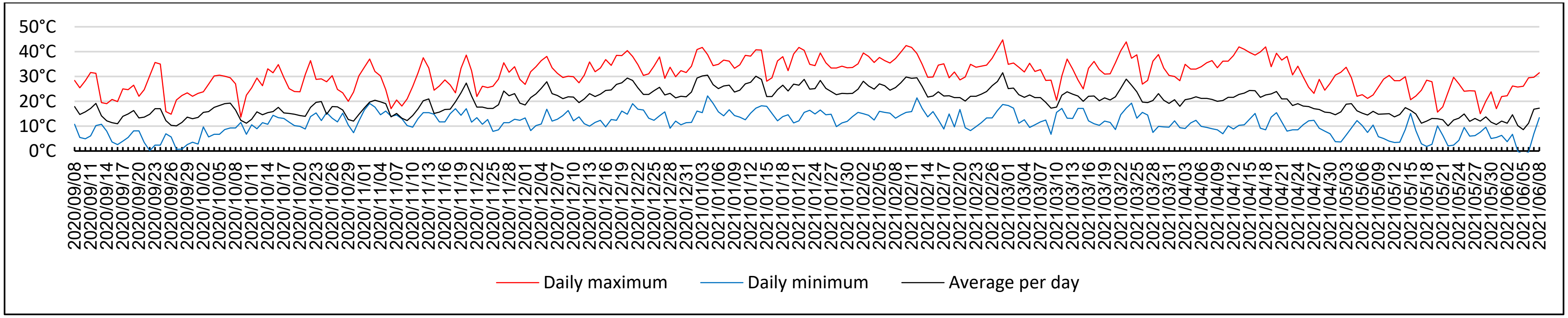


Figure 2.6. The daily maximum, minimum and average temperature during the 2020/2021 season for Modderfontein.

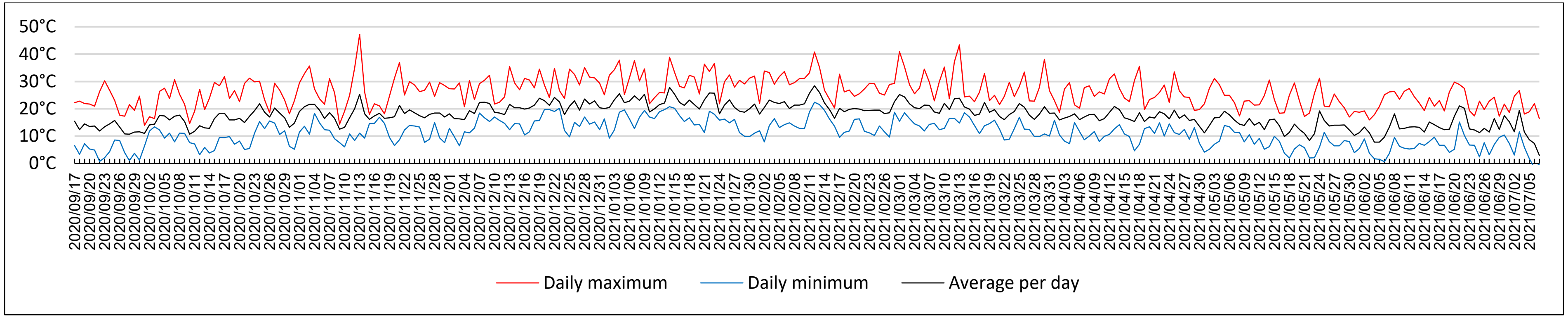


Figure 2.7. The daily maximum, minimum and average temperature during the 2020/2021 season for Mandaryn.

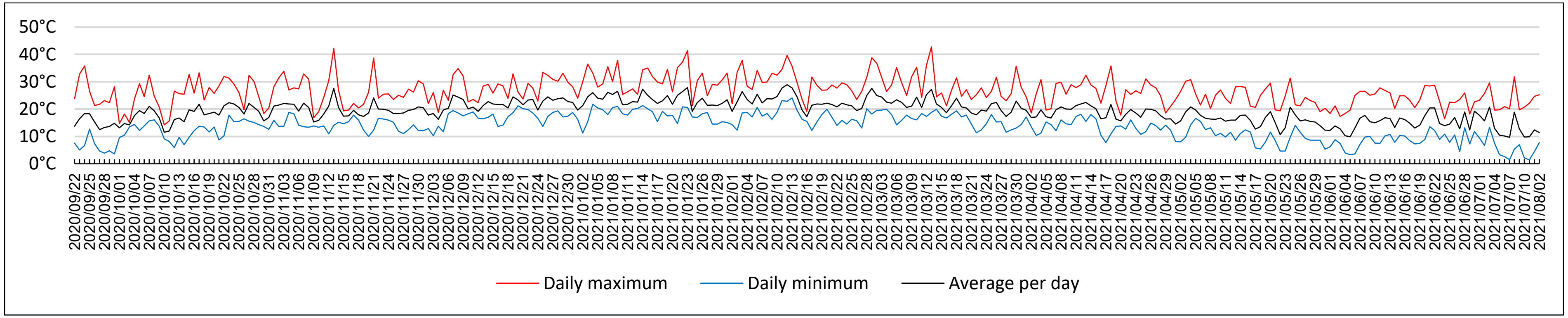


Figure 2.8. The daily maximum, minimum and average temperature during the 2020/2021 season for Step Aside.

2.2.6.3. The 2021/2022 season

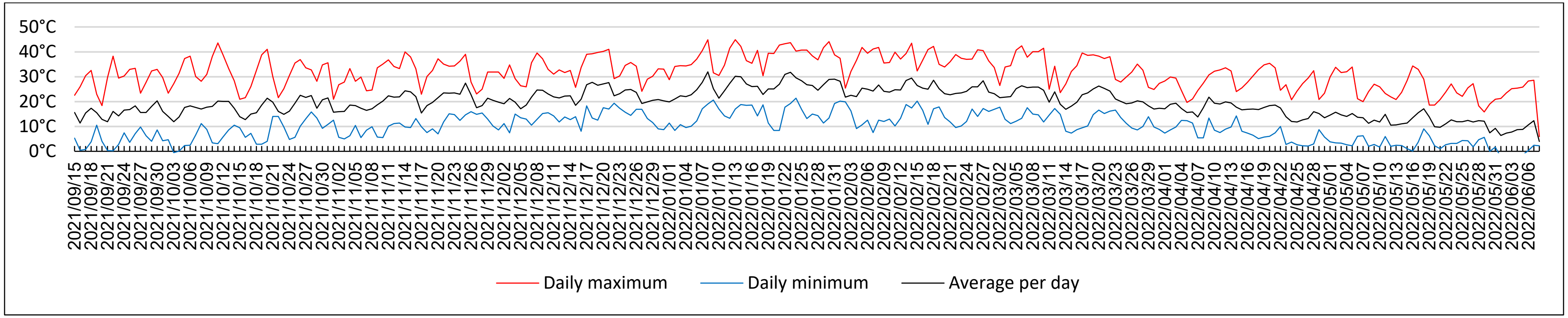


Figure 2.9. The daily maximum, minimum and average temperature during the 2021/2022 season for Hexfarm.

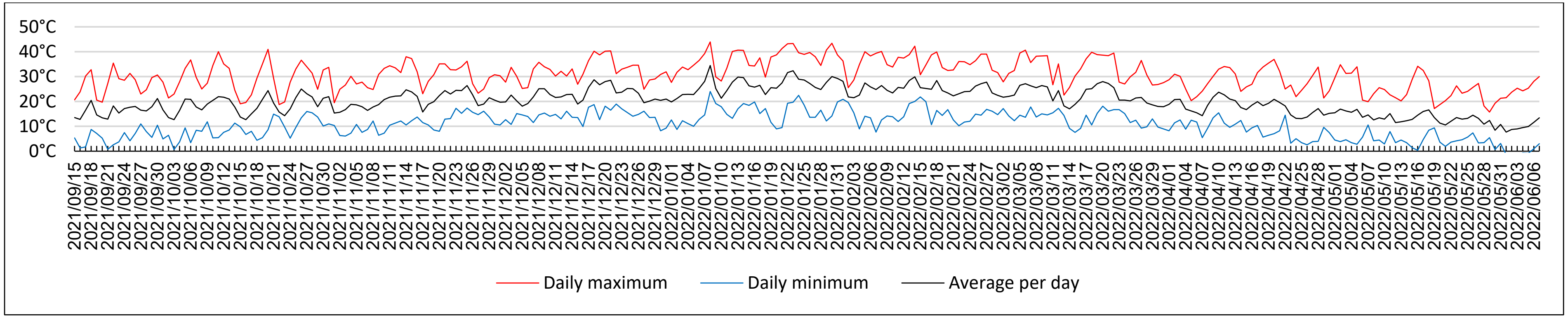


Figure 2.10. The daily maximum, minimum and average temperature during season 2021/2022 for Modderfontein.

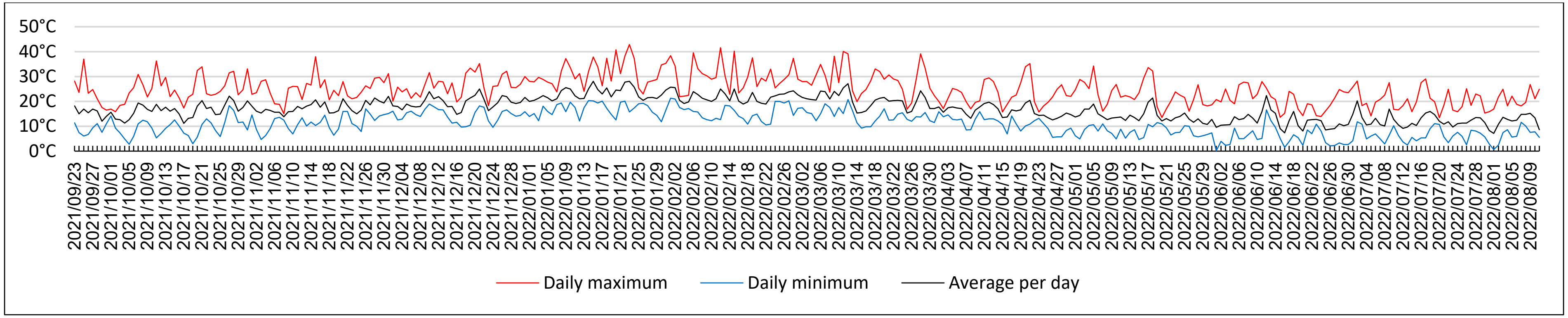


Figure 2.11. The daily maximum, minimum and average temperature during season 2021/2022 for Mandaryn.

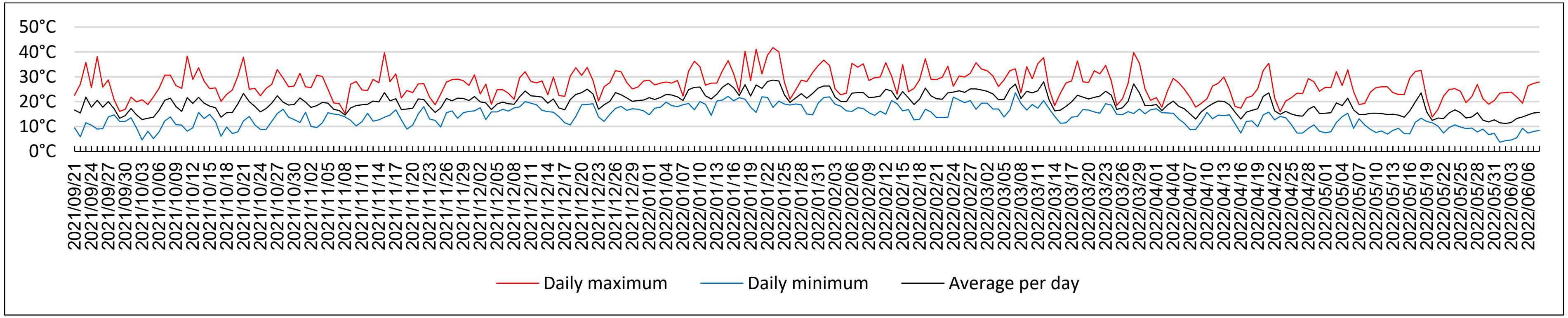


Figure 2.12. The daily maximum, minimum and average temperature during season 2021/2022 for Normandale and Step Aside.

2.2.7. Release programmes

Aphytis melinus wasps from an overseas insectary were imported to South Africa in the same manner as when supplied to commercial citrus growers. The local insectary based in South Africa, could only supply us for releases to Hexfarm 1371 orchard in the 2020/2021 season. Neither insectary shared rearing methods and techniques with us. Wasps were released in treatment orchards as set out in Tables 2.2., 2.3. and 2.4., four days after exiting the overseas insectary and two days from the local insectary. Release techniques and dose rates per hectare were adjusted after the first year of the augmentation study. *Aphytis melinus* wasps were released later in the season in orchards where pesticides were applied, to protect them from the potential harmful effect of the pesticides (Grout et al., 2011).

Wasps were sent in paper cups containing 10 000 wasps each with sawdust and cotton wool swabs, as well as honey as a food source. The cups were packed in polystyrene boxes and kept cool with ice packs. The shipping methods were the same as those used for growers.

2.2.7.1. The 2019/2020 season

The aim of the approach in the first year was to release high numbers as early as possible over a relatively short period of time, that is, four to five weeks (Table 2.16).

Table 2.16. Number of *Aphytis melinus* wasps released during the 2019/2020 season.

Release date	**Orchard**						
	Sunad 6	**Sunad 60 T**	**Step Aside 26**	**Mandaryn T**	**Modderfontein 401**	**Hexfarm 1371**	**Hexfarm 1350 T**
04-Nov-19					70 000		
11-Nov-19					70 000	70 000	
18-Nov-19					70 000	70 000	
25-Nov-19					70 000	70 000	
02-Dec-19					70 000	70 000	
09-Dec-19						70 000	
06-Jan-20							50 000
13-Jan-20							50 000
20-Jan-20	70 000	60 000	50 000	50 000			50 000
27-Jan-20	70 000	60 000	50 000	50 000			50 000
03-Feb-20	70 000	60 000	50 000	50 000			50 000
10-Feb-20	70 000	60 000	50 000	50 000			

17-Feb-20	70 000	60 000	50 000	50 000			
Total	350 000	300 000	250 000	250 000	350 000	350 000	250 000

2.2.7.2. The 2020/2021 season

The approach in the second year was altered to release the same final dose but divided up into smaller numbers per release over a longer period. The idea behind this approach was to spread the releases out to test whether a longer period during which releases were made would be more effective (Grafton-Cardwell & Stewart-Leslie, 1998). Unfortunately, the overseas insectary had supply and logistical problems due to the worldwide Covid-19 pandemic and some of the weeks for which releases were planned had to be postponed and thus combined with the subsequently planned releases at all orchards except for Modderfontein (Table 2.17).

Table 2.17. Number of *Aphytis melinus* wasps released during the 2020/2021 season.

Release date	**Orchard**				
	Step Aside 23	**Mandaryn T**	**Modderfontein 401**	**Hexfarm 1371**	**Hexfarm 1350 T**
28-Sep-20			30 000	30 000	
05-Oct-20			30 000	30 000	
12-Oct-20			30 000	30 000	
19-Oct-20			30 000	30 000	
26-Oct-20			30 000	30 000	
02-Nov-20			30 000	30 000	
09-Nov-20			30 000		
16-Nov-20			30 000		
23-Nov-20			30 000		20 000
30-Nov-20			30 000	90 000	20 000
07-Dec-20			30 000	30 000	20 000
14-Dec-20					20 000
21-Dec-20					20 000
28-Dec-20					20 000
04-Jan-21					20 000
01-Feb-21					60 000
08-Feb-21	150 000	20 000			20 000
15-Feb-21	50 000	140 000			20 000

22-Feb-21	50 000				
08-Mar-21		50 000			
15-Mar-21	50 000	50 000			
Total	300 000	260 000	330 000	300 000	240 000

2.2.7.3. The 2021/2022 season

The aim of the release programme during the third season was, again, to augment wasps in as high numbers as possible, as early as possible to provide the augmented *A. melinus* with more time for possible establishment in release orchards (Table 2.18).

Table 2.18. Number of *Aphytis melinus* wasps released during the 2021/2022 season.

Release date	**Orchard**					
	Step Aside 23	**Step Aside 26**	**Mandaryn T**	**Modderfontein 401**	**Hexfarm 1371**	**Hexfarm 1350 T**
20-Sep-21				30 000		
27-Sep-21				30 000	30 000	
04-Oct-21				30 000	30 000	
11-Oct-21				30 000	30 000	
25-Oct-21				60 000	60 000	
01-Nov-21				30 000	30 000	10 000
08-Nov-21				30 000	40 000	30 000
22-Nov-21					60 000	40 000
29-Nov-21					30 000	20 000
06-Dec-21					30 000	40 000
13-Dec-21						20 000
20-Dec-21						20 000
03-Jan-22						20 000
10-Jan-22	50 000	50 000	40 000			20 000
17-Jan-22	50 000	50 000	40 000			
31-Jan-22	50 000	50 000	40 000			
07-Feb-22	50 000	50 000	40 000			
14-Feb-22	50 000	50 000	40 000			
Total	250 000	250 000	200 000	240 000	340 000	220 000

2.2.8. Data analyses

All statistical analyses were conducted in Statistica version 14.0.0.15. Differences in red scale status (dead, healthy, mutilated or parasitised) over time, between release and control orchards were analysed using a generalised linear model with a Poisson/log link. The regressions were then compared using a homogeneity of slopes model.

The number of adult *Aphytis* spp. caught per orchard per week were visualised by calculating weighted marginal means over time for release and control orchards.

The number of fruit that were infested with red scale out of 10 fruit were recorded and visualised by calculating the weighted marginal means over time for release and control orchards. The differences were then compared using a univariate test of significance.

2.3. Results

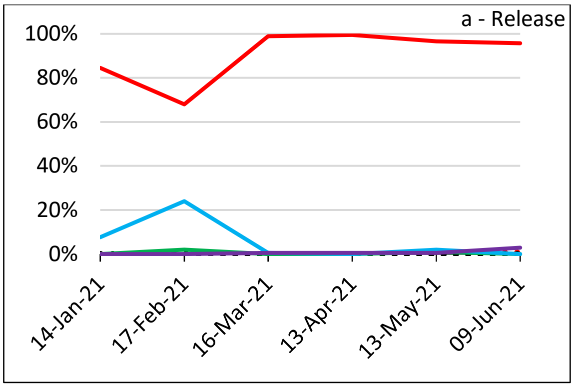
2.3.1. The 2019/2020 season

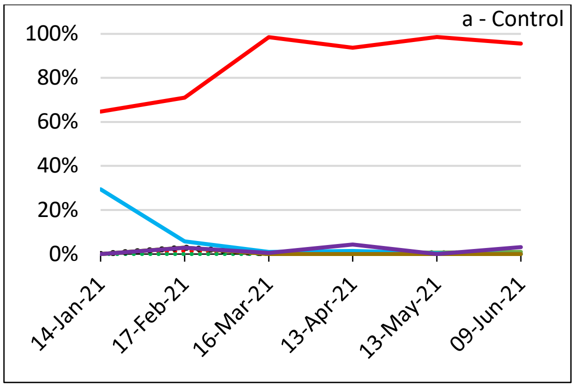
2.3.1.1. Red scale infestation and parasitoid activity trends during the 2019/2020 season in release and control orchards.

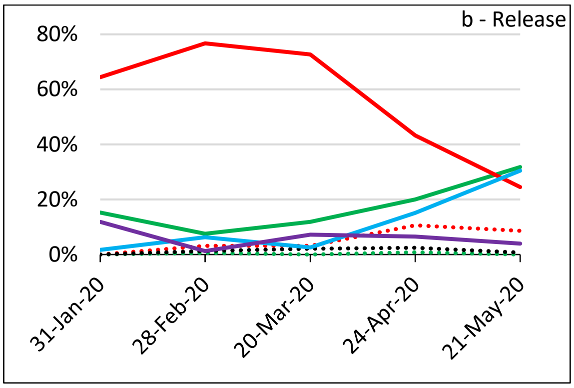
There was an initial reduction in the number of healthy red scales at the release and control orchards of Hexfarm 1350, but then very little parasitism or host mutilation occurred further on until harvest (Fig. 2.13 a). This lack of *Aphytis* spp. activity might be ascribed to the negative effect of the consecutive kaolin sprays on these orchards (Table 2.3). A clear trend is evident at the release and control orchards of Hexfarm 1371 and 1272 where the numbers of healthy red scales declined as parasitism and host mutilation increased (Fig. 2.13b).

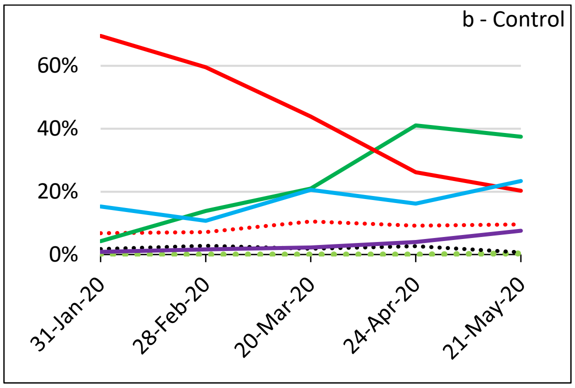
Healthy red scale numbers declined as parasitism and host mutilation increased in all release and control orchards except for at Hexfarm 1350, as mentioned earlier. *Aphytis africanus* parasitism was the highest among all parasitoid species (Fig. 2.13).

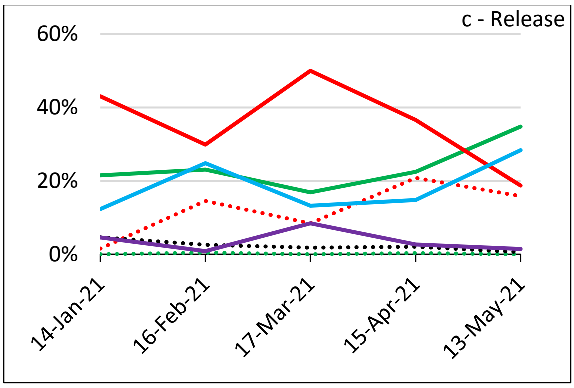
Very few healthy red scales remained on fruit at the release and control orchards at harvest because of increasing parasitism, mainly by *A. africanus,* and host mutilation as the season progressed at all orchards except for Hexfarm 1371 and 1272 (Fig. 2.13).

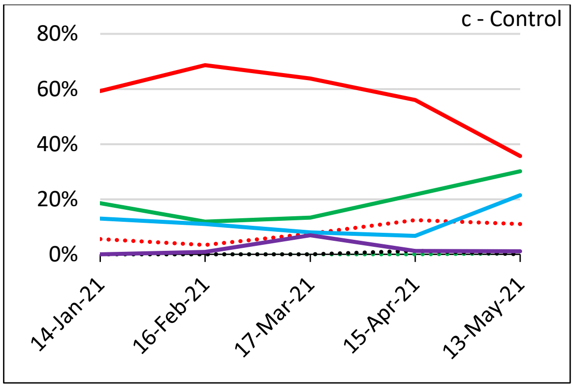

a - Release
100%
80%
60%
40%
20%
0%
14-Jan-21
17-Feb-21
16-Mar-21
13-Apr-21
13-May-21
09-Jun-21

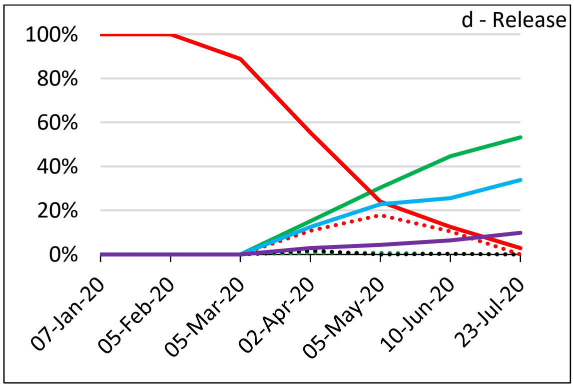

a - Control
100%
80%
60%
40%
20%
0%
14-Jan-21
17-Feb-21
16-Mar-21
13-Apr-21
13-May-21
09-Jun-21

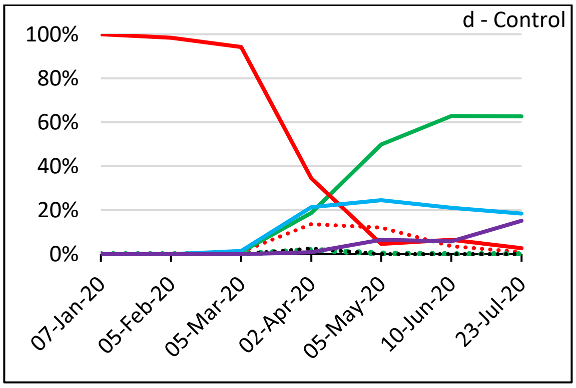

b - Release
80%
60%
40%
20%
0%
31-Jan-20
28-Feb-20
20-Mar-20
24-Apr-20
21-May-20


b - Control
60%
40%
20%
0%
31-Jan-20
28-Feb-20
20-Mar-20
24-Apr-20
21-May-20


c - Release
60%
40%
20%
0%
14-Jan-21
16-Feb-21
17-Mar-21
15-Apr-21
13-May-21


c - Control
80%
60%
40%
20%
0%
14-Jan-21
16-Feb-21
17-Mar-21
15-Apr-21
13-May-21


d - Release
100%
80%
60%
40%
20%
0%
07-Jan-20
05-Feb-20
05-Mar-20
02-Apr-20
05-May-20
10-Jun-20
23-Jul-20


d - Control
100%
80%
60%
40%
20%
0%
07-Jan-20
05-Feb-20
05-Mar-20
02-Apr-20
05-May-20
10-Jun-20
23-Jul-20

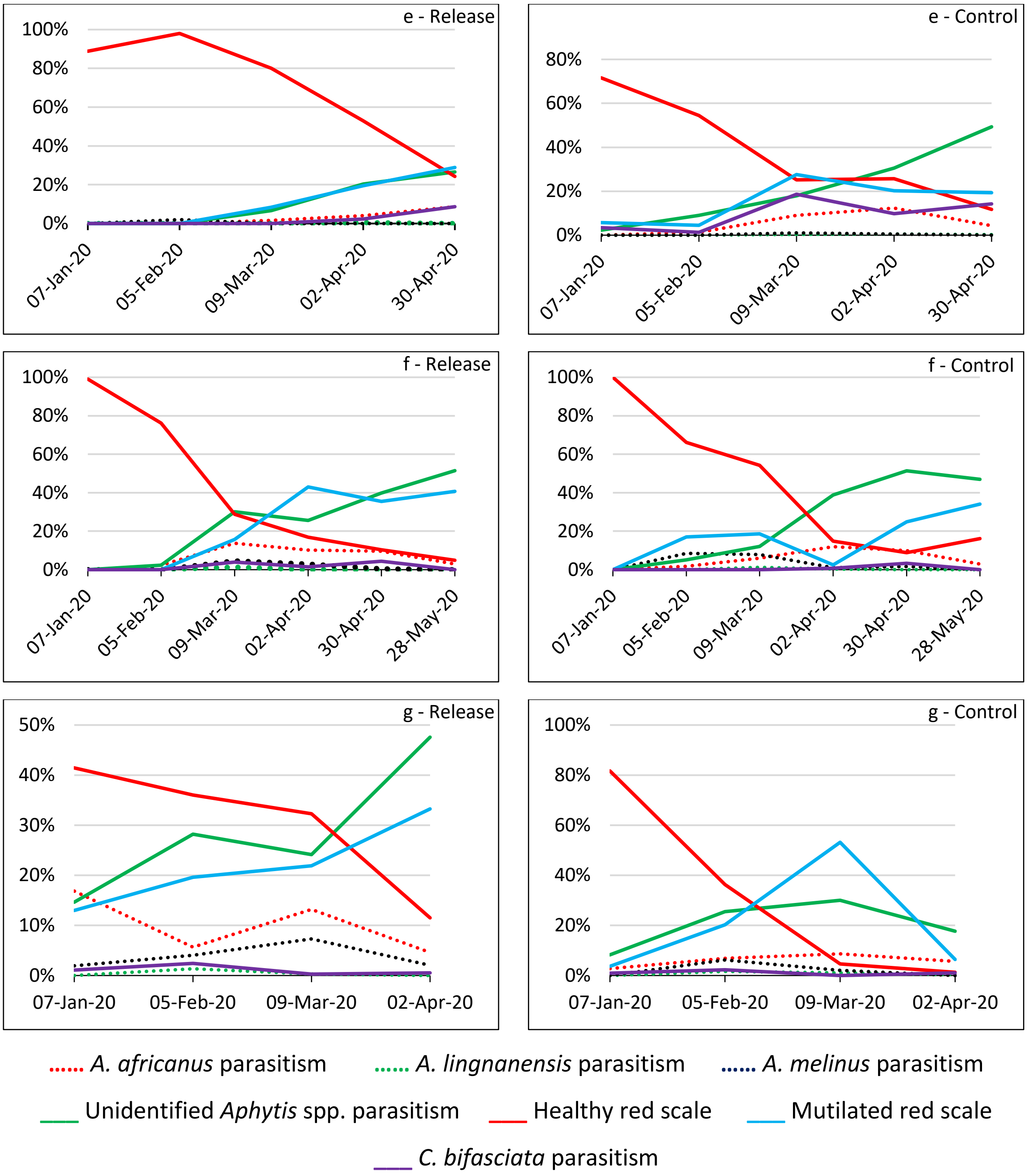


Figure 2.13. Red scale infestation and parasitoid activity trends during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b), Modderfontein 401 and 405 (c) Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e), Sunad 60 T and C (f) and Sunad 6 and 61 (g).

2.3.1.2. Microscopic assessments of red scales during the 2019/2020 season

No significant differences between release and control orchards could be found in terms of the mean number of healthy scales left on fruit at the time of harvest (Fig. 2.14 and Table 2.19). In all the trial orchards, the increase or decrease of healthy scale during the season followed the same trend for release and control orchards. Although *A. melinus* had a significant interaction with healthy red scales at Hexfarm 1350, Hexfarm 1371 and 1272 and Sunad 60 T and C (Table 2.19), it did not result in less healthy scale at the time of harvest compared to the untreated control.

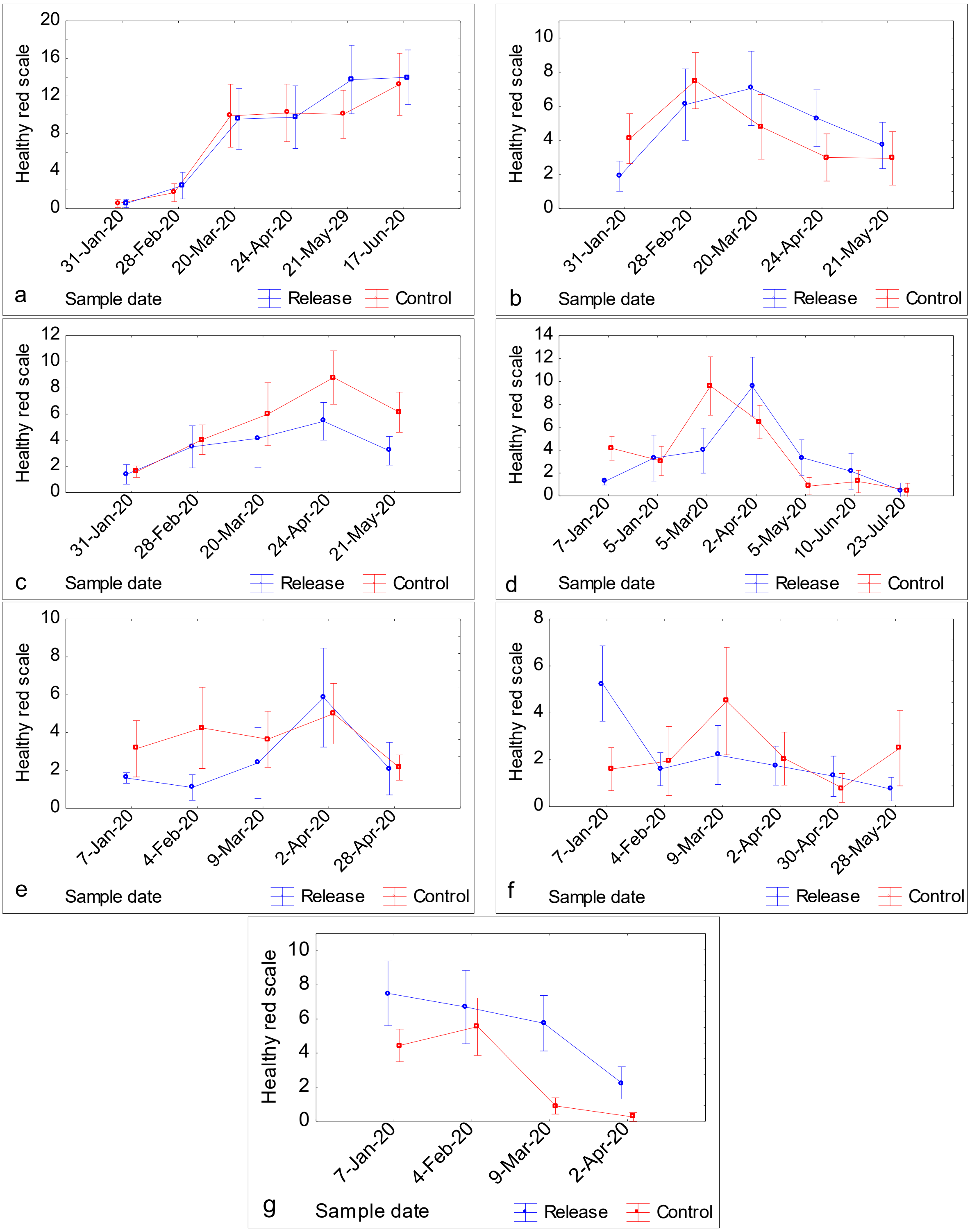


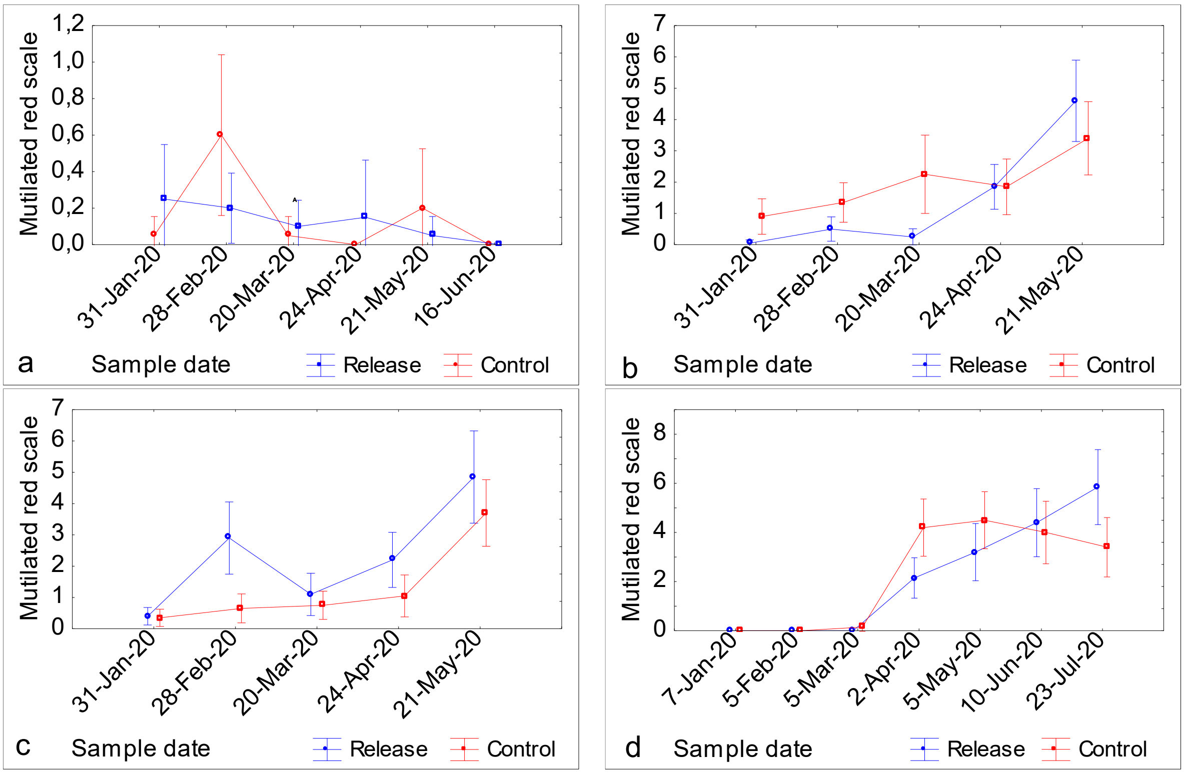
Figure 2.14. The mean number of healthy red scales per fruit during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Sunad 60 T and C (f); Sunad 6 and 61 (g). Refer to Table 2.19 for results of statistical analyses. Error bars represent SE.

Table 2.19. Results of generalised linear models indicating significant interactions between parasitism and healthy red scales during the 2019/2020 season (Wald test statistic).

Orchard	***A. africanus***	***A. lingnanensis***	***A. melinus***	***C. bifasciata***
Hexfarm 1350 T and B	0.131	-	4.068*	2.14
Hexfarm 1371 and 1272	0.957	0.118	11.434*	2.855
Modderfontein 401 and 405	8.724*	0.206	0.029	0.209
Mandaryn T and B	4.813*	0.00	0.0071	10.723*
Normandale 33 and Step Aside 26	2.96	9.713*	0.465	0.258
Sunad 60 T and C	31.478*	0.054	22.319*	0.779
Sunad 6 and 61	0.064	0.819	1.139	1.772

* Denotes a significant interaction at a 95% confidence level.

No significant differences in scale mutilation between release and control orchards were evident in these trials (Fig. 2.15). The number of mutilated red scale was the same for both release and control in each set of trial orchards throughout the season. *Aphytis africanus* and *C. bifasciata* had more interactions at a significant level than did *A. melinus* (Table 2.20).



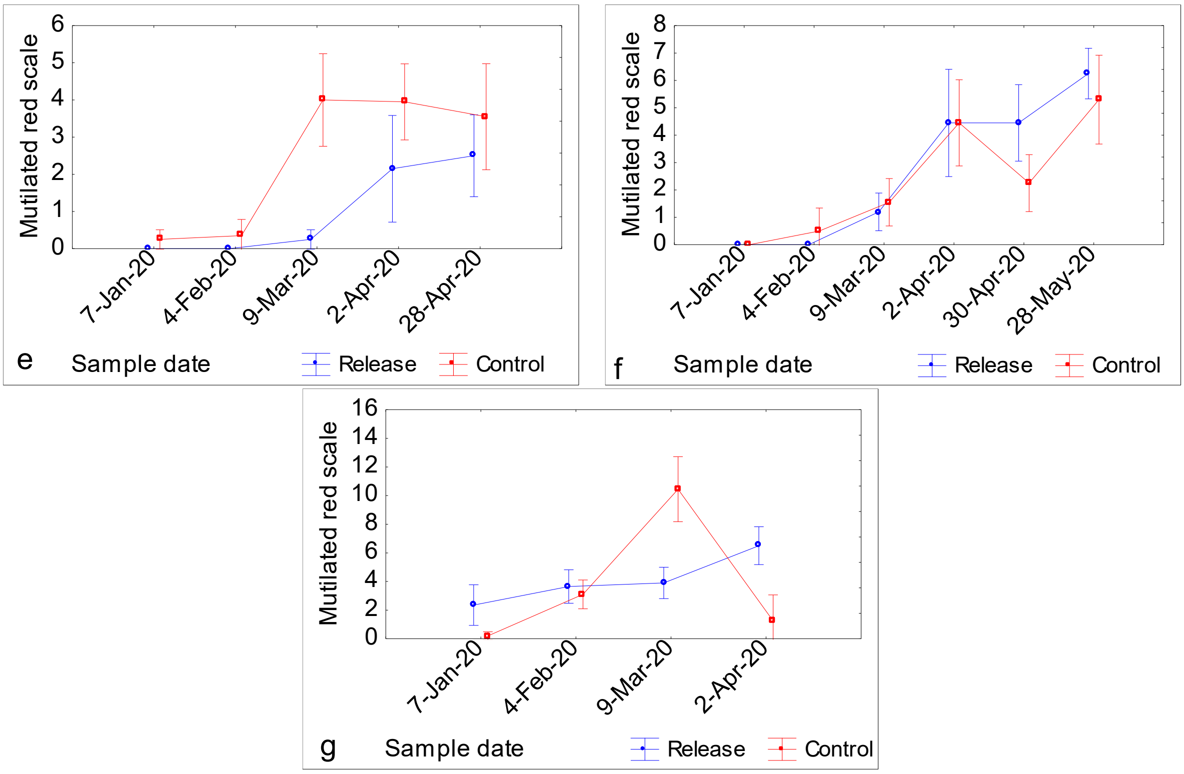


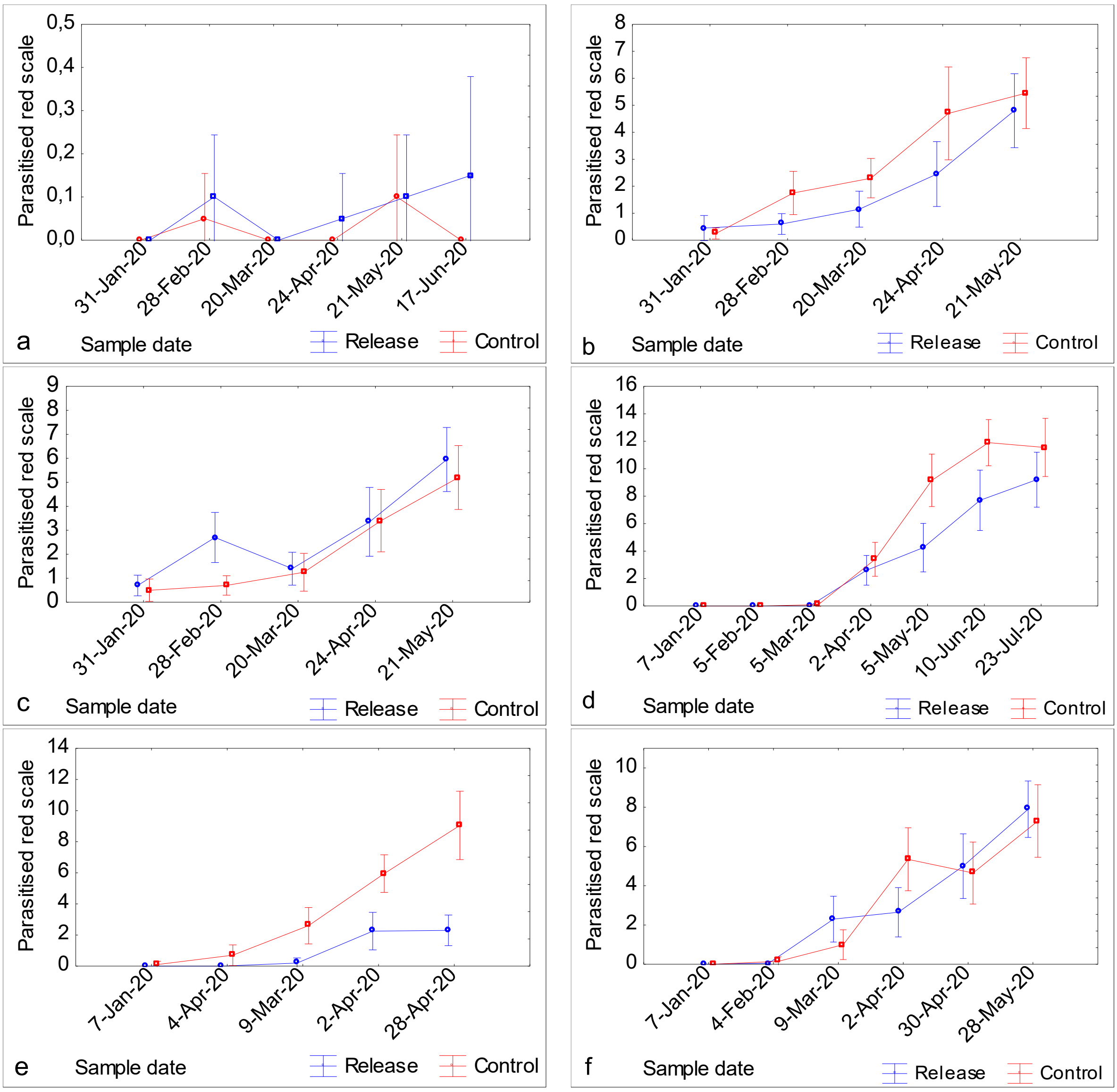
Figure 2.15. The mean number of mutilated red scales per fruit during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Sunad 60 T and C (f); Sunad 6 and 61 (g). Refer to Table 2.20 for results of statistical analyses. Error bars represent SE.

Table 2.20. Results of generalised linear models indicating significant interactions between parasitism and mutilated red scales during the 2019/2020 season (Wald test statistic).

Orchard	*A. africanus*	*A. lingnanensis*	*A. melinus*	*C. bifasciata*
Hexfarm 1350 T and B	0.000	-	0.012	0.259
Hexfarm 1371 and 1272	18.316*	0.396	0.121	8.081*
Modderfontein 401 and 405	1.626	0.028	0.828	0.761
Mandaryn T and B	2.860	0.005	0.013	12.865*
Normandale 33 and Step Aside 26	0.000	0.019	2.094	1.269
Sunad 60 T and C	14.680*	1.994	44.71*	0.206
Sunad 6 and 61	0.722	1.256	1.509	0.211

* Denotes a significant interaction at a 95% confidence level.

No significant differences between release and control orchards in terms of red scale parasitised by *Aphytis* spp. could be detected when the unidentifiable life stages of *Aphytis* spp. were used as indicators of *Aphytis* spp. parasitism (Fig. 2.16). Of all the red scale parasitoids, *Aphytis africanus* had the most interactions of significant value (Table 2.21).



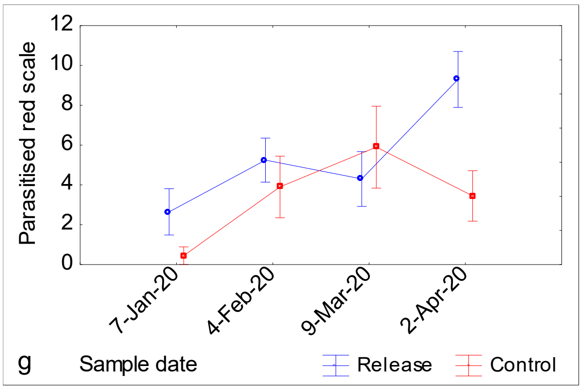


Figure 2.16. The mean number of red scales per fruit parasitised by unidentified *Aphytis* spp. during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Sunad 60 T and C (f); Sunad 6 and 61 (g). Refer to Table 2.21 for results of statistical analyses. Error bars represent SE.

Table 2.21. Results of generalised linear models indicating significant interactions between parasitism of red scales by known *Aphytis* spp. and unidentified *Aphytis* spp. with red scales during the 2019/2020 season (Wald test statistic).

Orchard	*A. africanus*	*A. lingnanensis*	*A. melinus*
Hexfarm 1350 T and B	1.209	-	1.128
Hexfarm 1371 and 1272	18.531*	1.831	2.178
Modderfontein 401 and 405	17.461*	1.852	11.250*
Mandaryn T and B	7.944*	0.509	0.160
Normandale 33 and Step Aside 26	13.021*	0.196	0.356
Sunad 60 T and C	18.269*	5.296*	2.510
Sunad 6 and 61	4.002*	0.061	0.226

* Denotes a significant interaction at a 95% confidence level.

The largest contribution to unhealthy red scales was *A. africanus* parasitism (Fig. 2.17). Of all the parasitoid species, parasitism of red scale by *A. melinus* yielded the fewest regressions of significance between unhealthy red scales and parasitised red scale, particularly relative to *A. africanus* and *C. bifasciata* (Table 2.22). It is clear from these data that the augmented *A. melinus* did not result in any differences between the release and control orchards in terms of unhealthy red scale.

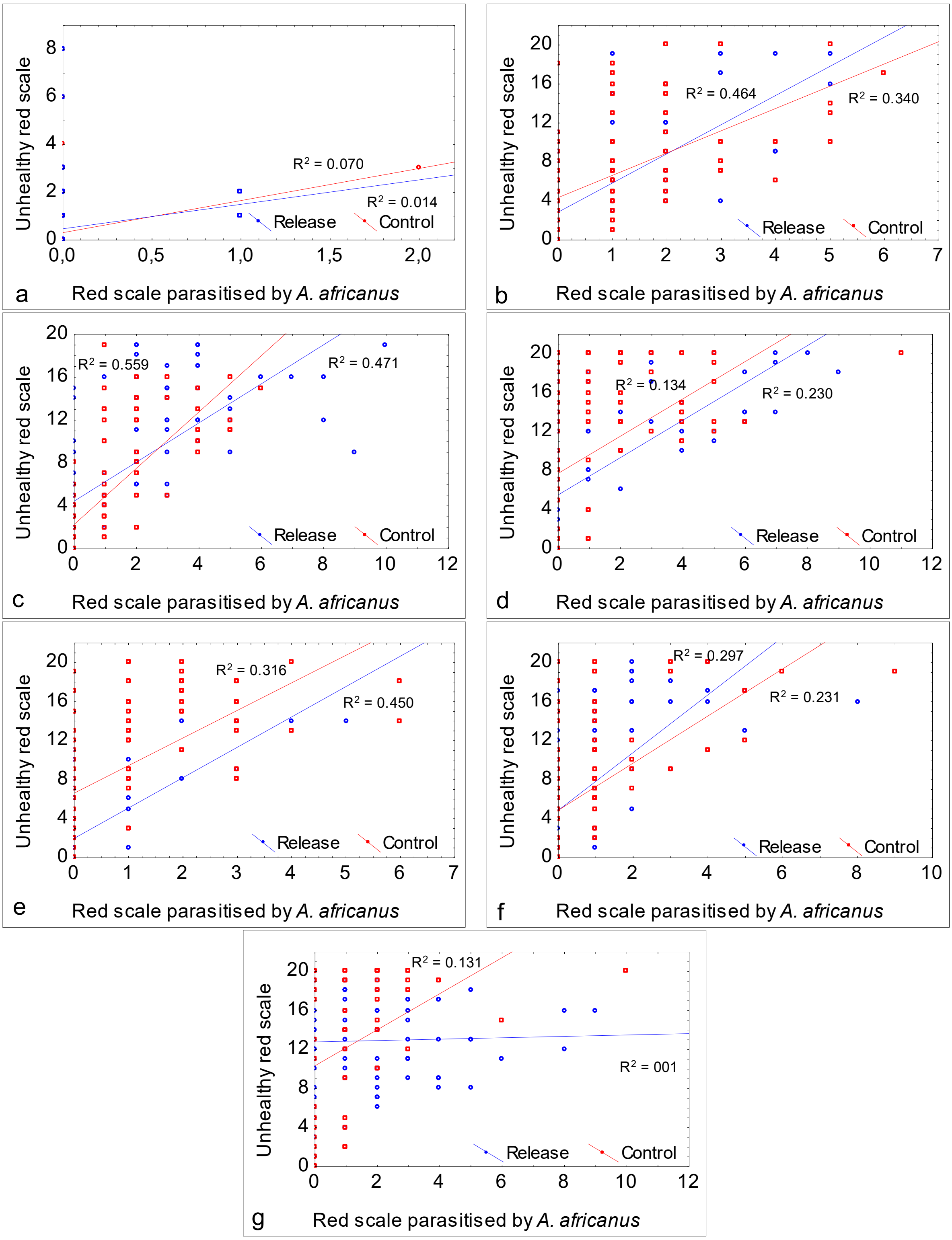


Figure 2.17. Number of unhealthy red scales per fruit correlated with red scales parasitised by *A. africanus* during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Sunad 60 T and C (f); Sunad 6 and 61 (g). Refer to Table 2.22 for results of statistical analyses.

Aphytis lingnanensis and *A. melinus* did not yield any strong regressions with red scale parasitism in any orchard during the 2019/2020 season as can be seen in Figs. 2.18 and 2.19.

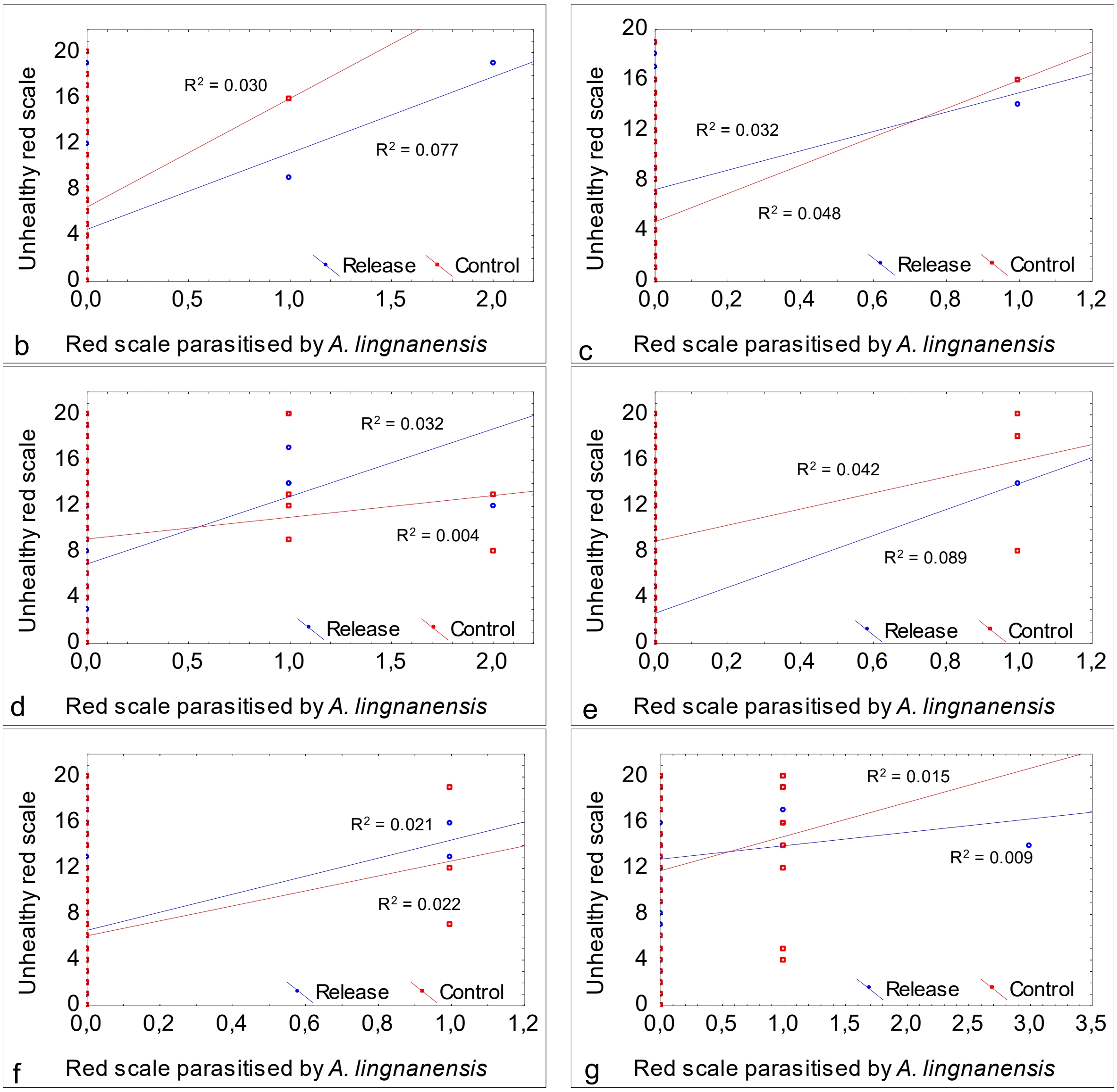


Figure 2.18. Number of unhealthy red scales per fruit correlated with red scales parasitised by *A. lingnanensis* during the 2019/2020 season in release and control orchards at Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Sunad 60 T and C (f); Sunad 6 and 61 (g). Refer to Table 2.22 for results of statistical analyses.

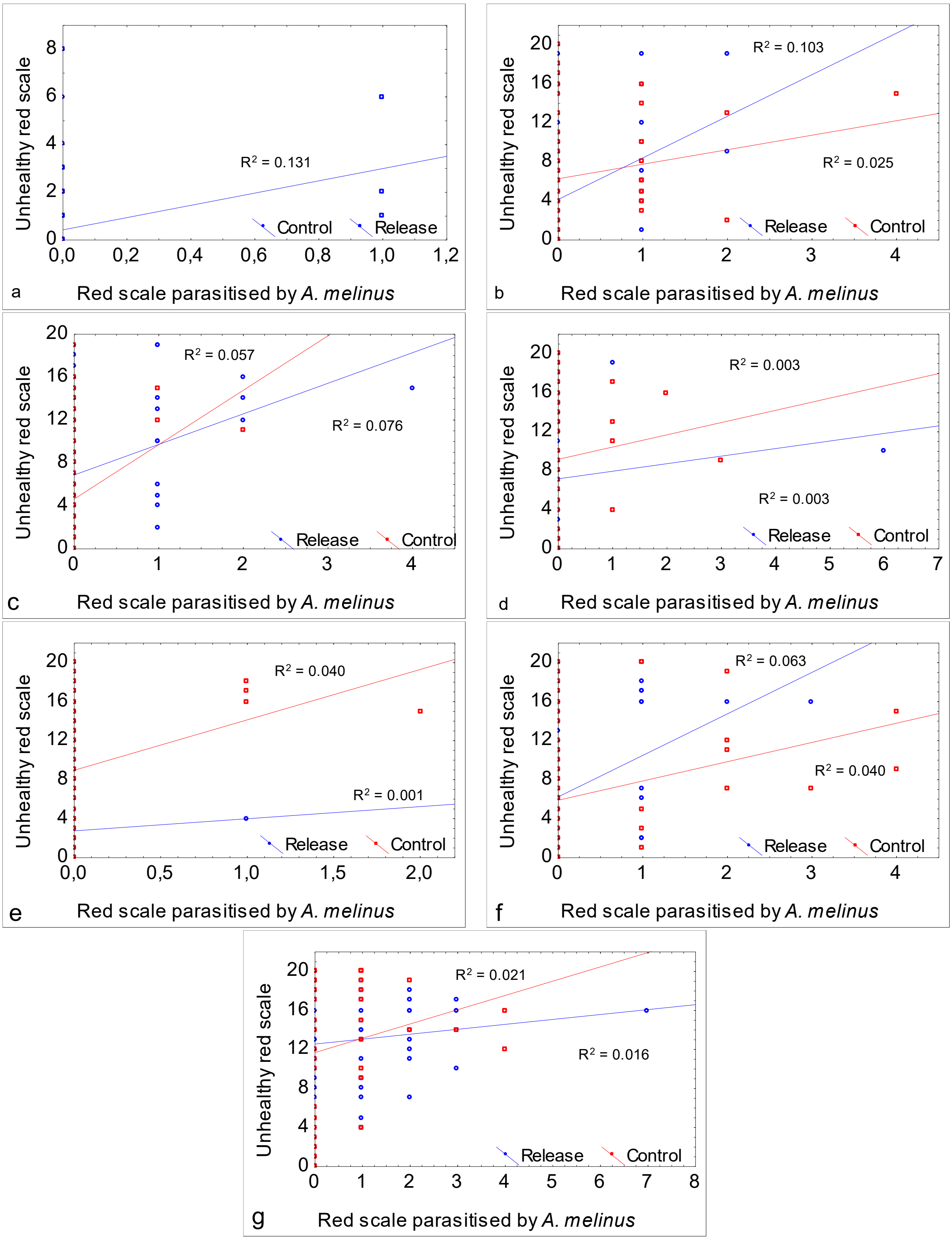


Figure 2.19. Number of unhealthy red scales per fruit correlated with red scales parasitised by *A. melinus* during the 2019/2020 season in release and control orchards at Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Sunad 60 (f); Sunad 6 and 61 (g). Refer to Table 2.22 for results of statistical analyses.

Comperiella bifasciata showed stronger regressions than both *A. lingnanensis* and *A. melinus* and thus impacted the red scale more (Fig. 2.20 and Table 2.22).

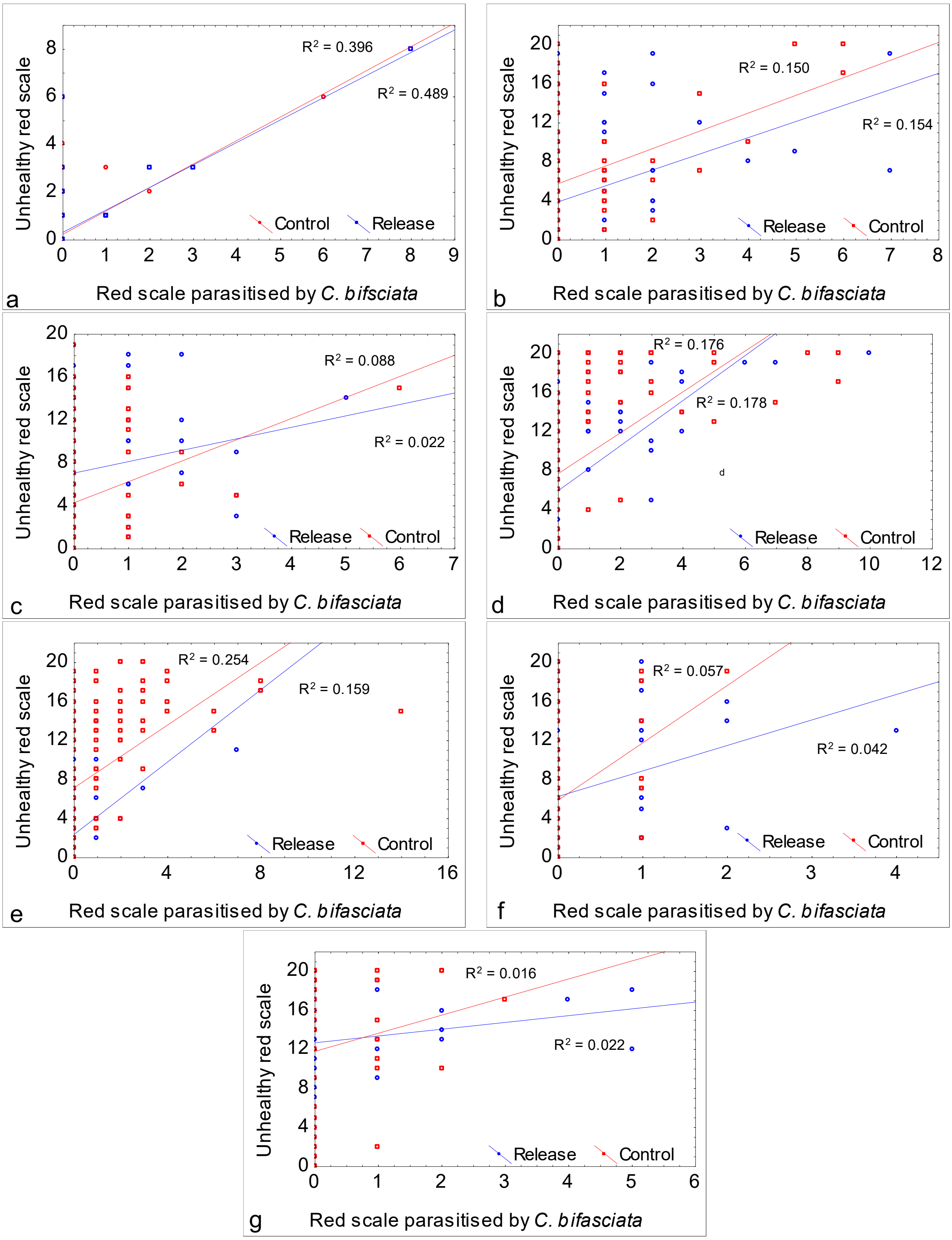


Figure 2.20. Number of unhealthy red scales per fruit correlated with red scales parasitised by *C. bifasciata* during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26(e); Sunad 60 T and C (f); Sunad 6 and 61 (g). Refer to Table 2.22 for results of statistical analyses.

Table 2.22. Univariate test of significance of the regressions between the number of unhealthy red scales per fruit and red scales parasitised by the various parasitoid species during the 2019/2020 season.

Orchards	*A. africanus*	*A. lingnanensis*	*A. melinus*	*C. bifasciata*
Hexfarm 1350 T and B	F = 7.219 *P* = 0.007*	-	-	F = 176.290 *P* = 0.001*
Hexfarm 1371 and 1272	F = 131.419 *P* = < 0.001*	F = 7.72 *P* = 0.005*	F = 12.874 *P* = < 0.001*	F = 35.168 *P* = < 0.001*
Modderfontein 401 and 405	F = 190.045 *P* = 0.001* Treatment × *A. africanus*: F = 6.145 *P* = 0.014*	F = 7.718 *P* = 0.005*	F = 10.520 *P* = 0.001*	F = 9.782 *P* = 0.002*
Mandaryn T and B	F = 57.80 *P* = < 0.001*	F = 4.216 *P* = 0.040*	F = 0.691 *P* = 0.406	F = 58.193 *P* = < 0.001*
Normandale 33 and Step Aside 26	F = 86.505 *P* = < 0.001*	F = 9.244 *P* = 0.002*	F = 1.200 *P* = 0.274	F = 29.480 *P* = < 0.001*
Sunad 60 T and C	F = 84.867 *P* = < 0.001*	F = 5.171 *P* = 0.023*	F = 12.698 *P* = < 0.001*	F = 11.723 *P* = < 0.001*
Sunad 6 and 61	F = 11.504 *P* = < 0.001* Treatment × *A. africanus*: F = 9.876 *P* = 0.002*	F = 1.989 *P* = 0.160	F = 3.088 *P* = 0.080	F = 2.813 *P* = 0.095

* Result of ANCOVA indicating significant differences between the regression slopes at a 95% confidence level.

Parasitism by all *Aphytis* spp. (combined data from *A. africanus*, *A. lingnanensis* and *A. melinus* parasitism and mutilation) strongly correlated with the presence of unhealthy red scales on fruit (Fig. 2.21 and Table 2.23). This indicates that *Aphytis* spp. were largely responsible for high levels of red scale parasitism. The strong regression can also be explained by the notable auto-regression formed when limited data sources are used to analyse the events. No significant differences were recorded between the release and control orchards.

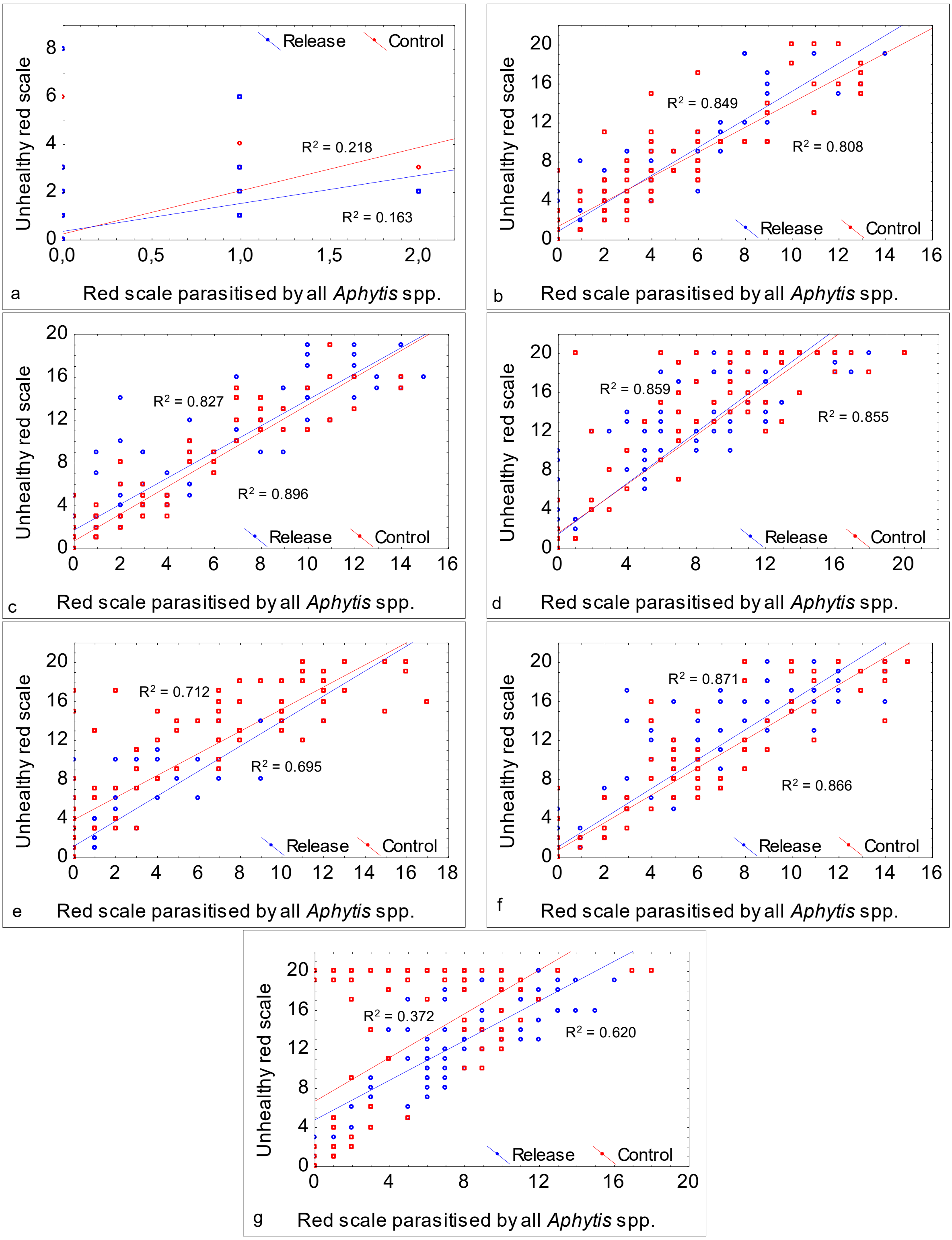


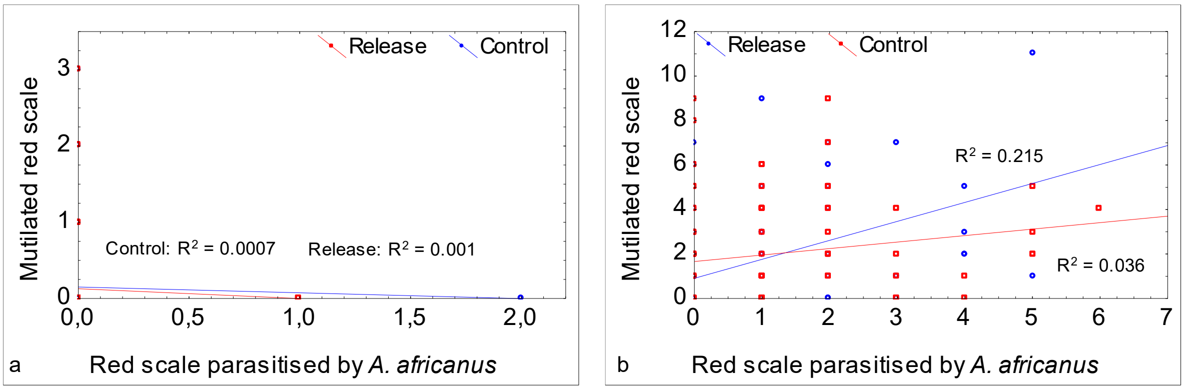
Figure 2.21. Number of unhealthy red scales per fruit correlated with red scales parasitised by all *Aphytis* spp. during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Sunad 60 T and C (f); Sunad 6 and 61 (g). Refer to Table 2.23 for results of statistical analyses.

Table 2.23. Univariate test of significance of the regressions between the number of unhealthy red scales per fruit and red scales parasitised by all *Aphytis* spp. during the 2019/2020 season.

Orchard	**All *Aphytis* spp.**
Hexfarm 1350 T and B	F = 50.56 *P* = < 0.001 *
Hexfarm 1371 and 1272	F = 941.41 *P* = < 0.001*
Modderfontein 401 and 405	F = 1140.42 *P* = < 0.001* Treatment: F = 6.00 *P* = 0.015*
Mandaryn T and B	F = 1624.24 *P* = < 0.001*
Normandale 33 and Step Aside 26	F = 319.346 *P* = < 0.001* Treatment: F = 26.340 *P* = < 0.001*
Sunad 60 T and C	F = 1558.608 *P* = < 0.001*
Sunad 6 and 61	F = 109.877 *P* = < 0.001*

* Denotes a significant interaction at a 95% confidence level.

Aphytis africanus parasitism (Fig. 2.22) showed the strongest correlation with host mutilation, when compared to *A. lingnanensis* (Fig. 2.23), *A. melinus* (Fig. 2.24) and *C. bifasciata* (Fig. 2.25), in release and control orchards during the 2019/2020 season (Table 2.24).



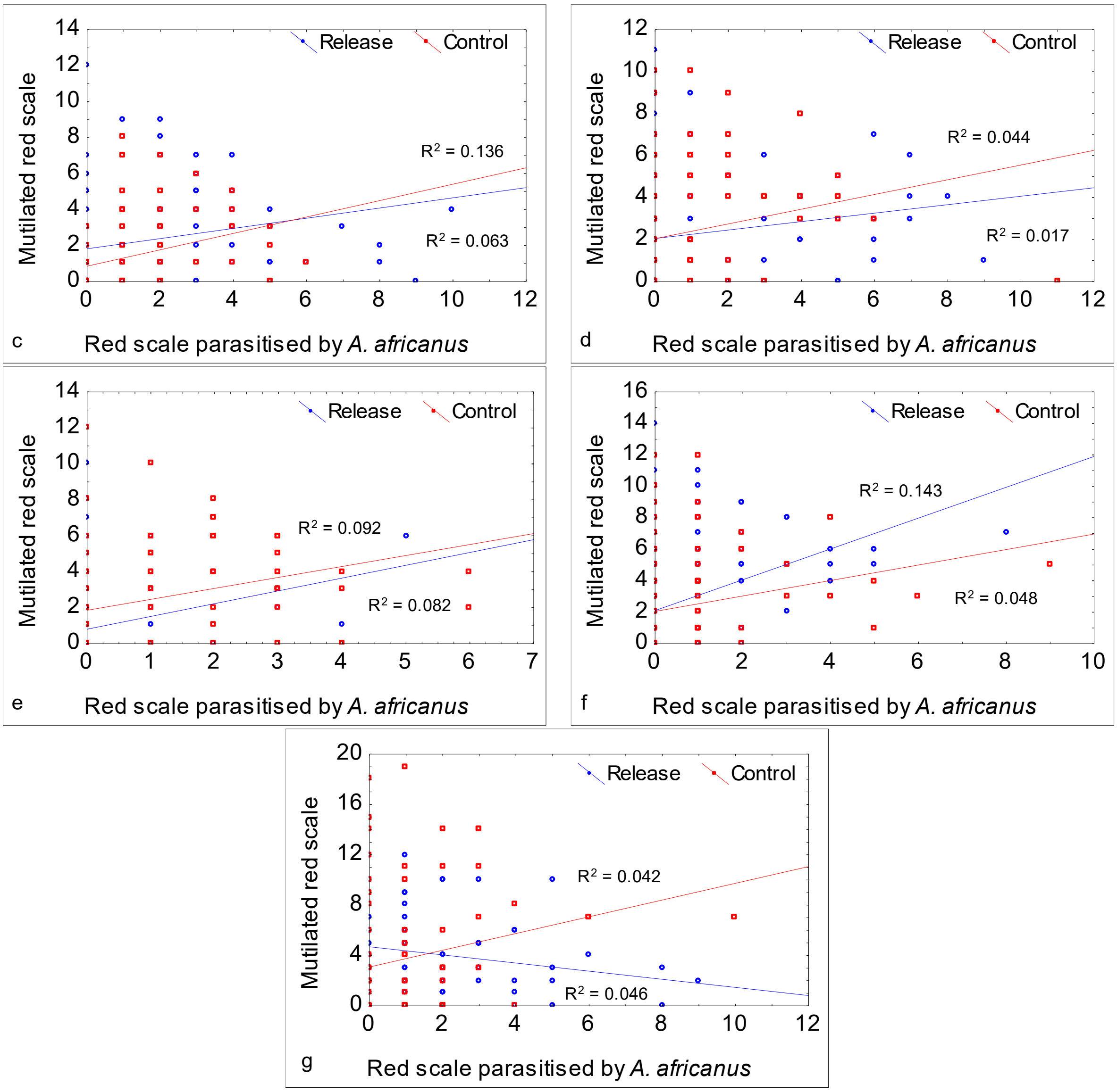
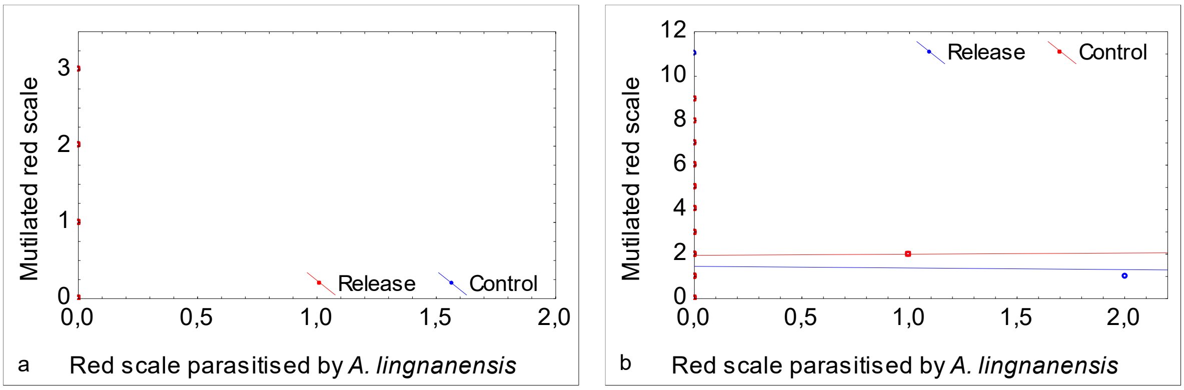


Figure 2.22. Number of mutilated red scales per fruit correlated with red scales parasitised by *A. africanus* during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Sunad 60 T and C (f); Sunad 6 and 61 (g). Refer to Table 2.24 for results of statistical analyses.



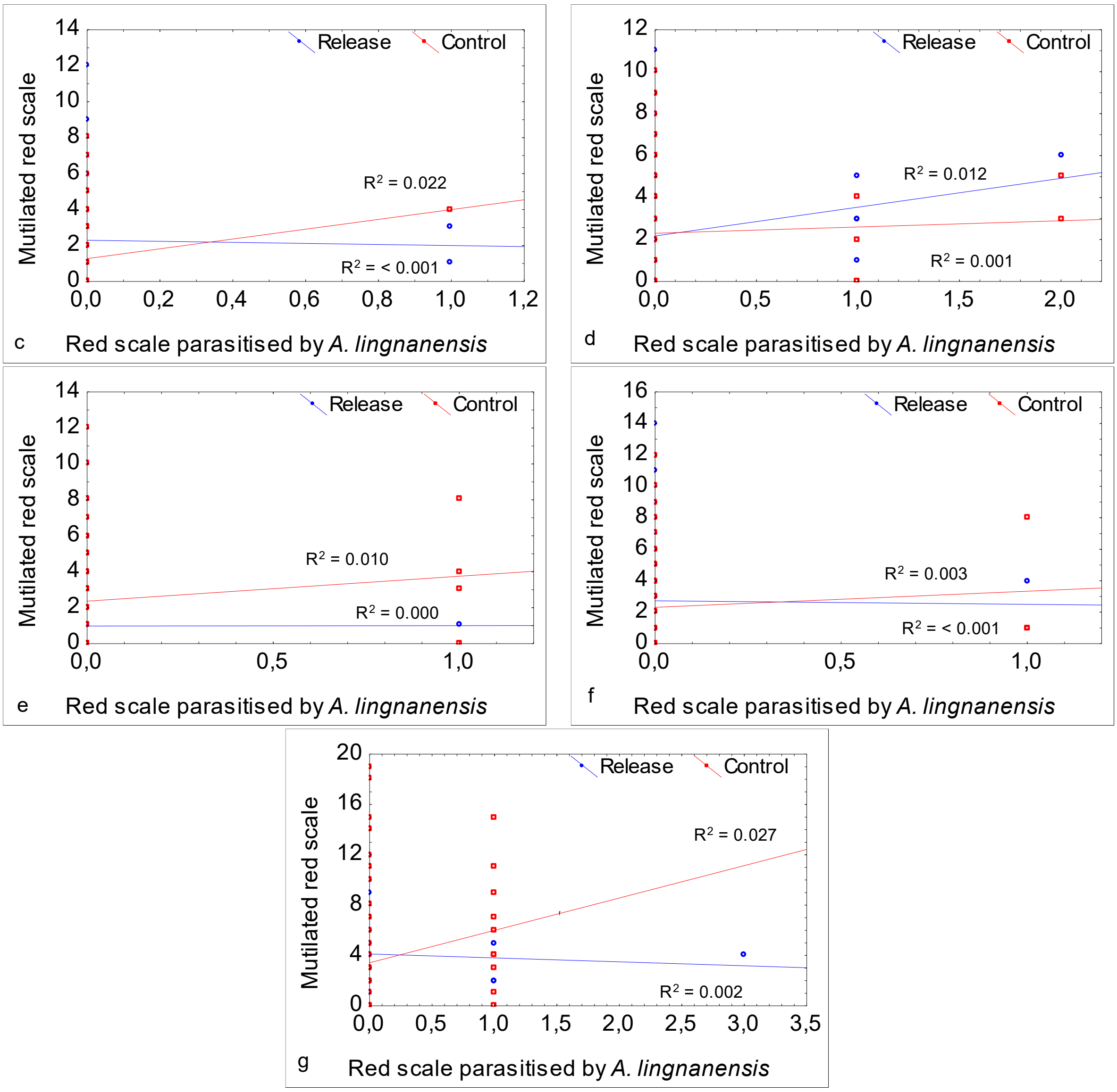
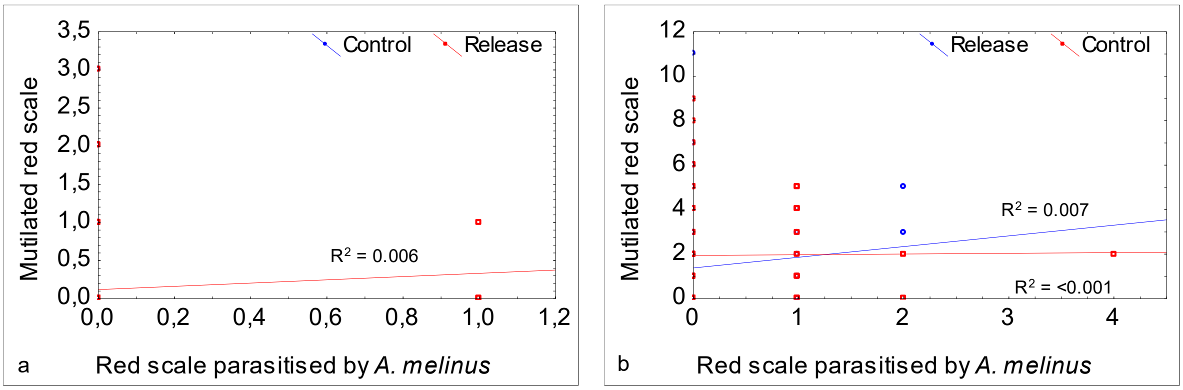


Figure 2.23. Number of mutilated red scales per fruit correlated with red scales parasitised by *A. lingnanensis* during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Sunad 60 T and C (f); Sunad 6 and 61 (g). Refer to Table 2.24 for results of statistical analyses.



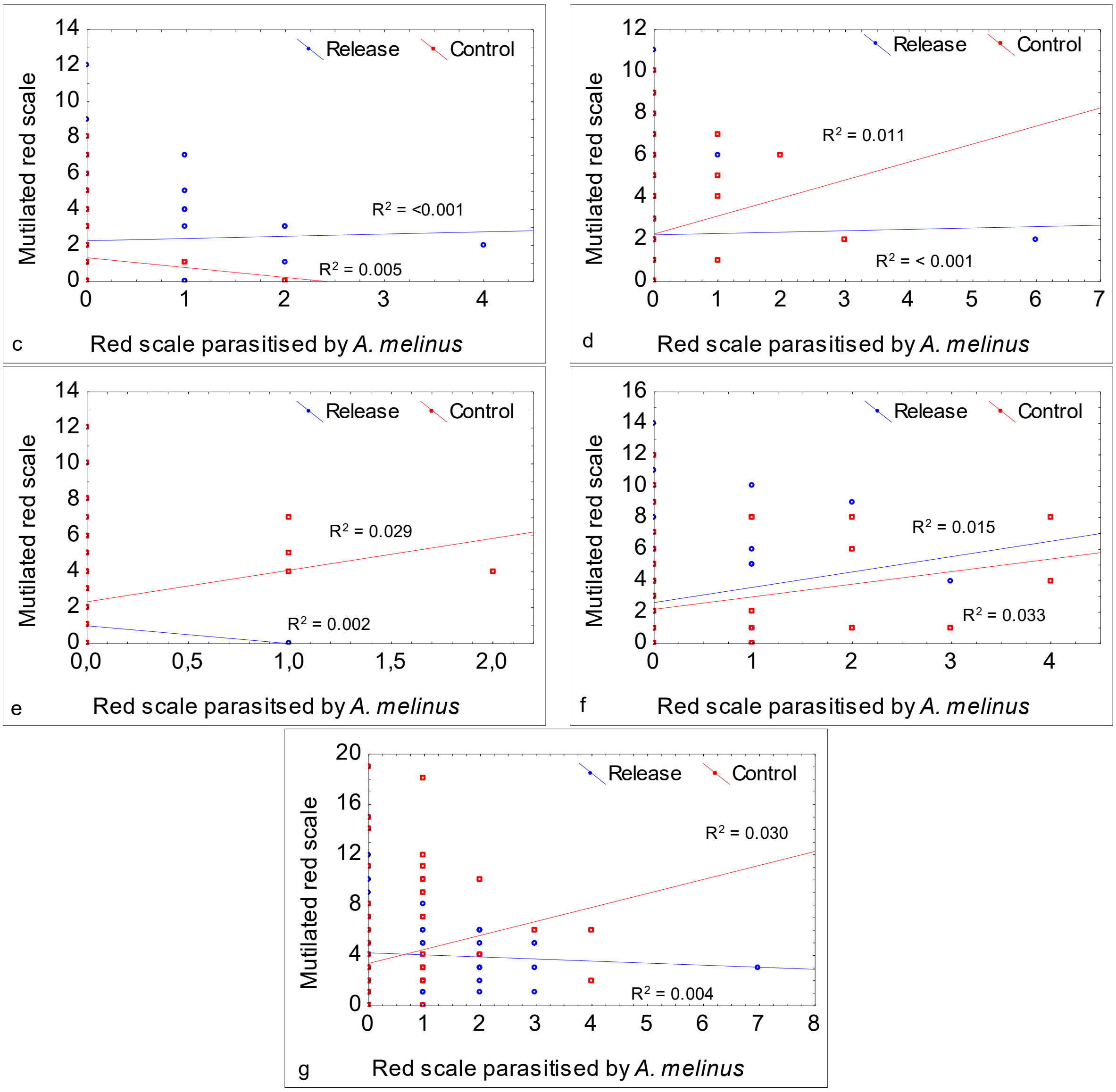
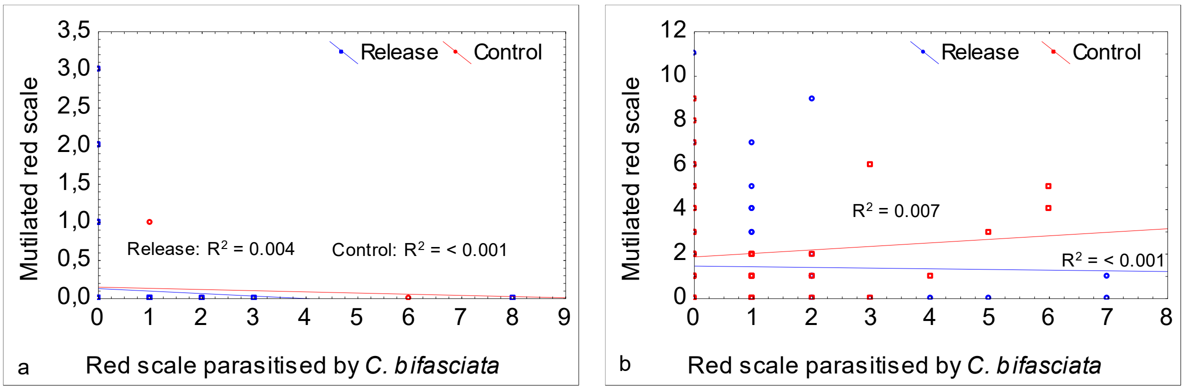


Figure 2.24. Number of mutilated red scales per fruit correlated with red scales parasitised by *A. melinus* during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Sunad 60 T and C (f); Sunad 6 and 61 (g). Refer to Table 2.24 for results of statistical analyses.



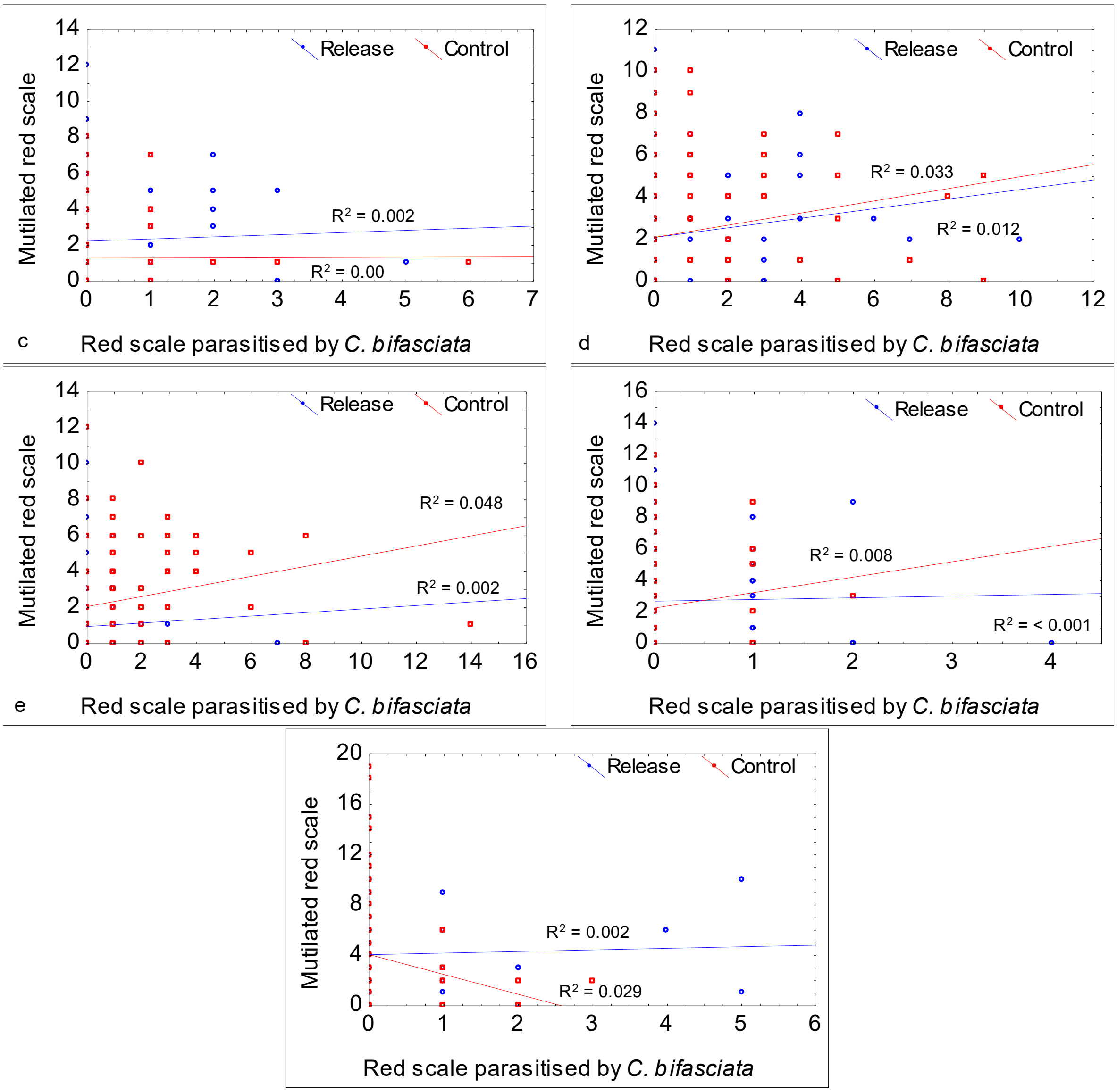


Figure 2.25. Number of mutilated red scales correlated with red scales parasitised by *C. bifasciata* during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Sunad 60 T and C (f); Sunad 6 and 61 (g). Refer to Table 2.24 for results of statistical analyses.

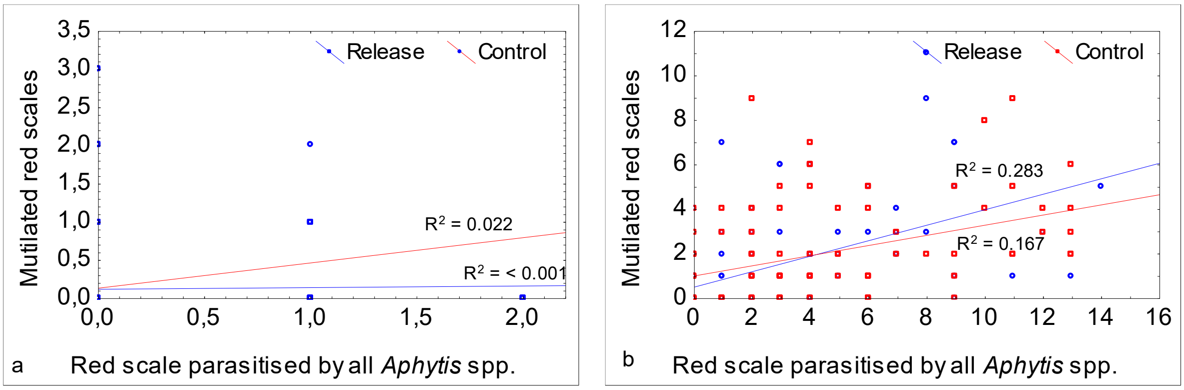
Table 2.24. Univariate test of significance of the regressions between the number of mutilated red scales per fruit and red scales parasitised by the various red scale parasitoid species during the 2019/2020 season.

Orchards	*A. africanus*	*A. lingnanensis*	*A. melinus*	*C. bifasciata*
Hexfarm 1350 T and B	-	-	-	-
Hexfarm 1371 and 1272	F = 25.641 *P* = < 0.001* Treatment: F = 4.798	-	-	-

	P = 0.029* Treatment × *A. africanus*: F = 6.181 *P* = 0.013*			
Modderfontein 401 and 405	F = 17.967 *P* = < 0.001* Treatment: F = 6.876 *P* = 0.009*	Treatment: F = 10.332 *P* = 0.002*	Treatment: F = 8.270 *P* = 0.004*	Treatment: F = 7.696 *P* = 0.006*
Mandaryn T and B	F = 8.401 *P* = 0.004*	-	-	F = 5.562 *P* = 0.019*
Normandale 33 and Step Aside 26	F = 16.040 *P* = < 0.001* Treatment: F = 8.223 *P* = 0.004*	Treatment: F = 16.198 *P* = < 0.001*	Treatment: F = 15.444 *P* = < 0.001*	Treatment: F = 8.914 *P* = 0.003*
Sunad 60 T and C	F = 24.250 *P* = < 0.001*	-	F = 4.676 *P* = 0.031*	-
Sunad 6 and 61	Treatment × *A. africanus* F = 6.920 *P* = 0.009*	-	-	-

* Result of ANCOVA indicating significant differences between the regression slopes at a 95% confidence level.

The number of red scales mutilated for every red scale that had been parasitised did not differ between release and control orchards (Fig. 2.26 and Table 2.25).



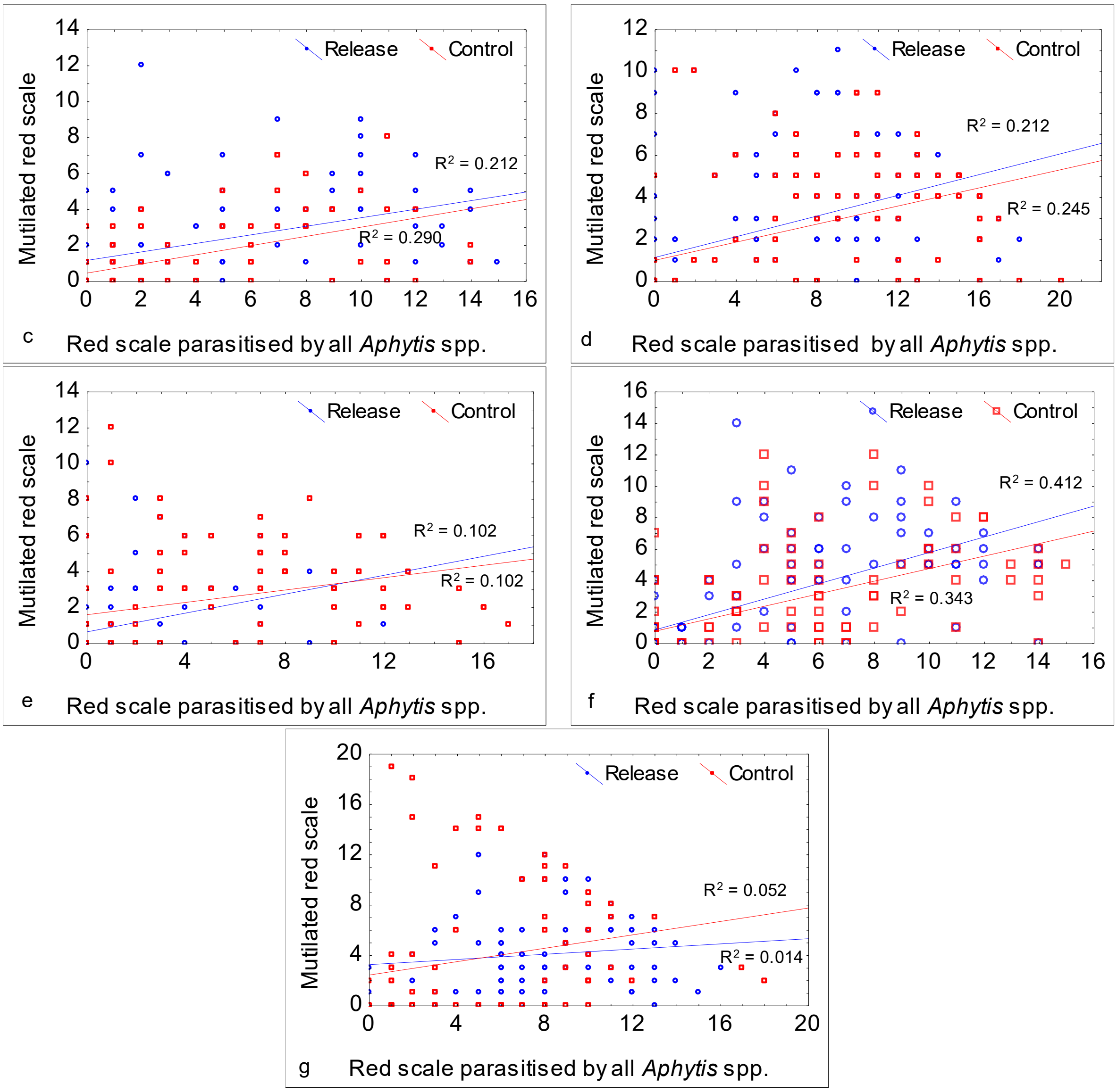


Figure 2.26. Number of mutilated red scales per fruit correlated with red scales parasitised by all *Aphytis* spp. during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Sunad 60 T and C (f); Sunad 6 and 61 (g). Refer to Table 2.25 for results of statistical analyses.

Table 2.25. Univariate test of significance of the regressions between the number of mutilated red scales per fruit and red scales parasitised by all *Aphytis* spp. during the 2019/2020 season.

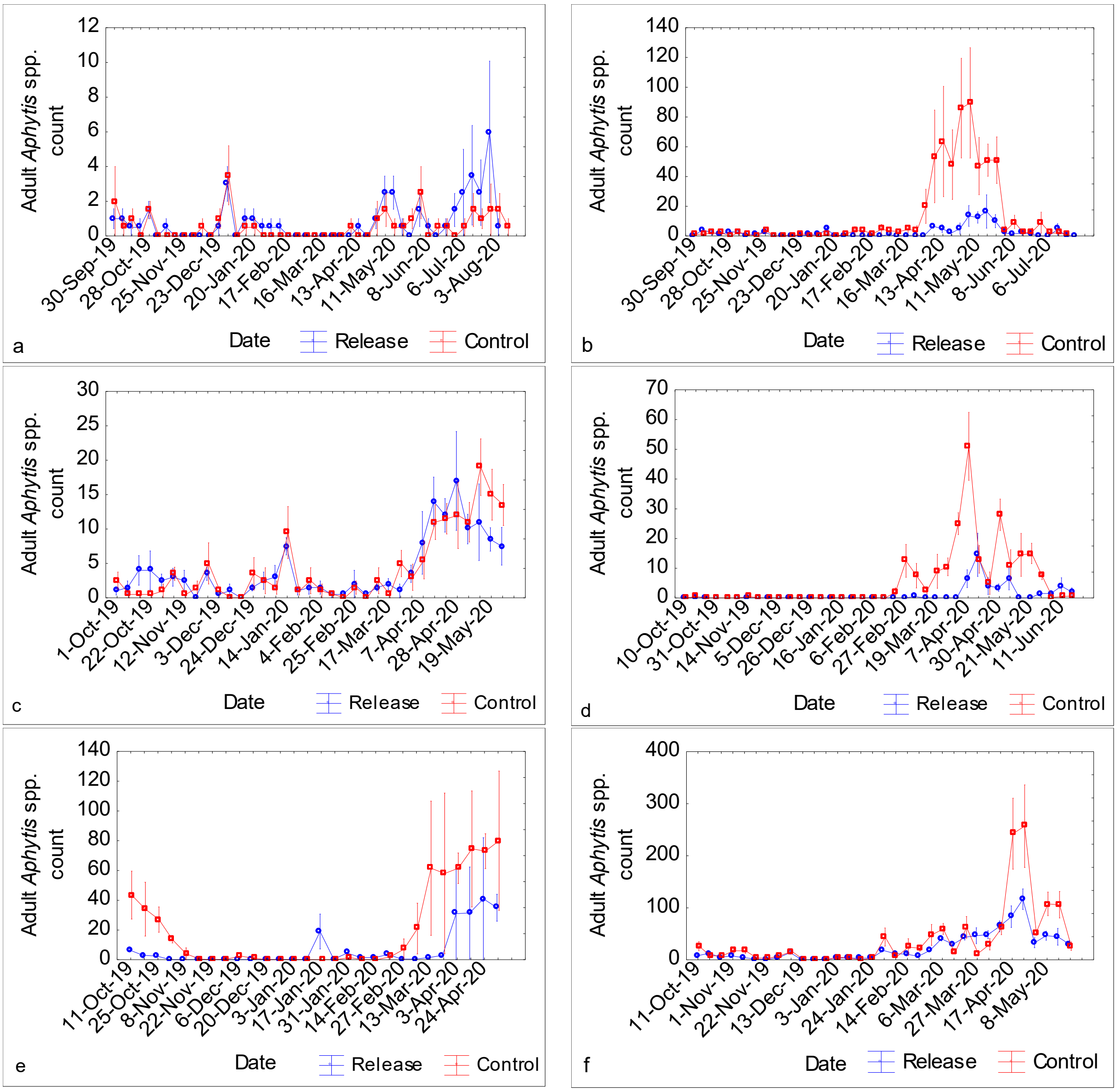
Orchard	**All *Aphytis* spp.**
Hexfarm 1350 T and B	-
Hexfarm 1371 and 1272	F = 57.143 *P* = < 0.001*
Modderfontein 401 and 405	F = 52.167 *P* = < 0.001*
Mandaryn T and B	F = 80.807

	P = < 0.001*
Normandale 33 and Step Aside 26	F = 17.653 *P* = < 0.001*
Sunad 60 T and C	F = 144.290 *P* = < 0.001*
Sunad 6 and 61	F = 4.690 *P* = 0.032*

* Denotes a significant interaction at a 95% confidence level.

2.3.1.3. Adult *Aphytis* spp. counts in traps during the 2019/2020 season

No significant differences were recorded between release and control orchards in the number of adult *Aphytis* spp. caught in the traps. Generally, wasp counts increased towards the end of the growing season when *Aphytis* spp. activity increased (Fig. 2.27).



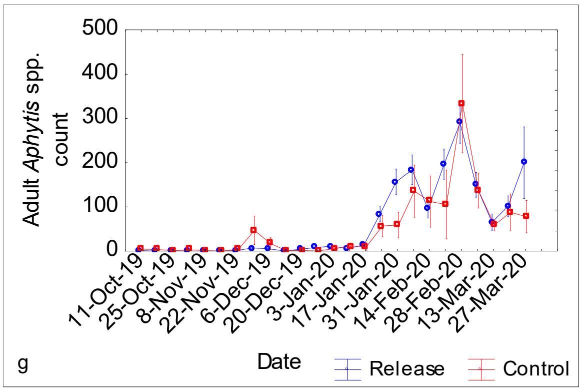


Figure 2.27. Mean counts of *Aphytis* spp. adult numbers caught on traps during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B orchards (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Sunad 60 T and C (f); Sunad 6 and 61 (g). Error bars represent SE.

2.3.1.4. Fruit infestation during the 2019/2020 season

Red scale fruit infestation increased significantly in all orchards through the season except for Hexfarm 1371 and 1272, as well as Modderfontein 401 and 405 where it remained fairly constant (effect of Date, Fig. 2.28, and Table 2.26). In all the trial orchards where red scale fruit infestation was significantly lower at the end of the season in the release orchards against the control orchards, the red scale fruit infestation was lower at the start of the assessment period, except for the release orchard at Modderfontein, where it was the same. Even in orchards where releases were made early in the season, like Hexfarm 1350, 1371 and Modderfontein, no significant difference in fruit infestation could be shown between release and control orchards where the red scale infestation was the same at the start of the season (Fig. 2.28 and Table 2.26). When there were significant differences between the release and control orchards, the red scale infestation was lower in the release orchard at the onset of the trials like at (b) Hexfarm 1272 and 1371, (d) Mandaryn T and B and (e) Normandale 33 and Step Aside 26 (Fig. 2.28).

Red scale, even when killed by either host mutilation or parasitism, amongst other factors, does not easily and quickly detach from the host plant surface. It is therefore accepted that many of the red scales counted during the assessment for fruit infestation, might not be alive and actively feeding and not available for parasitism by red scale parasitoids.

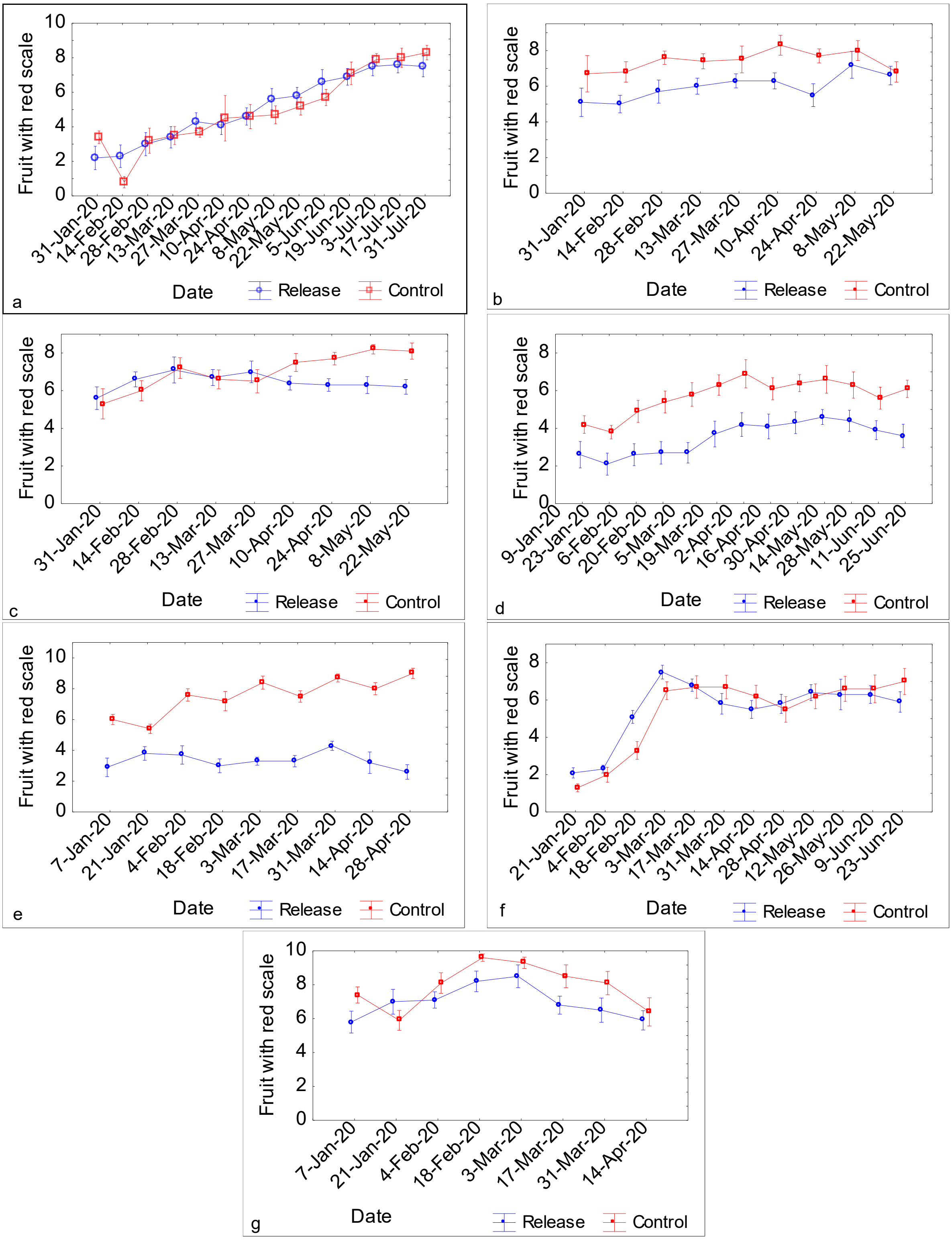


Figure 2.28. Mean number of fruit, out of 10 (10 fruit inspected on each of 10 trees), infested with red scale during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Sunad 60 T and C (f); Sunad 6 and 61 (g). Refer to Table 2.26 for results of statistical analyses. Error bars represent SE.

Table 2.26. Effect of date (assessment) and treatment (release or control) on red scale fruit infestation over time during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B orchards at Hexfarm 1371 and 1272; Modderfontein 401 and 405; Mandaryn T and B; Normandale 33 and Step Aside 26; Sunad 60 T and C; Sunad 6 and 61 (Wald test statistic).

Effect	**Df**	**F**	***P***
Hexfarm 1350 T and B			
Date	13, 252	23.161	< 0.001*
Treatment	1, 252	0.064	0.799
Date × Treatment	13, 252	0.779	0.682
Hexfarm 1371 and 1272			
Date	8, 162	1.740	0.092
Treatment	1, 162	26.515	< 0.001*
Date × Treatment	8, 162	0.567	0.803
Modderfontein 401 and 405			
Date	8, 162	2.532	0.127
Treatment	1, 162	5.249	0.023*
Date × Treatment	8, 162	2.076	0.040
Mandaryn T and B			
Date	12, 234	4.268	< 0.001*
Treatment	1, 234	93.158	< 0.001*
Date × Treatment	12, 234	0.315	0.986
Normandale 33 and Step Aside 26			
Date	8, 162	4.191	< 0.001*
Treatment	1, 162	401.873	< 0.001*
Date × Treatment	8, 162	4.488	< 0.001*
Sunad 60 T and C			
Date	11, 216	22.471	< 0.001*
Treatment	1, 216	0.211	0.646
Date × Treatment	11, 216	1.227	0.270
Sunad 6 and 61			
Date	7, 144	6.023	< 0.001*
Treatment	1, 144	9.623	0.002*
Date × Treatment	7, 144	1.181	0.317

* Denotes significant interaction at a 95% confidence level.

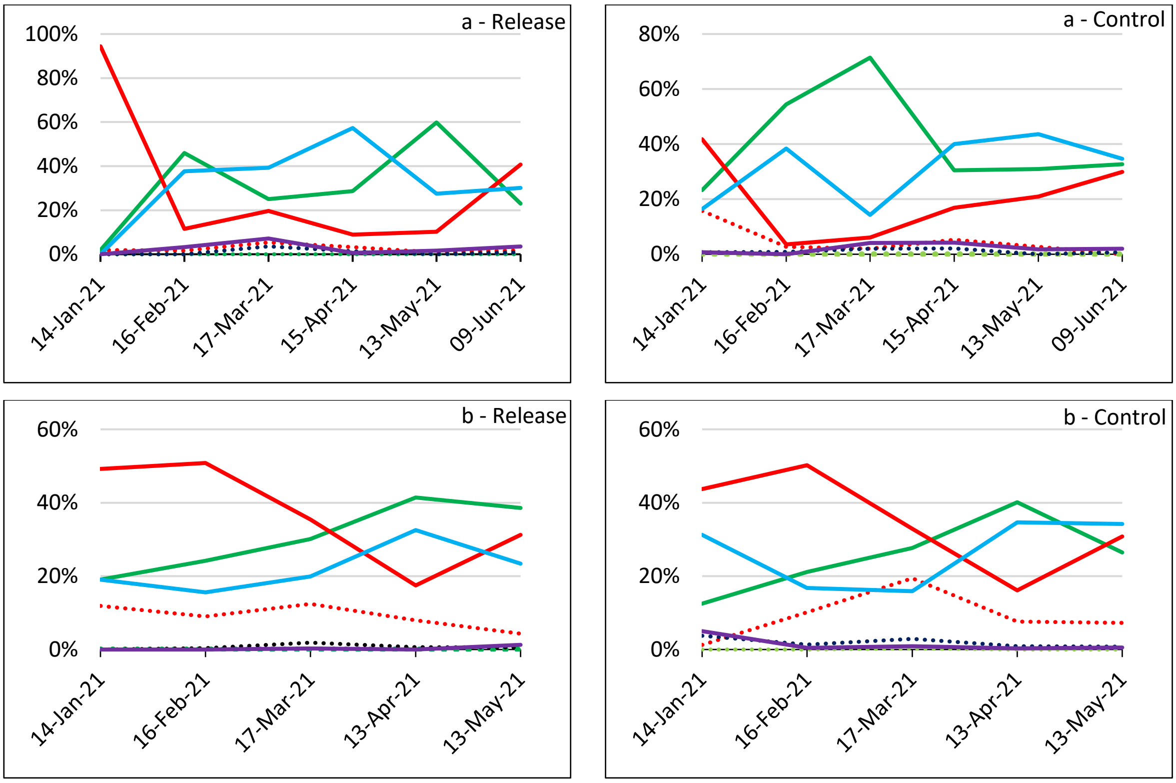
2.3.2. The 2020/2021 season

2.3.2.1. Red scale infestation and parasitoid activity trends during the 2020/2021 season in release and control orchards

Healthy red scale numbers decreased towards harvest time as red scale parasitism and host mutilation by various parasitoids increased at all orchards on Hexfarm 1350, Hexfarm 1371, Modderfontein, Mandaryn and Step Aside (Fig. 2.29). Red scale parasitism by *A. africanus* occurred the most amongst various red scale parasitoids apart from the release orchard Hexfarm 1350 T where parasitism by *C. bifasciata* were comparable to that of *A. africanus* (Fig. 2.29).

The increase of efficacy of the various red scale parasitoids increased notably from the previous season (Fig. 2.13 a) at orchard Hexfarm 1350 T and B towards this season (Fig. 2. 29 a) most probably due to the implementation of a compatible IPM programme.

Very effective red scale control by various red scale parasitoids concomitantly parasitising and mutilating hosts can be seen at the release and control orchards at Step Aside (Fig. 2.29 e).



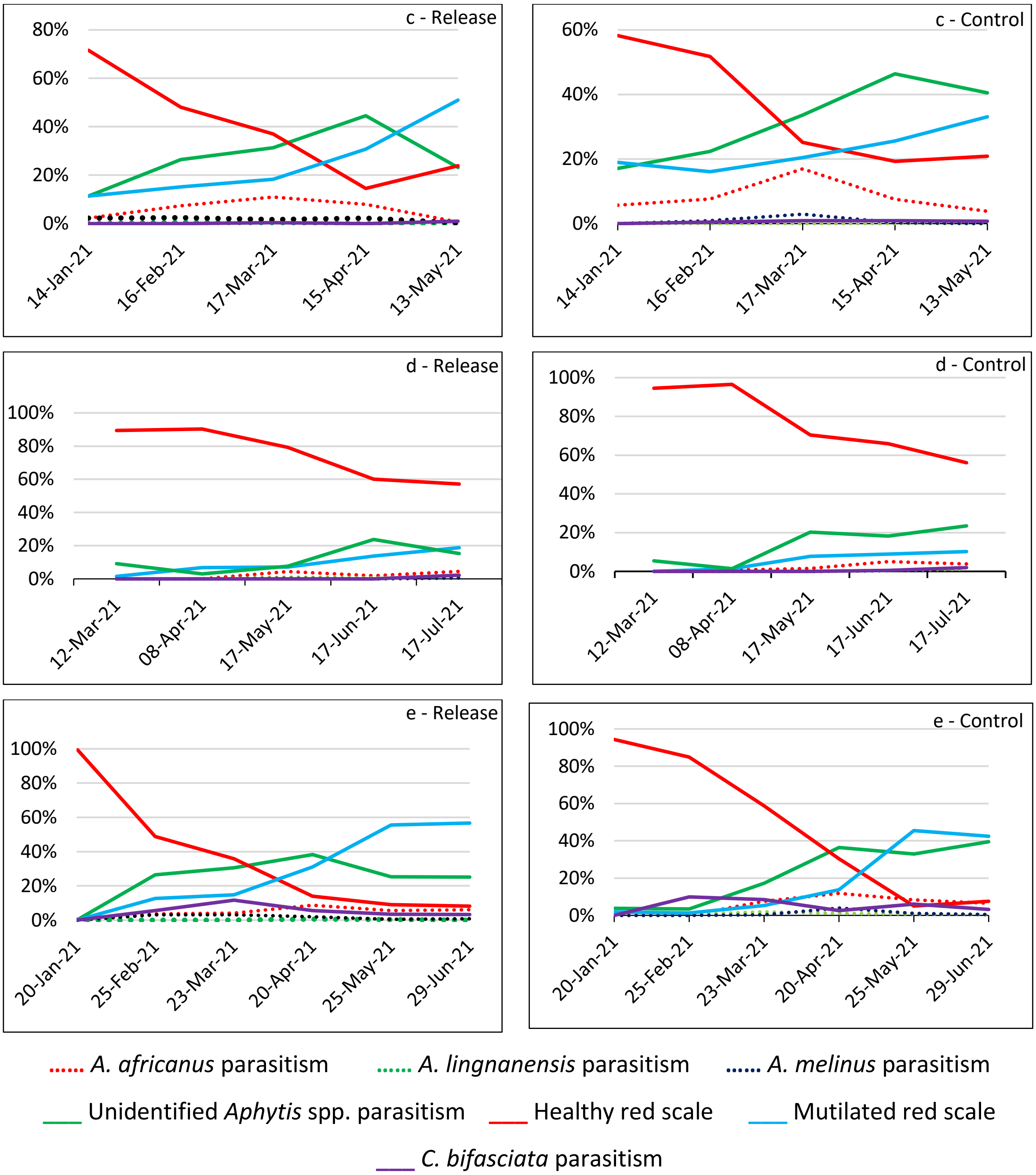


Figure 2.29. Red scale infestation and parasitoid activity trends during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c), Mandaryn T and B (d) and Step side 23 and 32 (e).

2.3.2.2. Microscopic assessments of red scales during the 2020/2021 season

No significant differences in the mean number of healthy scales remaining per fruit at the time of harvest were recorded between release and control orchards (Fig. 2.30). More significant interactions were recorded between *Aphytis africanus* parasitism and a lack of healthy red scales per fruit than for the other parasitoid species (Table 2.27).

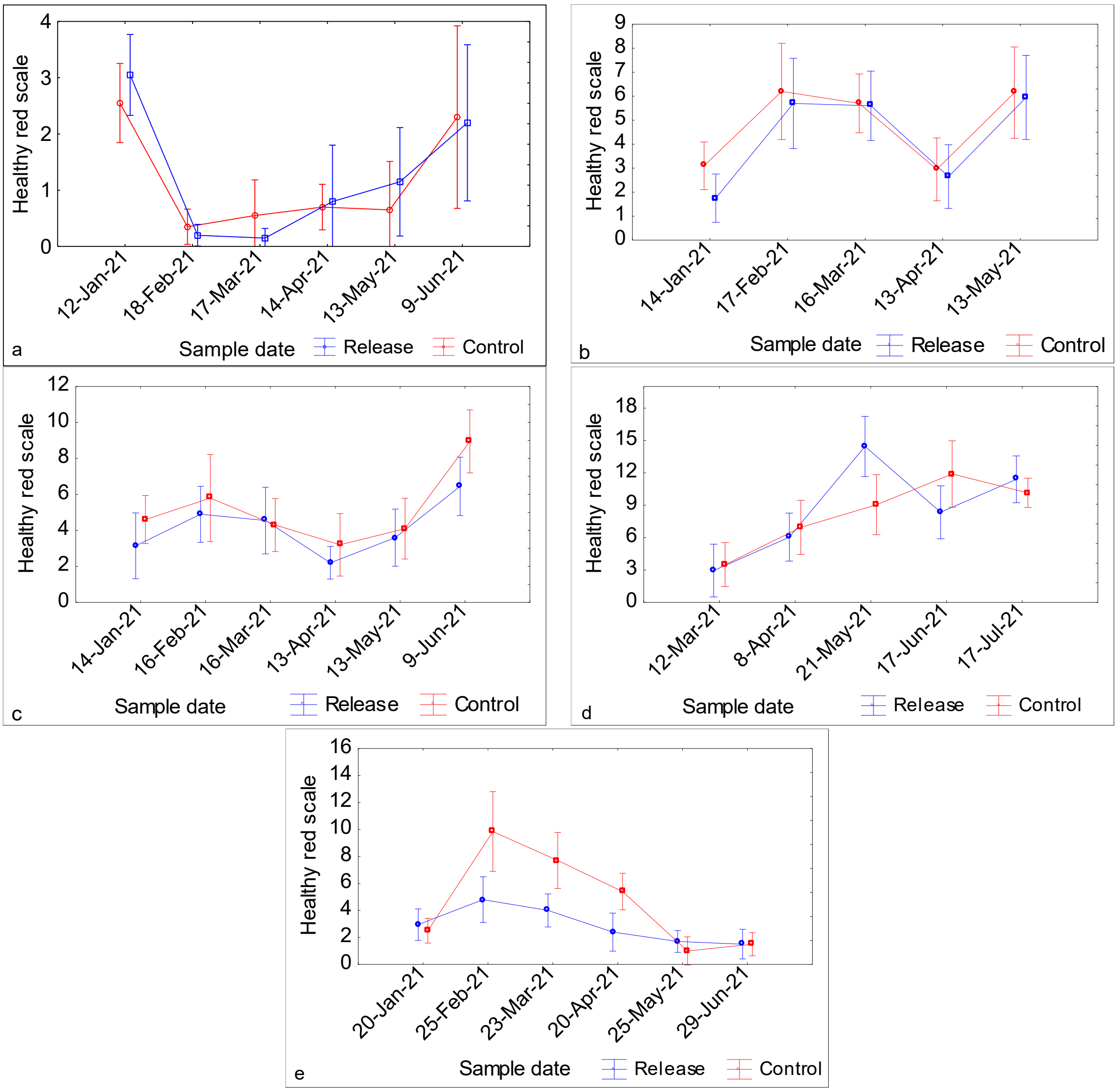


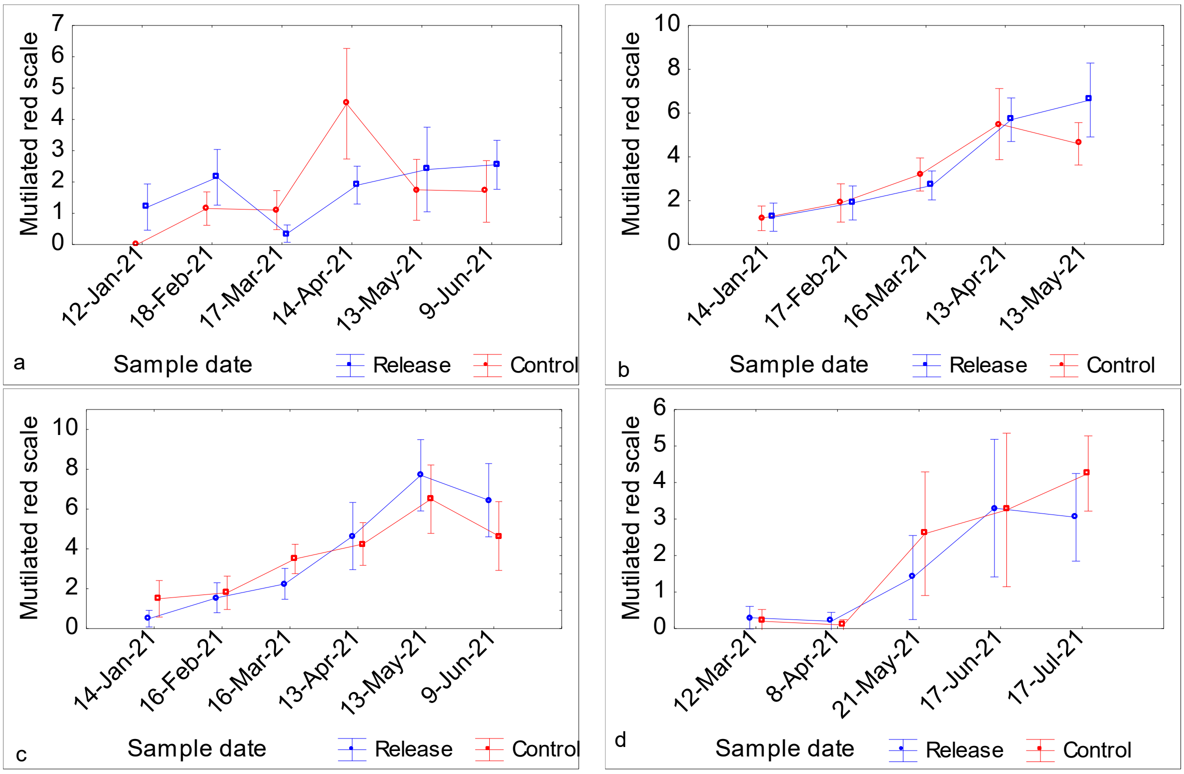
Figure 2.30. The mean number of healthy red scales per fruit during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.27 for results of statistical analyses. Error bars represent SE.

Table 2.27. Results of generalised linear models indicating significant interactions between parasitism and healthy red scales during the 2020/2021 season (Wald test statistic).

Orchard	*A. africanus*	*A. lingnanensis*	*A. melinus*	*C. bifasciata*
Hexfarm 1350 T and B	3.193	-	9.854*	0.206
Hexfarm 1371 and 1272	4.230*	0.659	2.250	0.858
Modderfontein 401 and 405	1.245	0.118	1.393	0.473
Mandaryn T and B	6.427*	2.910	0.000	1.676
Step Aside 23 and 32	0.009	0.290	0.023	2.9.9

* Denotes a significant interaction at a 95% confidence level.

No significant differences realised between release and control orchards with regards host mutilation (Fig. 2.31). *Aphytis africanus* contributed most to host mutilation when compared to the other parasitoids measured (Table 2.28).



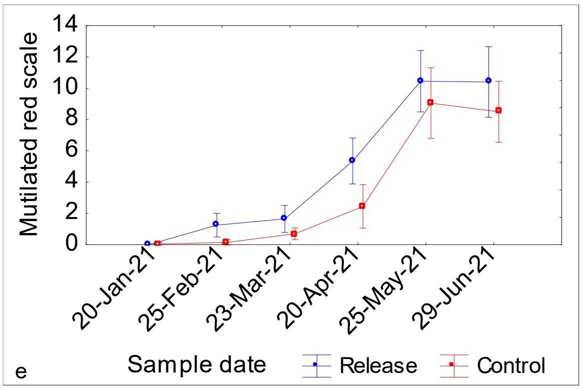


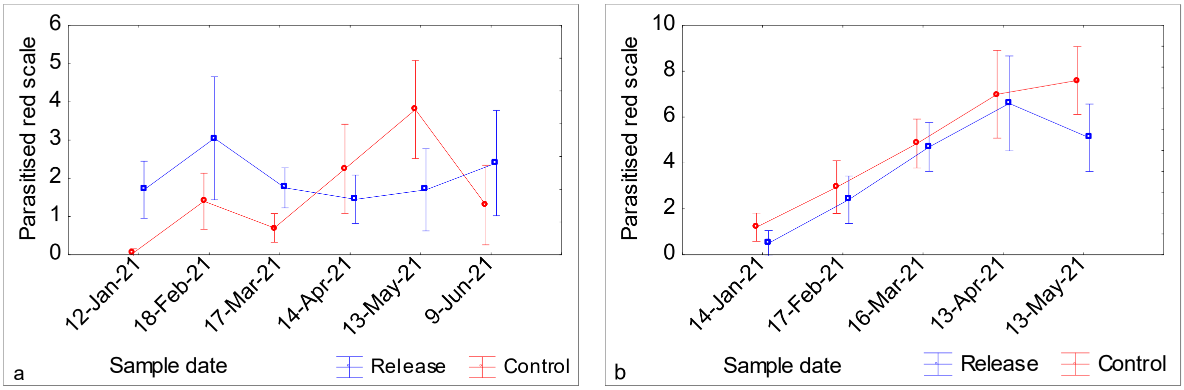
Figure 2.31. The mean number of mutilated red scales per fruit during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.28 for results of statistical analyses. Error bars represent SE.

Table 2.28. Results of generalised linear models indicating significant interactions between parasitism and mutilated red scales during the 2020/2021 season (Wald test statistic).

Orchard	*A. africanus*	*A. lingnanensis*	*A. melinus*	*C. bifasciata*
Hexfarm 1350 T and B	43.611*	-	15.51*	3.373
Hexfarm 1371 and 1272	0.142	0.068	0.441	3.223
Modderfontein 401 and 405	0.063	0.000	0.045	0.284
Mandaryn T and B	5.279*	1.843*	0.201	1.048
Step Aside 23 and 32	0.753	6.757*	0.003	6.236*

* Denotes a significant interaction at a 95% confidence level.

The mean number of red scales parasitised by unidentified *Aphytis* spp. per fruit in any control orchard did not differ significantly from any release orchard during the 2020/2021 season (Fig. 2.32). *Aphytis africanus* had the most interactions of significance with red scale parasitised by unidentified *Aphytis* spp. amongst all three *Aphytis* spp., while *A. melinus* significant interactions exceeded those of *A. lingnanensis*, during the 2020/2021 season (Table 2.29).



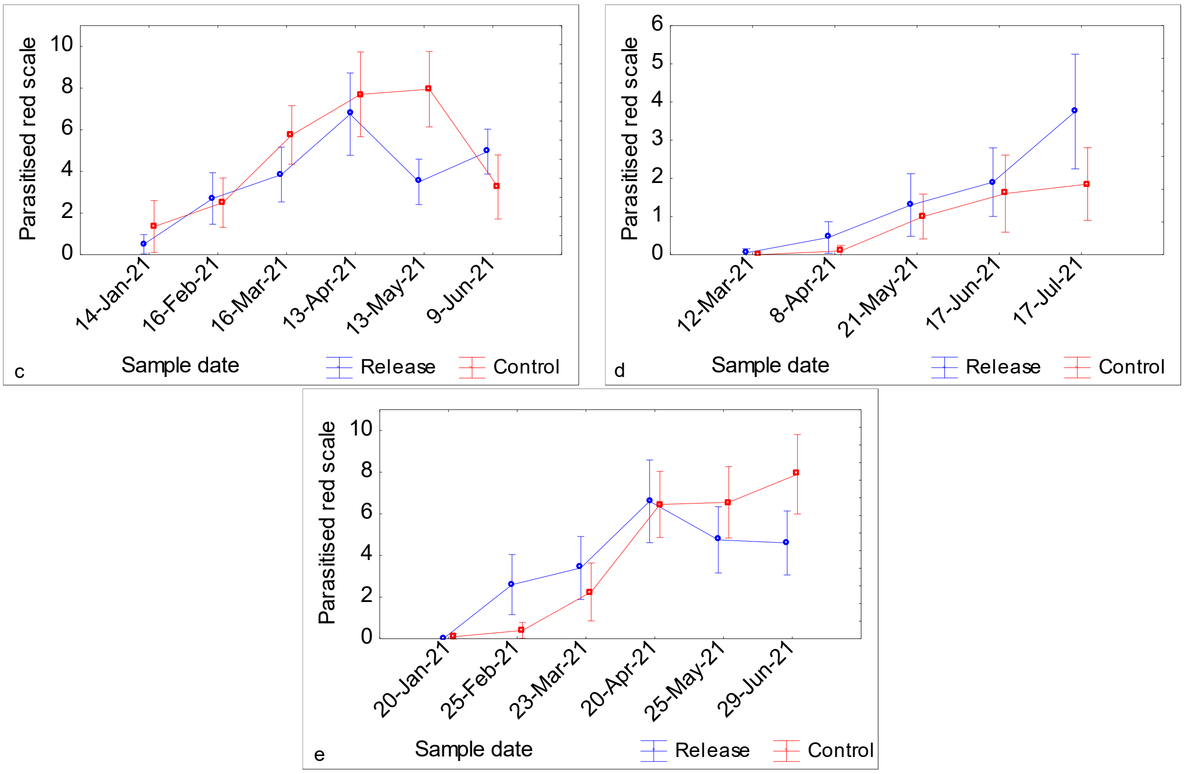


Figure 2.32. The mean number of red scales per fruit parasitised by unidentified *Aphytis* spp. during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.29 for results of statistical analyses. Error bars represent SE.

Table 2.29. Results of generalised linear models indicating significant interactions between parasitism of red scales by known *Aphytis* spp. and unidentified *Aphytis* spp. with red scales during the 2020/2021 season (Wald test statistic).

Orchard	*A. africanus*	*A. lingnanensis*	*A. melinus*
Hexfarm 1350 T and B	28.128*	-	7.234*
Hexfarm 1371 and 1272	8.449*	0.586	0.138
Modderfontein 401 and 405	9.818*	4.198*	5.012*
Mandaryn T and B	16.177*	2.256	0.483
Step Aside 23 and 32	1.418	0.047	6.169*

* Denotes a significant interaction at a 95% confidence level.

Red scale parasitism by *A. africanus* had the most significant correlations with the number of unhealthy red scale found per fruit when compared to other *Aphytis* spp. and *C. bifasciata* (Figs. 2.33, 2.34, 2.35, and 2.36; Table 2.30). *Aphytis melinus* had the second most significant interactions after *A. africanus*.

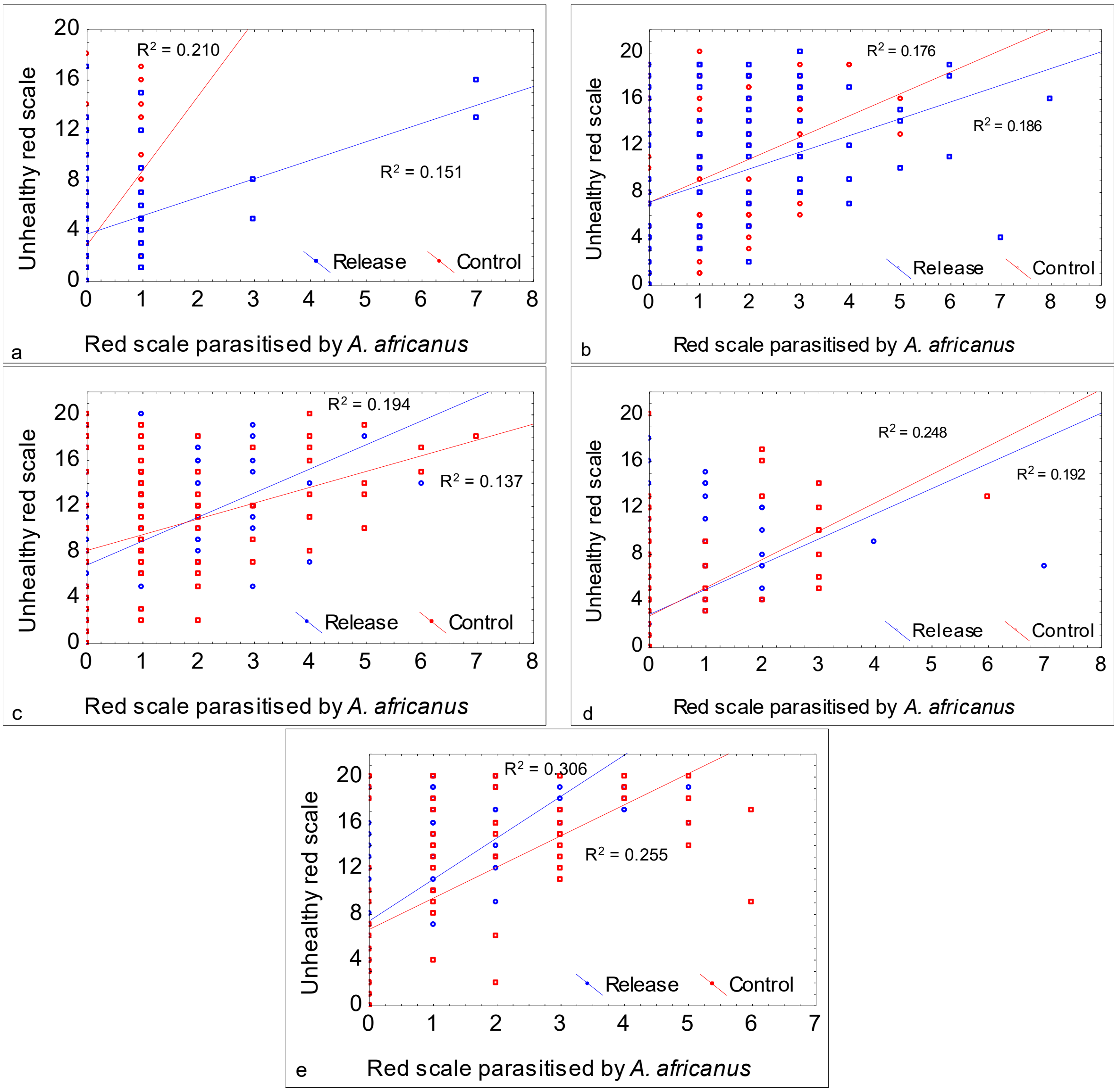


Figure 2.33. Number of unhealthy red scales per fruit correlated with red scales parasitised by *A. africanus* during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.30 for results of statistical analyses.

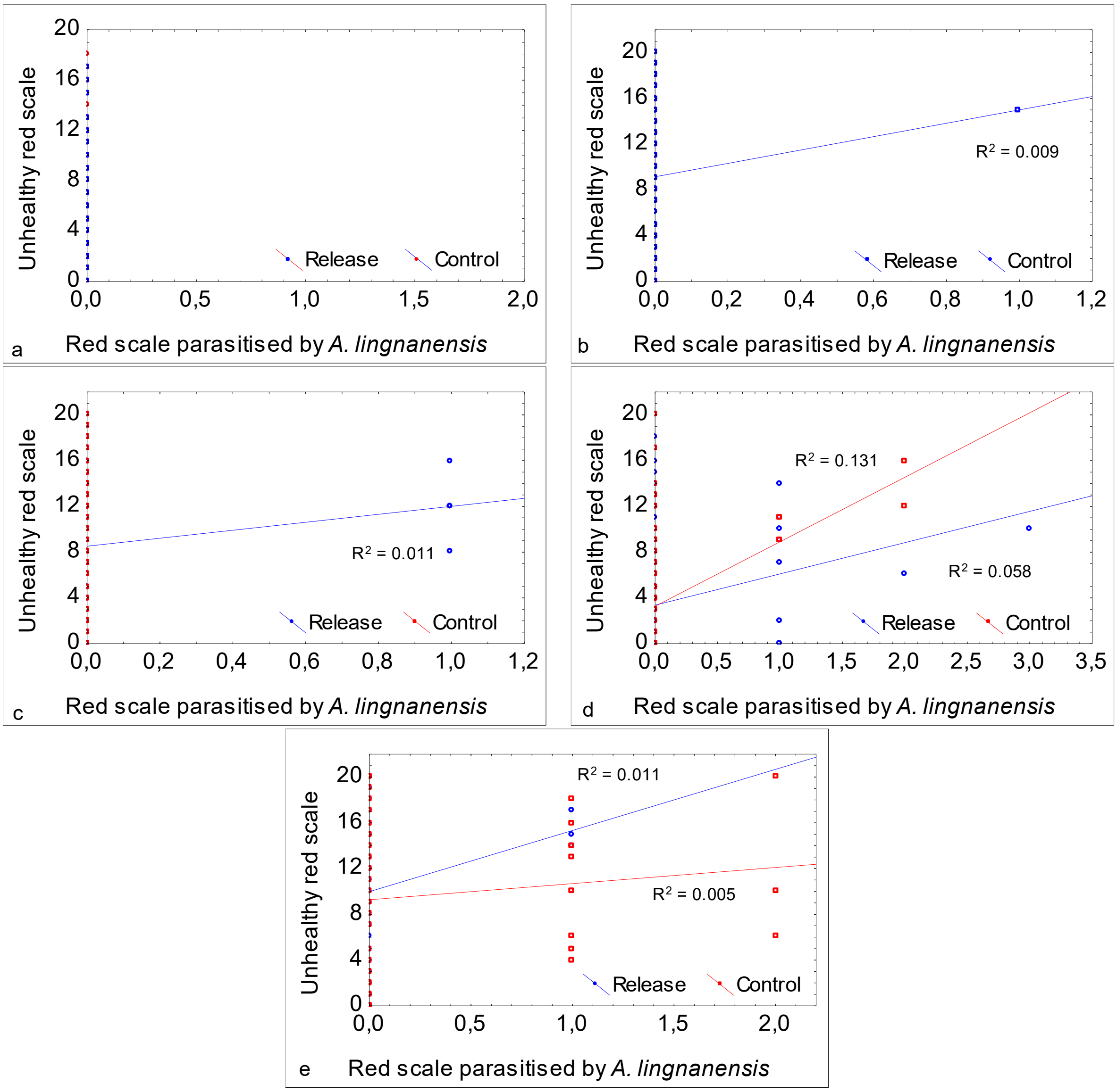
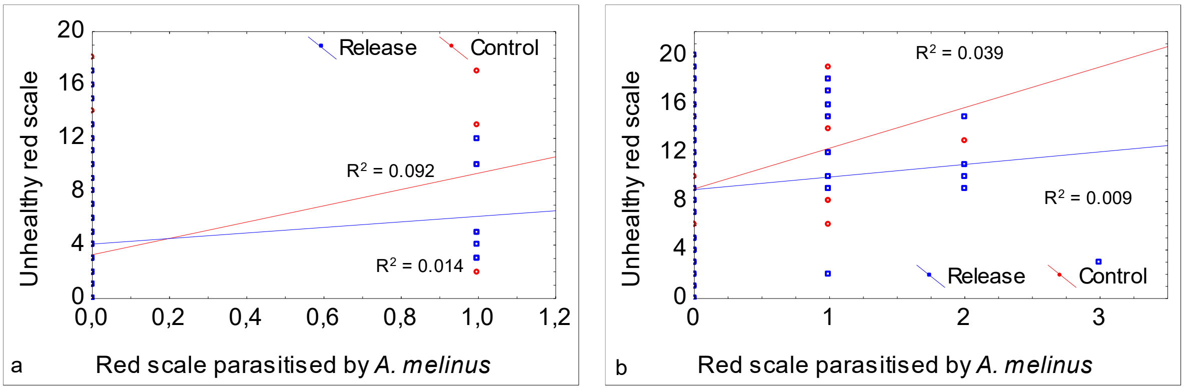


Figure 2.34. Number of unhealthy red scales per fruit correlated with red scales parasitised by *A. lingnanensis* during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.30 for results of statistical analyses.



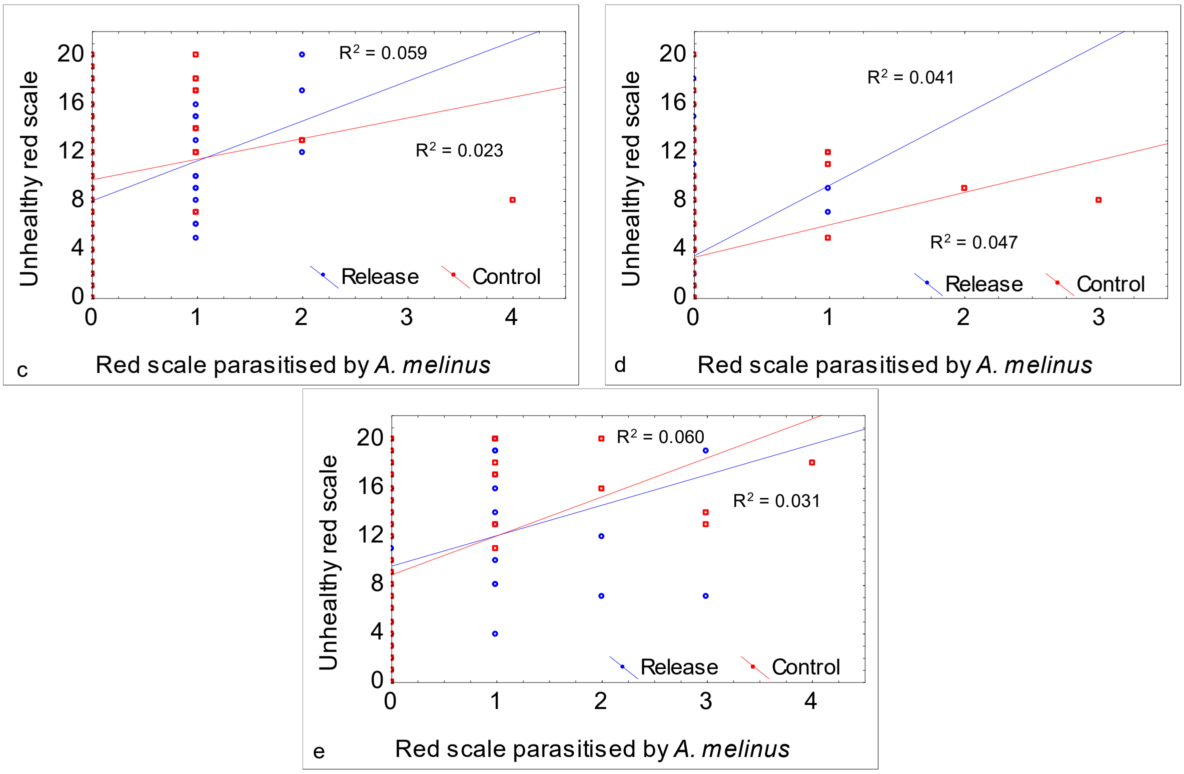
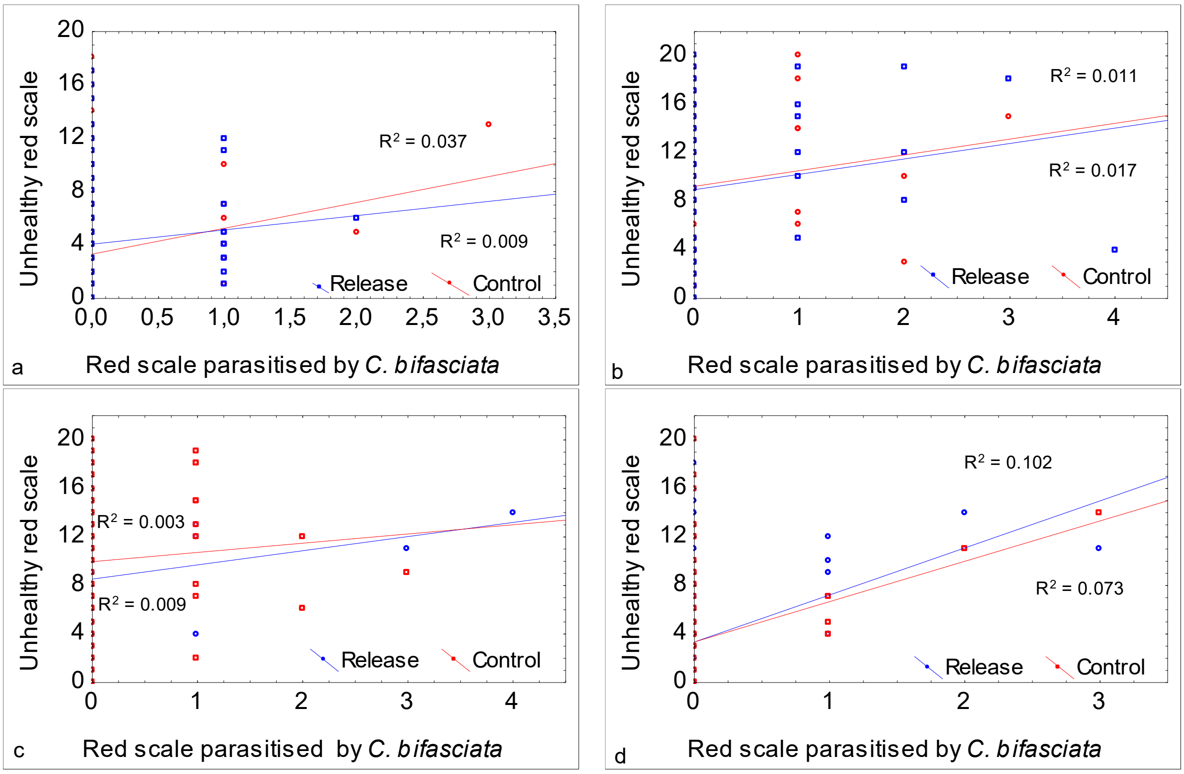


Figure 2.35. Number of unhealthy red scales per fruit correlated with red scales parasitised by *A. melinus* during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.30 for results of statistical analyses.



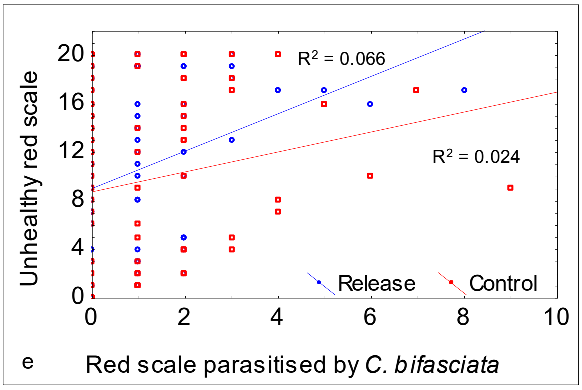


Figure 2.36. Number of unhealthy red scales per fruit correlated with red scales parasitised by *C. bifasciata* during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.30 for results of statistical analyses.

Table 2.30. Univariate test of significance of the regressions between the number of unhealthy red scales per fruit and red scales parasitised by the various red scale parasitoid species during the 2020/2021 season.

Orchards	***A. africanus***	***A. lingnanensis***	***A. melinus***	***C. bifasciata***
Hexfarm 1350 T and B	F = 45.556 *P* = 0.001* Treatment × *A. africanus*: F = 16.531 *P* = 0.001*	-	F = 11.845 *P* = < 0.001*	F = 4.756 *P* = 0.030*
Hexfarm 1371 and 1272	F = 41.472 *P* = < 0.001*	-	F = 4.656 *P* = 0.032*	F = 2.553 *P* = 0.111
Modderfontein 401 and 405	F = 46.864 *P* = < 0.001*	Treatment: F = 4.039 *P* = 0.045*	Treatment: F = 4.653 *P* = 0.032* *A. melinus*: F = 9.864 *P* = 0.002*	F = 1.347 *P* = 0.246
Mandaryn T and B	F = 55.090 *P* = < 0.001*	F = 20.352 *P* = < 0.001*	F = 7.661 *P* = 0.006*	F = 18.608 *P* = < 0.001*
Step Aside 23 and 32	F = 90.952 *P* = < 0.001*	F = 1.801 *P* = 0.1809	F = 10.687 *P* = 0.001*	F = 10.688 *P* = 0.001*

* Result of ANCOVA indicating significant differences between the regression slopes at a 95% confidence level.

Parasitism by all *Aphytis* spp. (combined data from *A. africanus*, *A. lingnanensis* and *A. melinus* parasitism and mutilation) strongly correlated with the presence of unhealthy red scales on fruit (Fig. 2.37 and Table 2.31). This indicates that *Aphytis* spp. were significantly responsible for high levels of red scale parasitism. The strong regression can also be explained by the notable auto-regression formed when limited data sources are used to analyse the events. No significant differences were recorded between the release and control orchards.

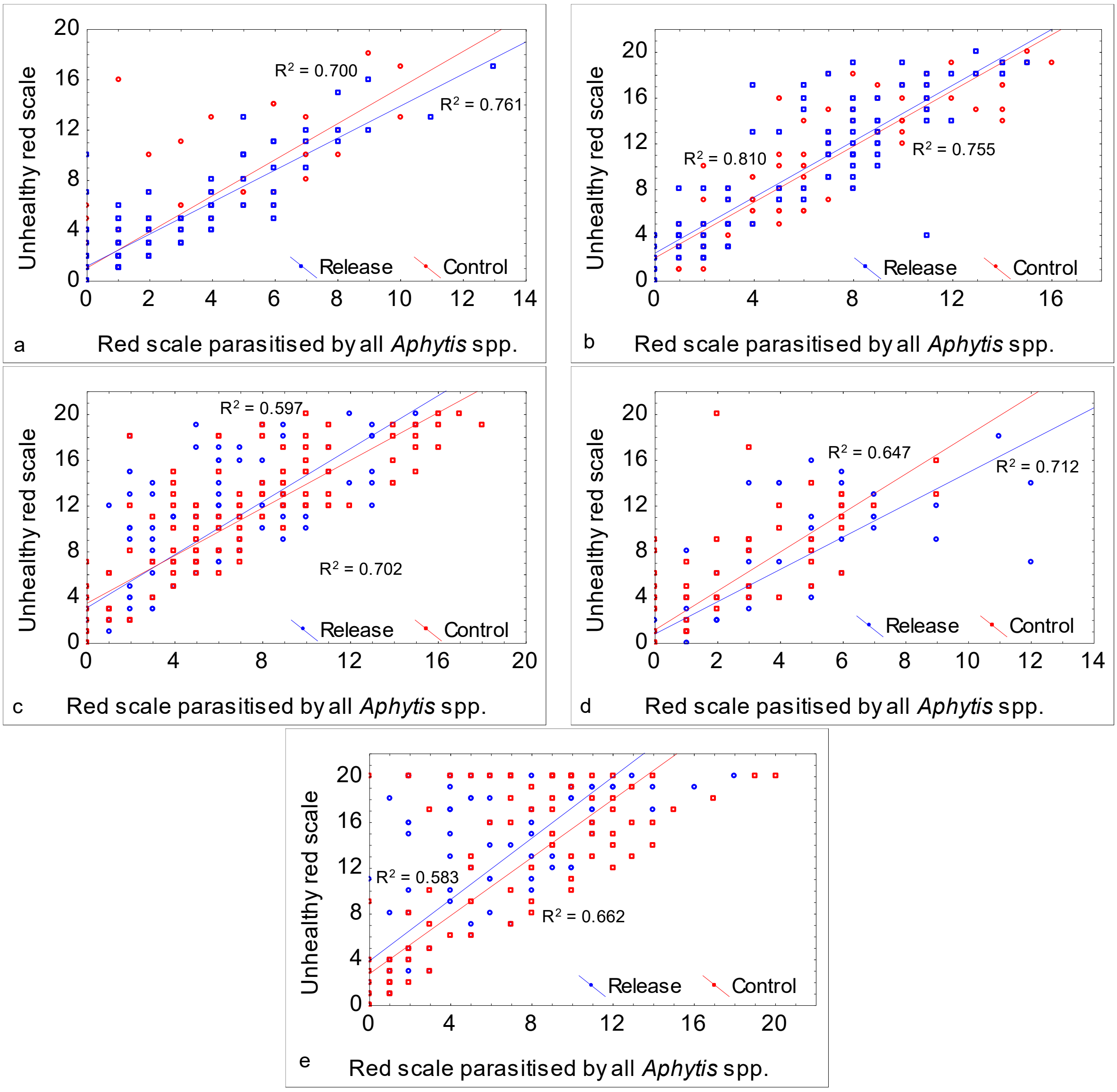


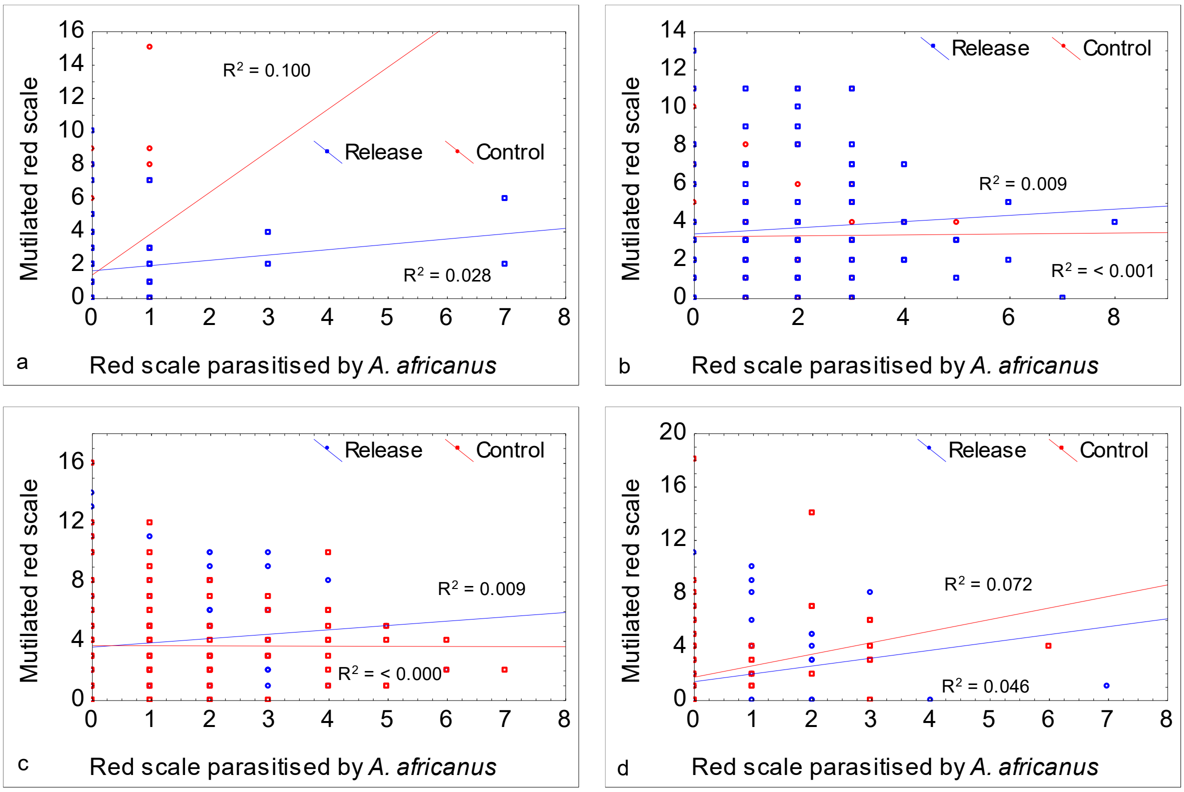
Figure 2.37. Number of unhealthy red scales per fruit correlated with red scales parasitised by all *Aphytis* spp. during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.31 for results of statistical analyses.

Table 2.31. Univariate test of significance of the regressions between the number of unhealthy red scales per fruit and red scales parasitised by all *Aphytis* spp. during the 2020/2021 season.

Orchard	**All *Aphytis* spp.**
Hexfarm 1350 T and B	F = 631.974 *P* = < 0.001*
Hexfarm 1371 and 1272	F = 690.911 *P* = < 0.001*
Modderfontein 401 and 405	F = 429.549 *P* = < 0.001*
Mandaryn T and B	F = 406.512 *P* = < 0.001*
Step Aside 23 and 32	F = 385.824 *P* = < 0.001*

* Result of ANCOVA indicating significant differences between the regression slopes at a 95% confidence level.

Parasitism by *A. africanus* showed the strongest correlation to red scale mutilation in all orchards while there were no significant differences between release and control orchards during the 2020/2021 season (Figs. 2.38, 2.39, 2.40 and 2.41 and Table 2.32).



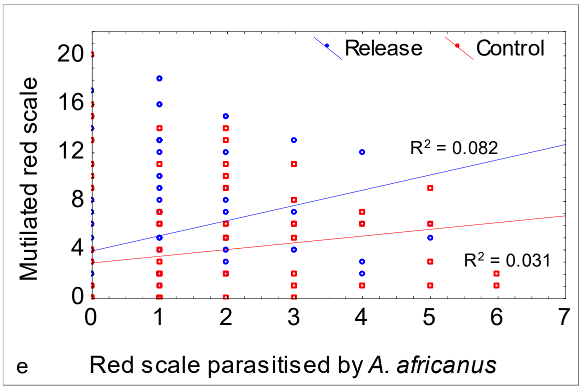
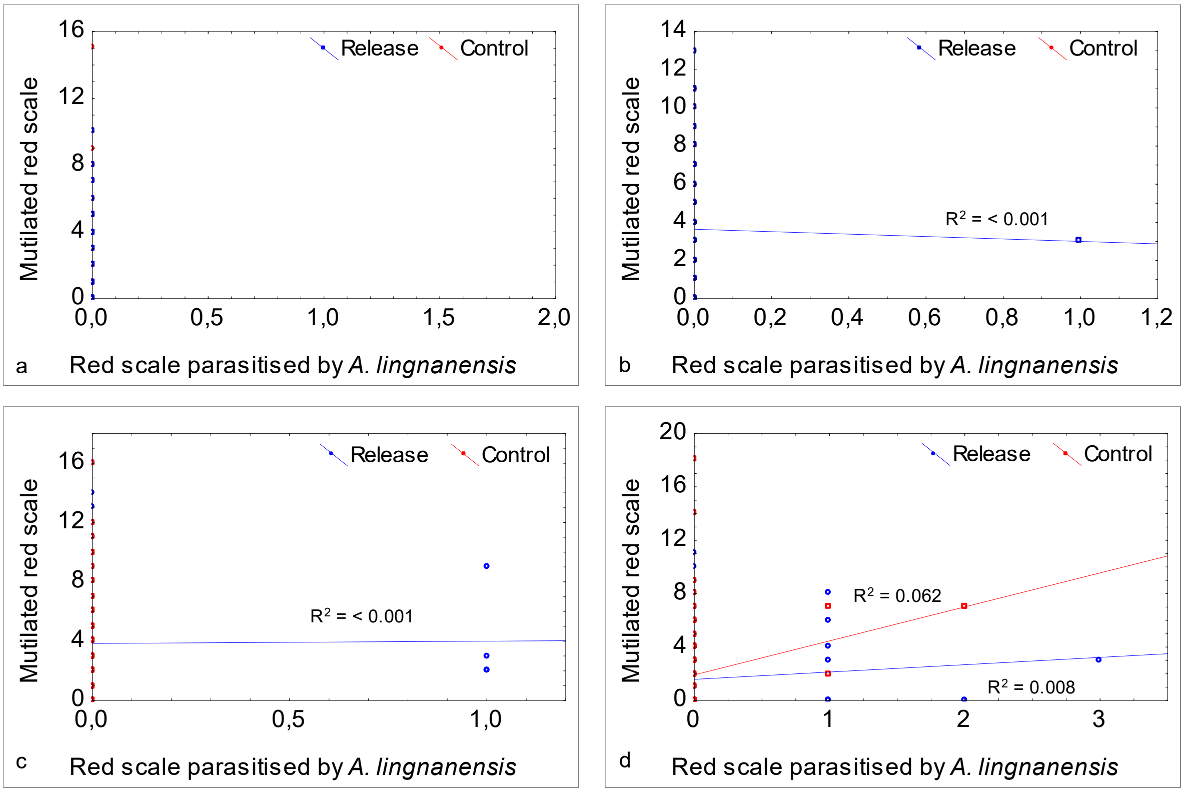


Figure 2.38. Number of mutilated red scales per fruit correlated with red scales parasitised by *A. africanus* during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.32 for results of statistical analyses.



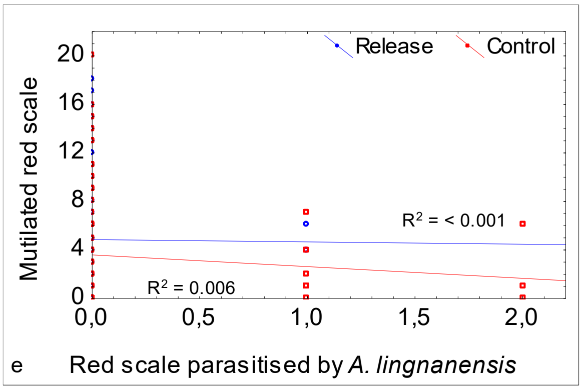


Figure 2.39. Number of mutilated red scales per fruit correlated with red scales parasitised by *A. lingnanensis* during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.32 for results of statistical analyses.

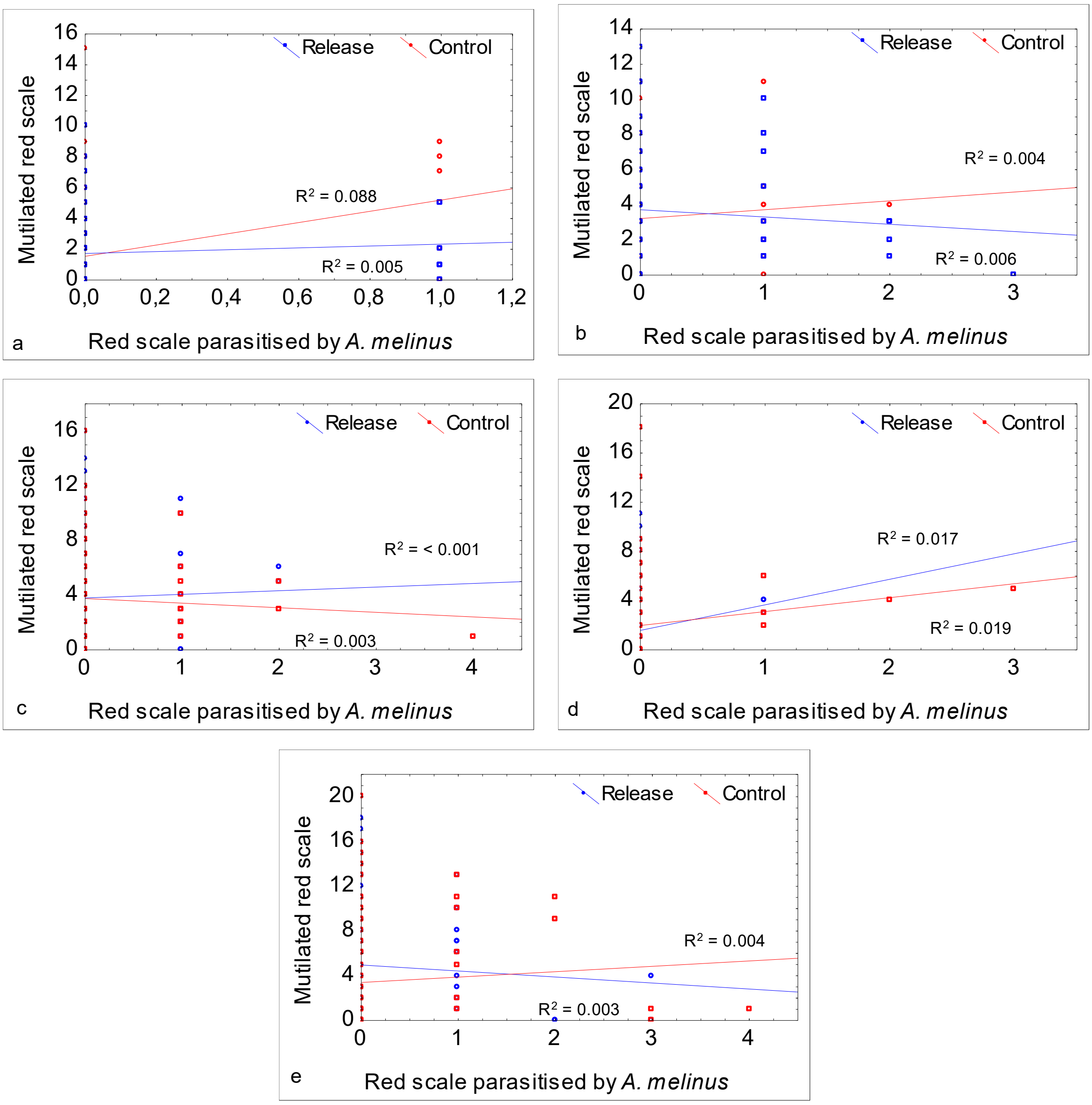


Figure 2.40. Number of mutilated red scales per fruit correlated with red scales parasitised by *A. melinus* during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.32 for results of statistical analyses.

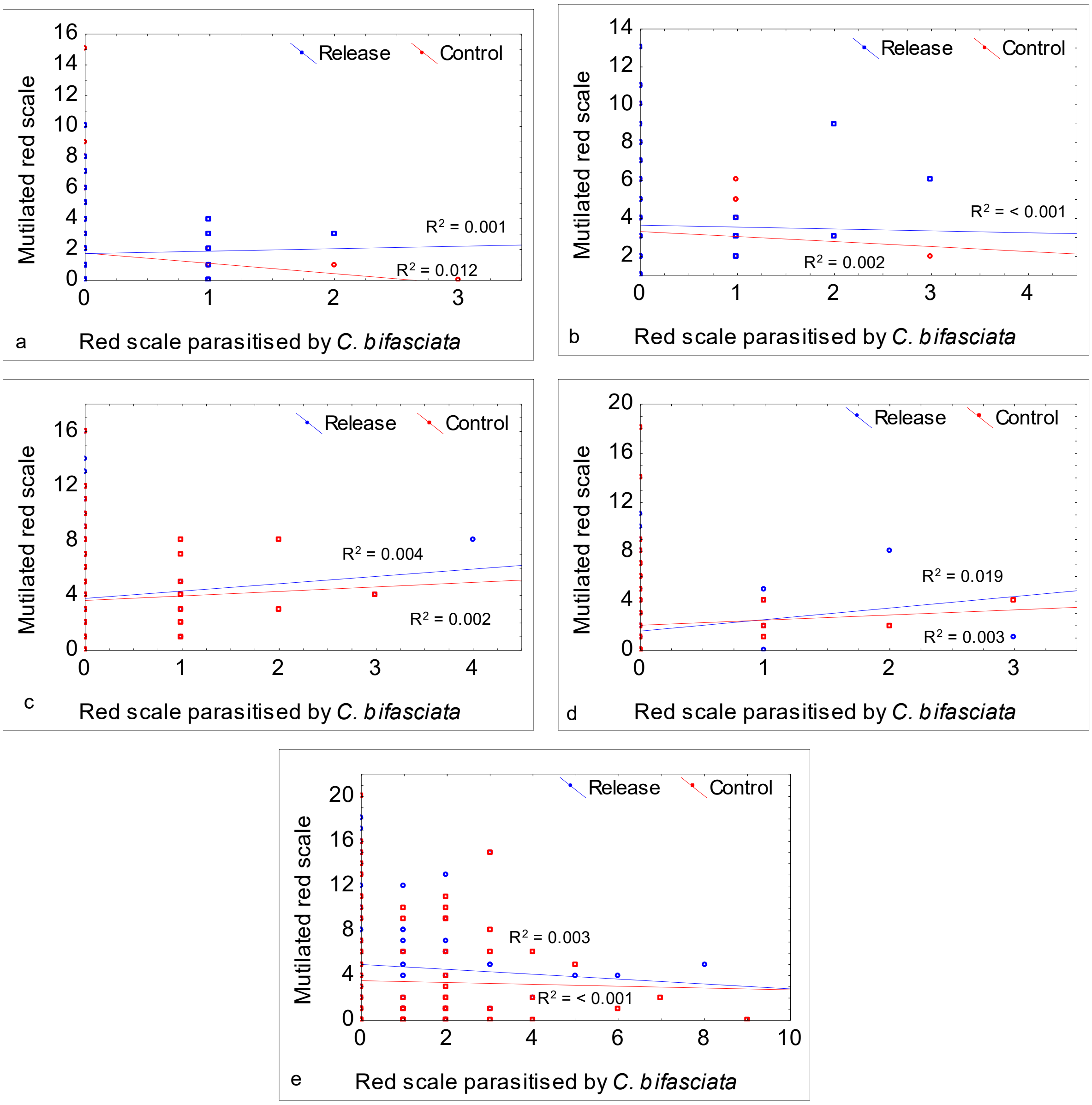


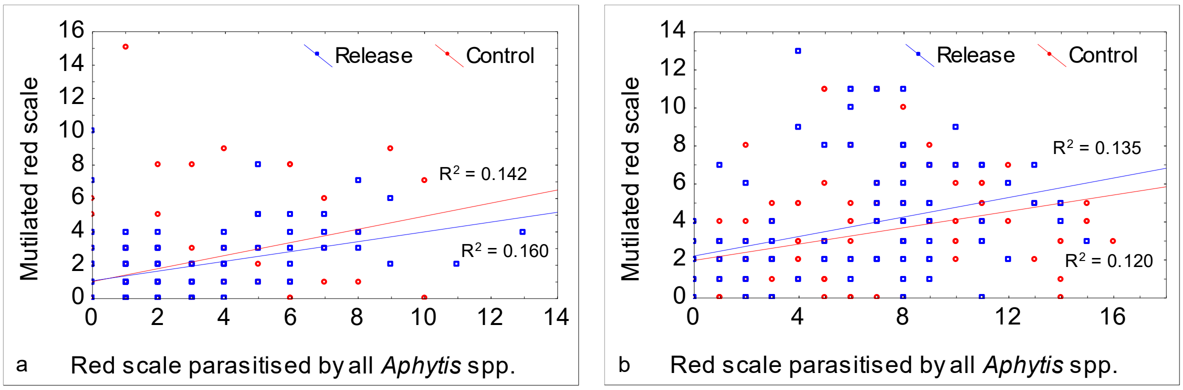
Figure 2.41. Number of mutilated red scales per fruit correlated with red scales parasitised by *C. bifasciata* during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.32 for results of statistical analyses.

Table 2.32. Univariate test of significance of the regressions between the number of mutilated red scales per fruit and red scales parasitised by the various red scale parasitoid species during the 2020/2021 season.

Orchards	***A. africanus***	***A. lingnanensis***	***A. melinus***	***C. bifasciata***
Hexfarm 1350 T and B	F = 18.357 *P* = < 0.001* Treatment × *A. africanus*: F = 10.983 *P* = 0.001*	-	F = 10.187 *P* = 0.002* Treatment × *A. melinus*: F = 5.217 *P* = 0.023*	-
Hexfarm 1371 and 1272	-	-	-	-
Modderfontein 401 and 405	-	-	-	-
Mandaryn T and B	F = 12.312 *P* = < 0.001*	F = 7.090 *P* = 0.008*	-	-
Step Aside 32 and 23	F = 14.374 *P* = < 0.001*	-	Treatment: F = 5.222 *P* = 0.023*	Treatment: F = 3.906 *P* = 0.049*

* Result of ANCOVA indicating significant differences between the regression slopes at a 95% confidence level.

A significant correlation exists between the number of mutilated red scales per fruit and red scales parasitised by all the *Aphytis* spp. assessed in all release and control orchards (Fig. 2.42 and Table 2.33).



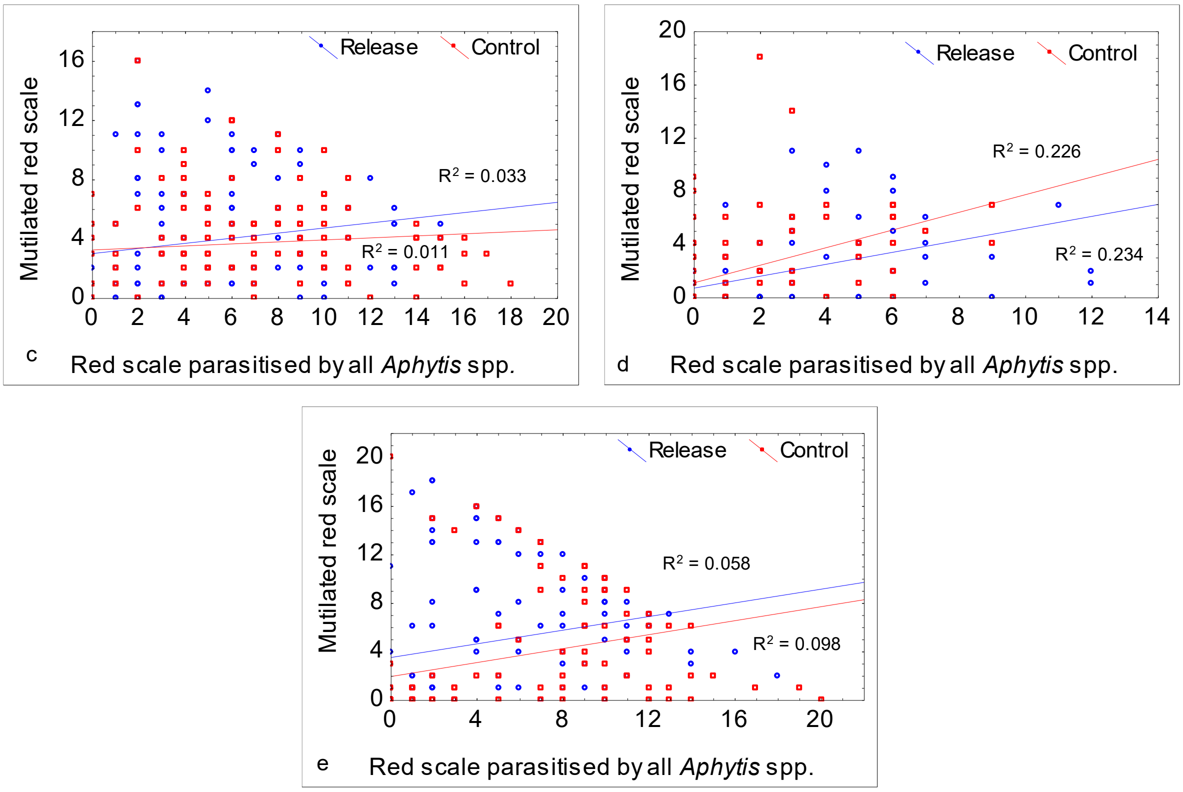


Figure 2.42. Number of mutilated red scales per fruit correlated with red scales parasitised by all *Aphytis* spp. during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.33 for results of statistical analyses.

Table 2.33. Univariate test of significance of the regressions between the number of mutilated red scales per fruit and red scales parasitised by all *Aphytis* spp. during the 2020/2021 season.

Orchard	All *Aphytis* spp.
Hexfarm 1350 T and B	F = 41.196 *P* = < 0.001*
Hexfarm 1371 and 1272	F = 28.593 *P* = < 0.001*
Modderfontein 401 and 405	F = 5.405 *P* = 0.021*
Mandaryn T and B	F = 58.140 *P* = < 0.001*
Step Aside 23 and 32	F = 18.943 *P* = < 0.001*

* Result of ANCOVA indicating significant differences between the regression slopes at a 95% confidence level.

2.3.2.3. Adult *Aphytis* spp. counts in traps during the 2020/2021 season

No significant differences were recorded in the number of adult *Aphytis* spp. caught between any release and control orchards during the 2020/2021 season, apart from the Hexfarm orchards, where more parasitoids were recorded for the control orchard (Fig. 2.43).

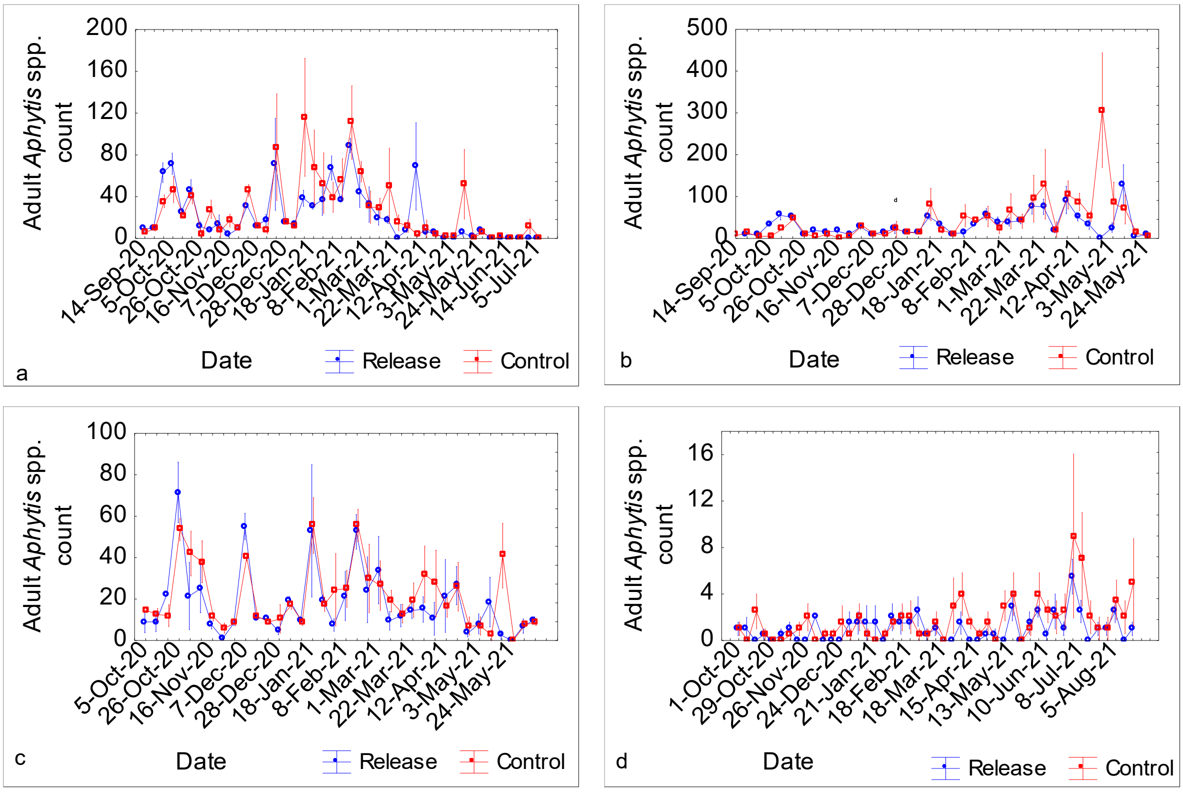


Figure 2.43. Mean counts of *Aphytis* spp. adult numbers caught on traps during the 2019/2020 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d). Error bars present standard error (SE).

2.3.2.4. Fruit infestation during the 2020/2021 season

Red scale fruit infestation increased significantly in all orchards during the season until harvest (Effect of Date, Fig. 2.44, and Table 2.34). In the release orchards, Hexfarm 1371 and Modderfontein 401, where the red scale infestation was significantly lower at harvest than the control orchards, the red scale infestation was lower at the start of the assessment period (Fig. 2.44 and Table 2.34).

Even in orchards where releases were made early in the season, like Hexfarm 1371 and Modderfontein 401, no significant difference in red scale infestation could be shown between release and control orchards at harvest. Although there were significant differences in terms of red scale infestation between release and control orchards in the Mandaryn and Step Aside orchards during the season, the infestation levels were comparable at harvest (Fig. 2.44 and Table 2.34).

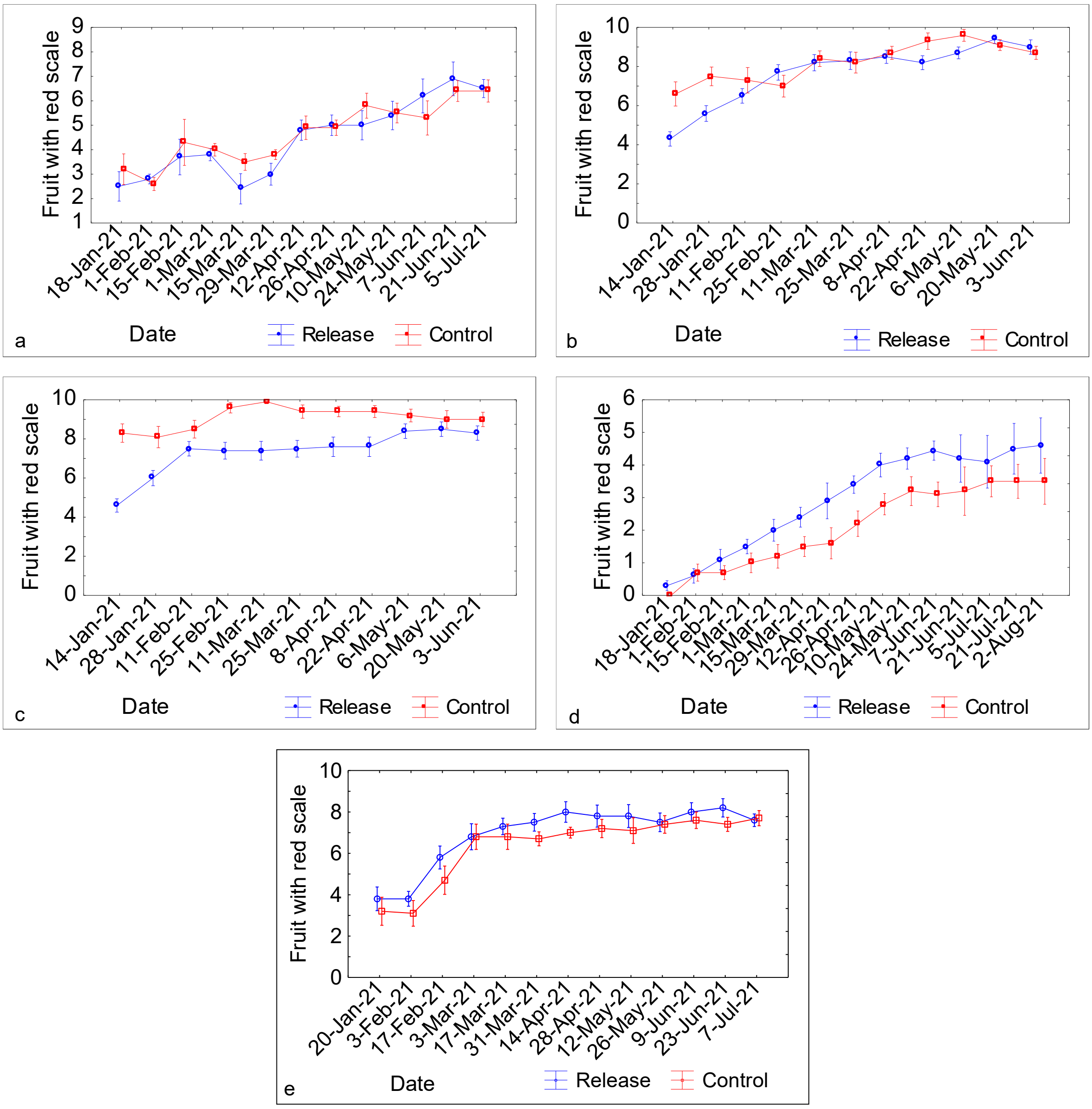


Figure 2.44. Mean number of fruit, out of 10 (10 fruit inspected on each of 10 trees), infested with red scale during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (e). Refer to Table 2.34 for results of statistical analyses. Error bars represent SE.

Table 2.34. Effect of date (assessment) and treatment (release or control) on red scale fruit infestation over time during the 2020/2021 season in release and control orchards at Hexfarm 1350 T and B orchards at Hexfarm 1371 and 1272; Modderfontein 401 and 405; Mandaryn T and B; Step Aside 23 and 32 (Wald test statistic).

Effect	**Df**	**F**	***P***
Hexfarm 1350 T and B			
Date	12, 234	13.846	< 0.001*
Treatment	1, 234	0.975	0.324
Date × Treatment	12, 234	0.625	0.820

Hexfarm 1371 and 1272			
Date	10, 198	17.039	< 0.001*
Treatment	1, 198	9.364	0.002*
Date × Treatment	10, 198	2.589	0.005*
Modderfontein 401 and 405			
Date	10, 198	7.449	< 0.001*
Treatment	1, 198	104.942	< 0.001*
Date × Treatment	10, 198	2.827	0.002*
Mandaryn T and B			
Date	14, 269	17.261	< 0.001*
Treatment	1, 269	24.393	< 0.001*
Date × Treatment	14, 269	0.401	0.974
Step Aside 23 and 32			
Date	12, 234	19.403	< 0.001*
Treatment	1, 234	8.039	0.004*
Date × Treatment	12, 234	0.274	0.992

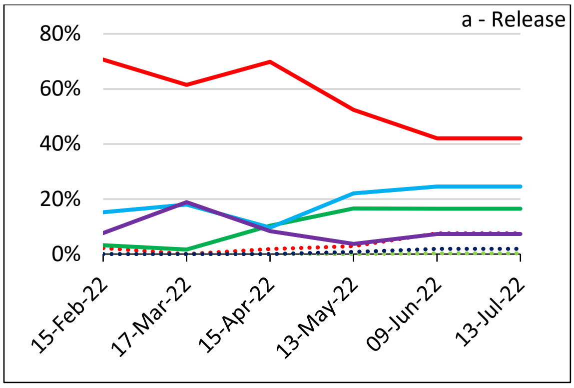
* Denotes significant difference at a 95% confidence level.

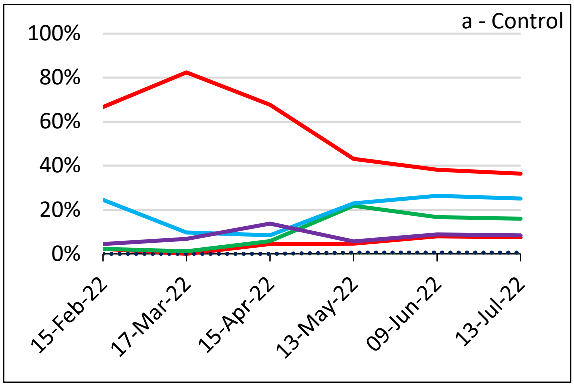
2.3.3. The 2021/2022 season

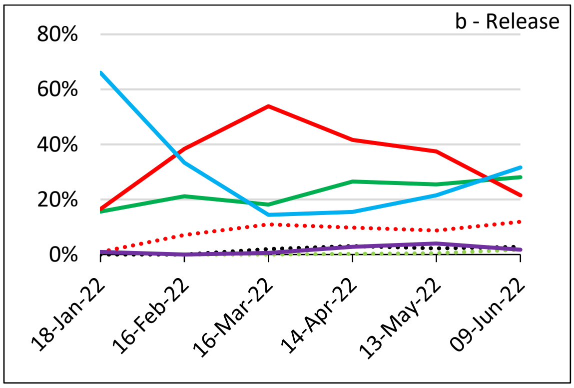
2.3.3.1. Red scale infestation and parasitoid activity trends during the 2020/2021 season in release and control orchards

Healthy red scale numbers increased as parasitism and host mutilation decreased and *vice versa* at all the farms, that is Hexfarm 1350, Hexfarm 1371 and 1272, Modderfontein, Mandaryn, Normandale and Step Aside. *Aphytis africanus* and *C. bifasciata* dominated red scale parasitism and host mutilation amongst the various red scale parasitoids (Fig. 2.45).

Very low red scale numbers infested fruit at the release and control orchards of Mandaryn during the 2021/2022 season. Red scale numbers also declined at the release and control orchards due to unknown reasons (unknown scale death) while it seems that host mutilation still took place (Fig. 2.45 d). Furthermore, the trend between healthy red scales and parasitism and mutilation is negatively correlated, as with the other orchards in the previous two seasons (Figs. 2.45, 2.29 (2020/2021 season), 2.13 (2019/2020 season)).


a - Release
80%
60%
40%
20%
0%
15-Feb-22
17-Mar-22
15-Apr-22
13-May-22
09-Jun-22
13-Jul-22


a - Control
100%
80%
60%
40%
20%
0%
15-Feb-22
17-Mar-22
15-Apr-22
13-May-22
09-Jun-22
13-Jul-22


b - Release
80%
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40%
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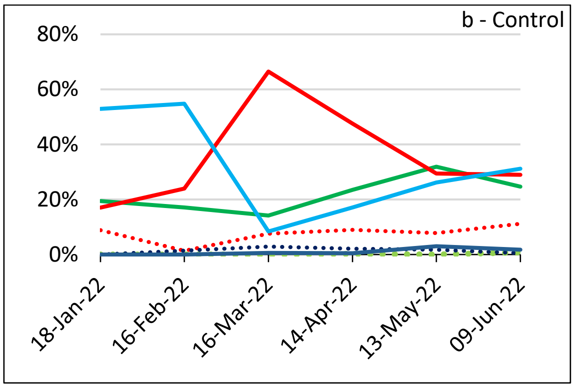
0%
18-Jan-22
16-Feb-22
16-Mar-22
14-Apr-22
13-May-22
09-Jun-22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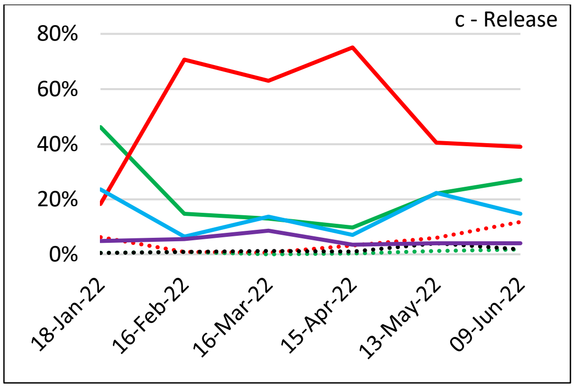
13-May-22
09-Jun-22


c - Release
80%
60%
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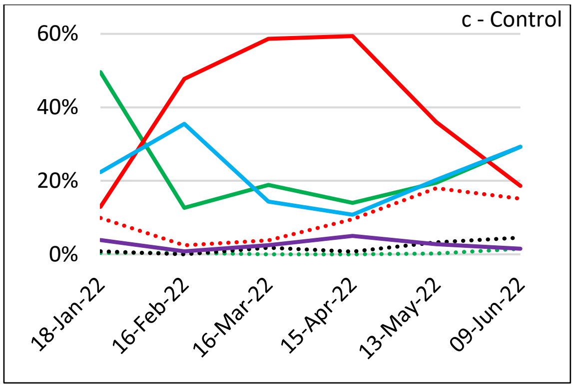
0%
18-Jan-22
16-Feb-22
16-Mar-22
15-Apr-22
13-May-22
09-Jun-22


c - Control
60%
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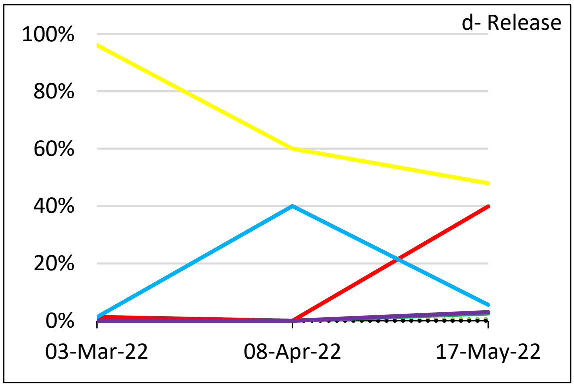
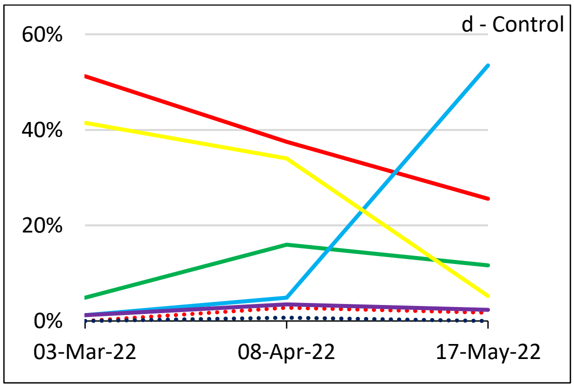
18-Jan-22
16-Feb-22
16-Mar-22
15-Apr-22
13-May-22
09-Jun-22


d- Release
100%
80%
60%
40%
20%
0%
03-Mar-22
08-Apr-22
17-May-22


d - Control
60%
40%
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03-Mar-22
08-Apr-22
17-May-22

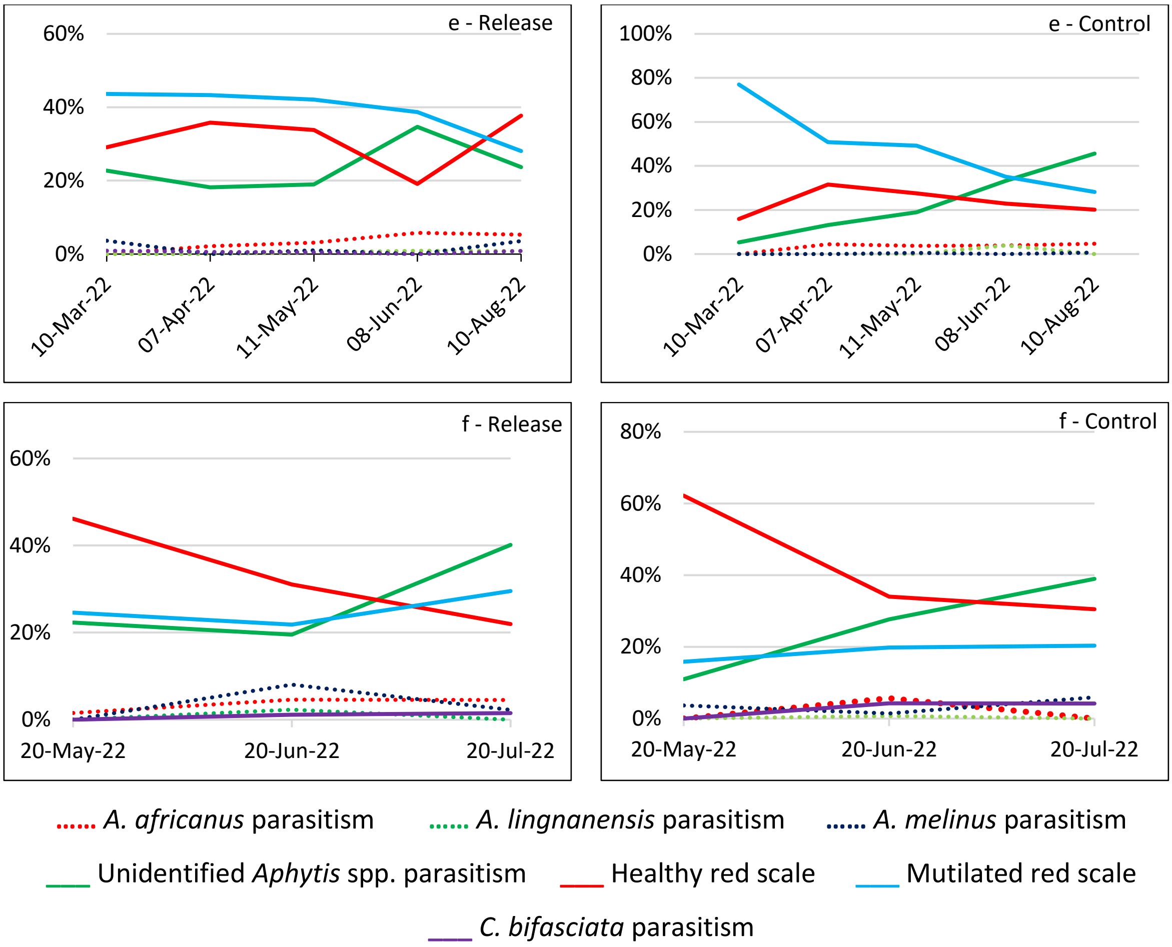


Figure 2.45. Red scale infestation and parasitoid activity trends during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e) and Step Aside 23 and 32 (f).

2.3.3.2. Microscopic assessments of red scales during the 2021/2022 season

During the 2021/22 season, no significant differences in the number of healthy red scale per fruit were recorded between release and control orchards, except for the Mandaryn orchards, where there were fewer healthy red scales at the start of the assessment and more at the end of the assessment period in the release orchard (Fig. 2.46). *Aphytis africanus* had the most significant interactions with red scale parasitism compared to *A. lingnanensis*, *A. melinus* and *C. bifasciata* (Table 2.35).

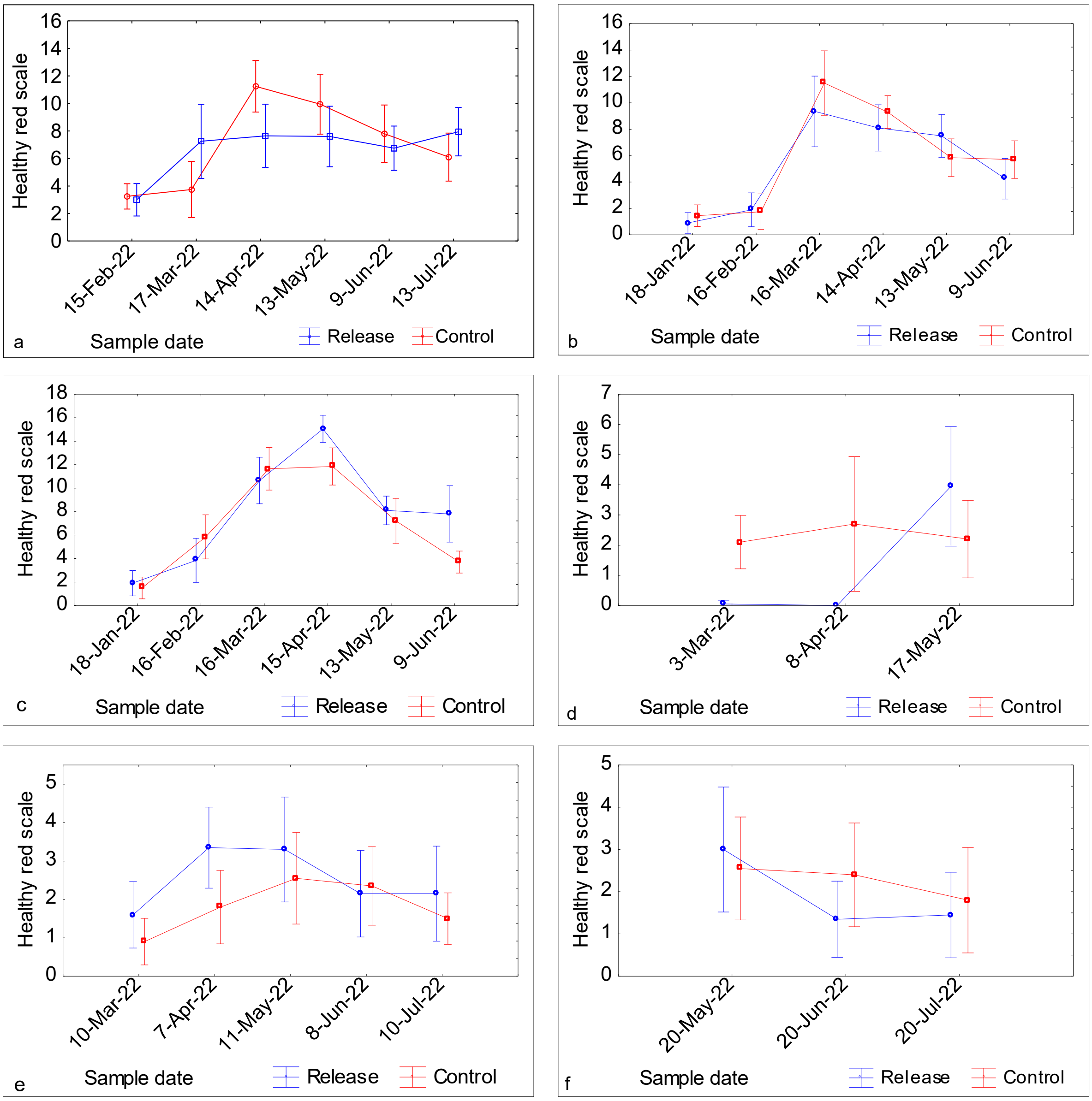


Figure 2.46. The mean number of healthy red scales per fruit during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.35 for results of statistical analyses. Error bars represent SE.

Table 2.35. Results of generalised linear models indicating significant interactions between parasitism and healthy red scales during the 2021/2022 season (Wald test statistic).

Orchard	*A. africanus*	*A. lingnanensis*	*A. melinus*	*C. bifasciata*
Hexfarm 1350 T and B	9.529*	3.021	0.085	3.025
Hexfarm 1371 and 1272	1.525	2.035	3.099	1.068
Modderfontein 401 and 405	53.431*	1.874	9.189*	0.027
Mandaryn T and B	10.761*	32.881*	0.000	-

Normandale 33 and Step Aside 26	9.563*	9.494*	16.531*	0.062
Step Aside 23 and 32	8.116*	0.012	8.488*	13.484*

* Denotes a significant interaction at a 95% confidence level.

There were no significant differences between release and control orchards for red scale mutilation during the 2021/2022 season (Fig. 2.47). *Aphytis africanus* yielded the most interactions of significance with red scale mutilation, when compared to the other parasitoid species (Table 2.36).

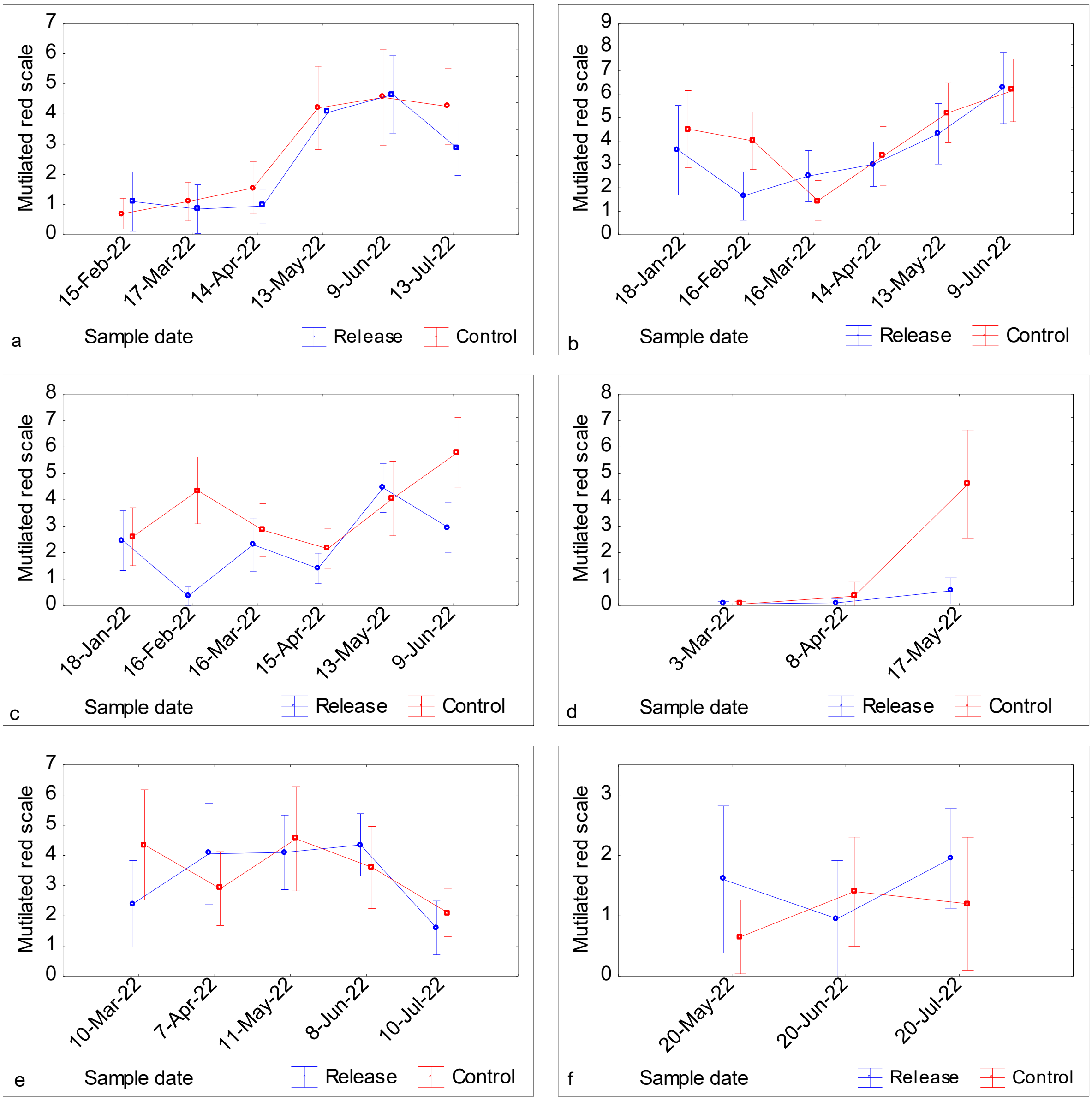


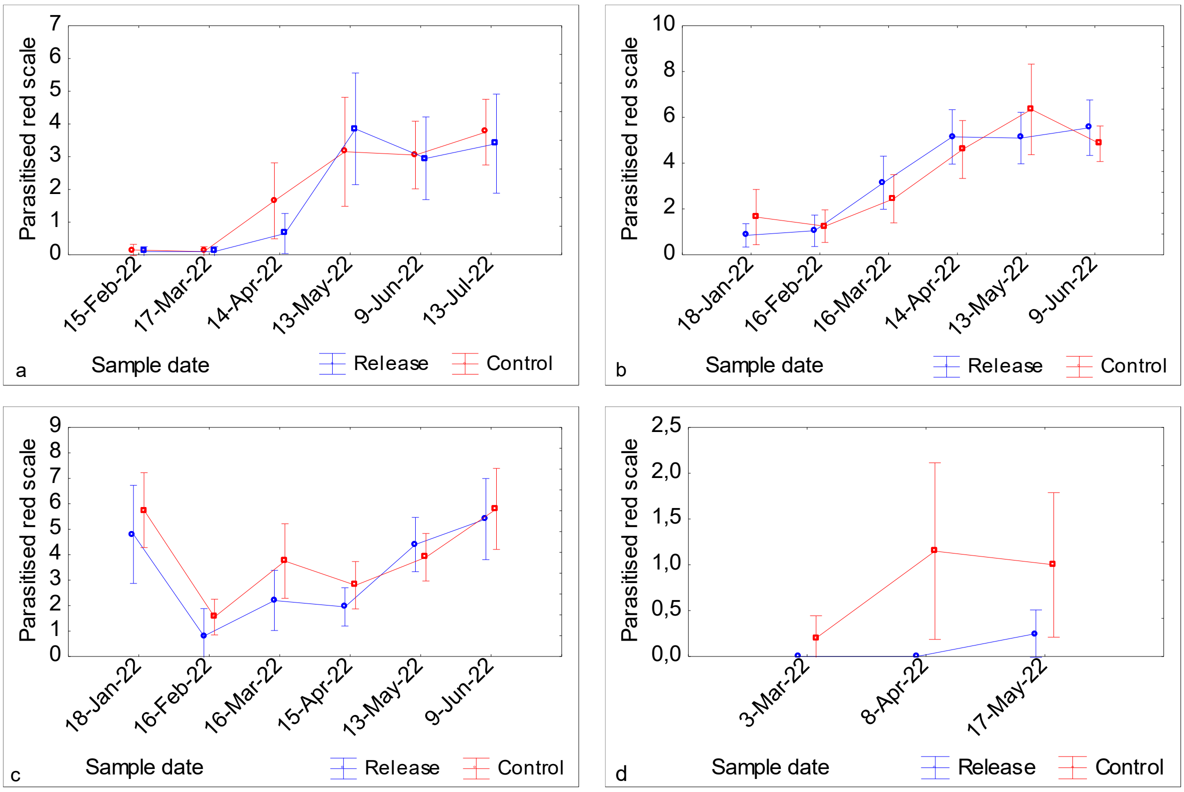
Figure 2.47. The mean number of mutilated red scales per fruit during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.36 for results of statistical analyses. Error bars represent SE.

Table 2.36. Results of generalised linear models indicating significant interactions between parasitism and mutilated red scales during the 2021/2022 season (Wald test statistic).

Orchard	*A. africanus*	*A. lingnanensis*	*A. melinus*	*C. bifasciata*
Hexfarm 1350 T and B	0.429	3.292	2.031	2.175
Hexfarm 1371 and 1272	0.052	1.163	3.386	1.171
Modderfontein 401 and 405	0.447	0.075	0.007	7.046*
Mandaryn T and B	12.352*	0.000	0.000	1.442
Normandale 33 and Step Aside 26	18.640*	0.034	0.638	0.947
Step Aside 23 and 32	8.397*	14.067*	29.106*	0.068

* Denotes a significant interaction at a 95% confidence level.

The number of red scales parasitised by unidentified *Aphytis* spp. did not differ significantly between the release and control orchards during the 2021/2022 season (Fig. 2.48). *Aphytis africanus* had more interactions of significance with red scale parasitism related to unidentified *Aphytis* spp. than did *A. lingnanensis* and *A. melinus* (Table 2.37).



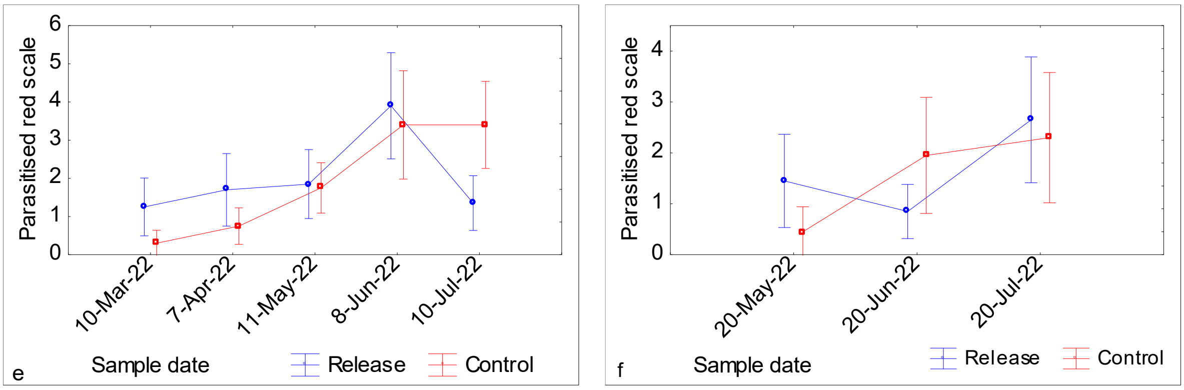


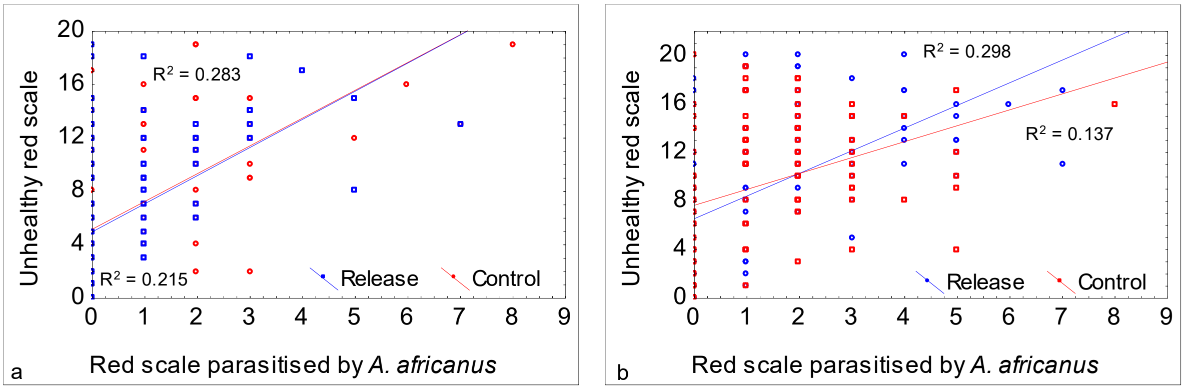
Figure 2.48. The mean number of red scales per fruit parasitised by unidentified *Aphytis* spp. during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.36 for results of statistical analyses. Error bars represent SE.

Table 2.37. Results of generalised linear models indicating significant interactions between parasitism of red scales by known *Aphytis* spp. and unidentified *Aphytis* spp. with red scales during the 2021/2022 season (Wald test statistic).

Orchard	***A. africanus***	***A. lingnanensis***	***A. melinus***
Hexfarm 1350 T and B	3.0267	0.204	0.076
Hexfarm 1371 and 1272	0.456	0.995	0.520
Modderfontein 401 and 405	1.930	0.077	0.083
Mandaryn T and B	0.103	0.557	0.000
Normandale 33 and Step Aside 26	20.215*	1.818	0.102
Step Aside 23 and 32	11.886*	34.184*	18.922*

* Denotes a significant interaction at a 95% confidence level.

During the 2021/2022 season, red scale parasitism by *A. africanus* correlated the strongest with the presence of unhealthy red scales, without any significant differences between release and control orchards (Figs. 2.49, 2.50, 2.51 and 2.52 and Table 2.38).



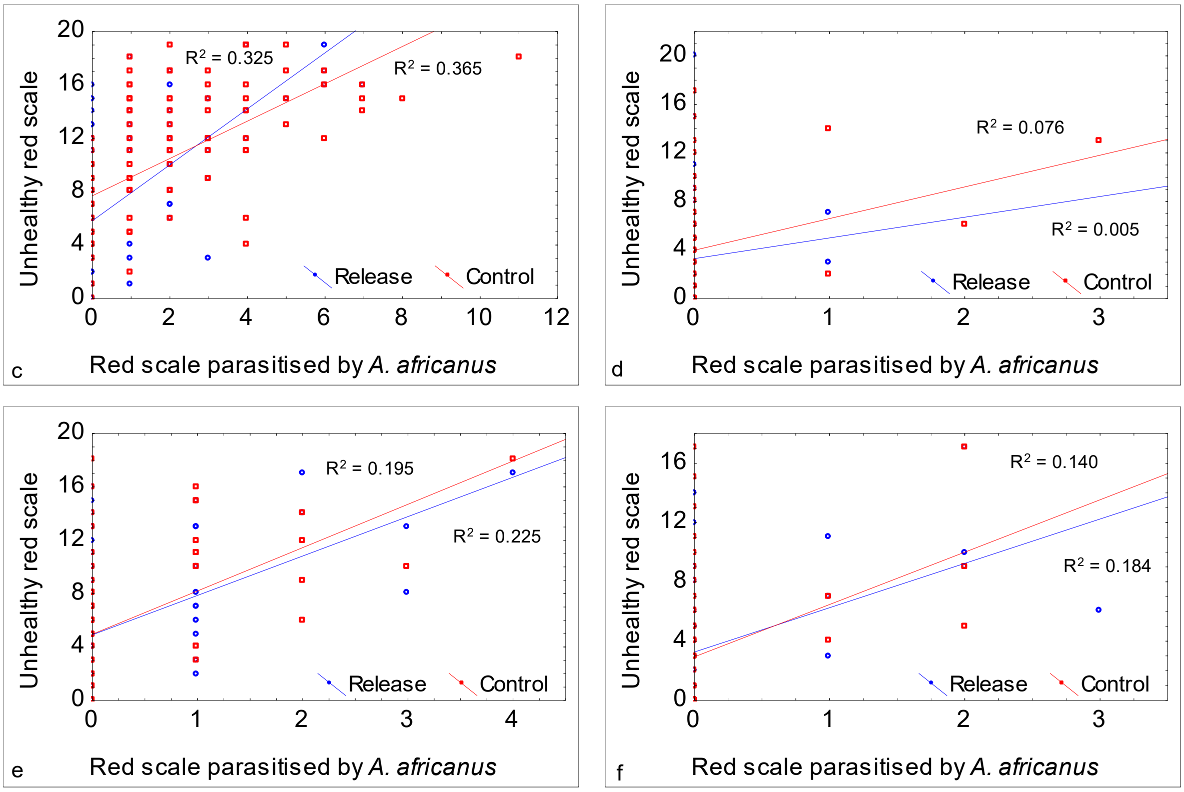
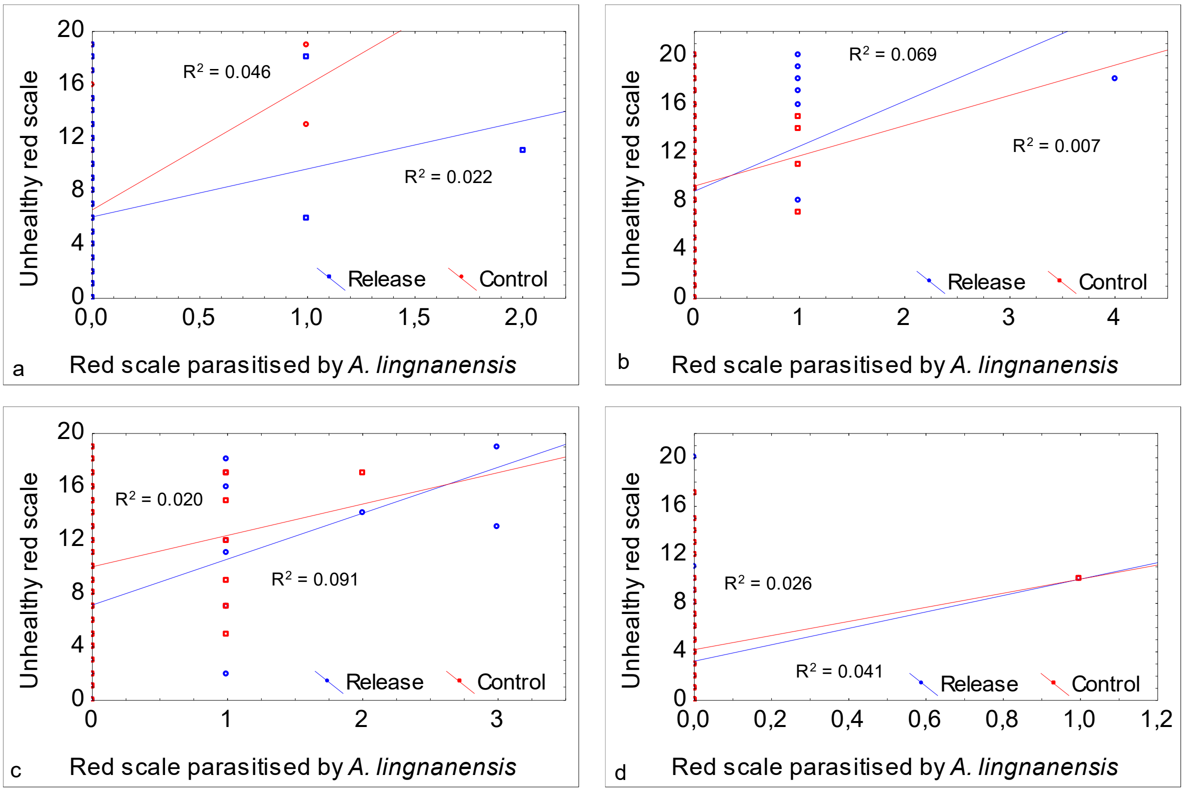


Figure 2.49. Number of unhealthy red scales per fruit correlated with red scales parasitised by *A. africanus* during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1731 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.38 for results of statistical analyses.



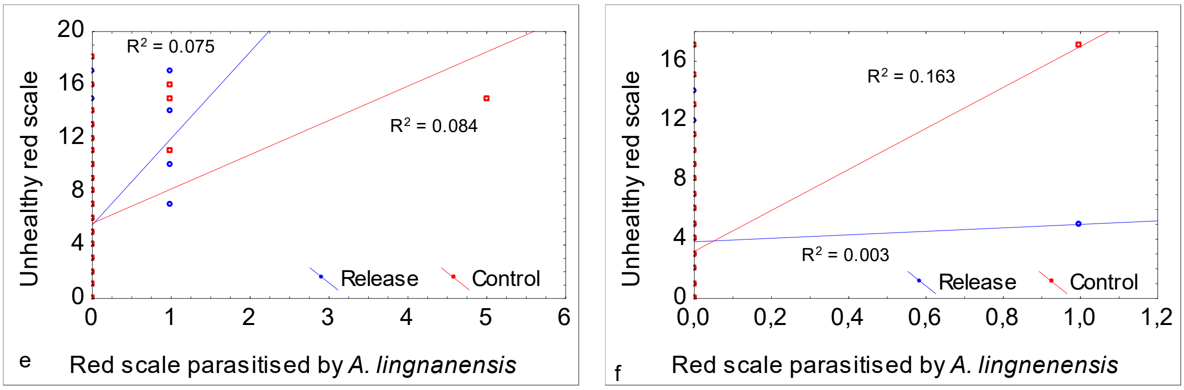
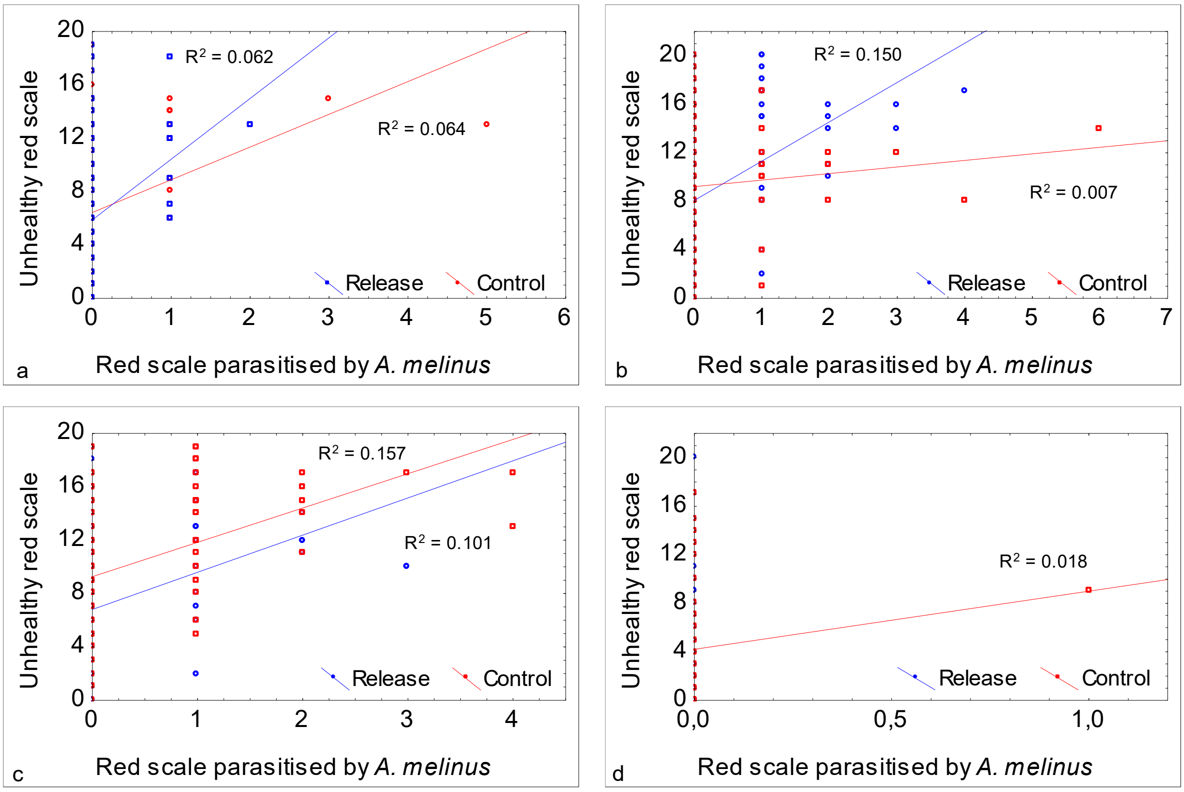


Figure 2.50. Number of unhealthy red scales per fruit correlated with red scales parasitised by *A. lingnanensis* during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1731 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.38 for results of statistical analyses.



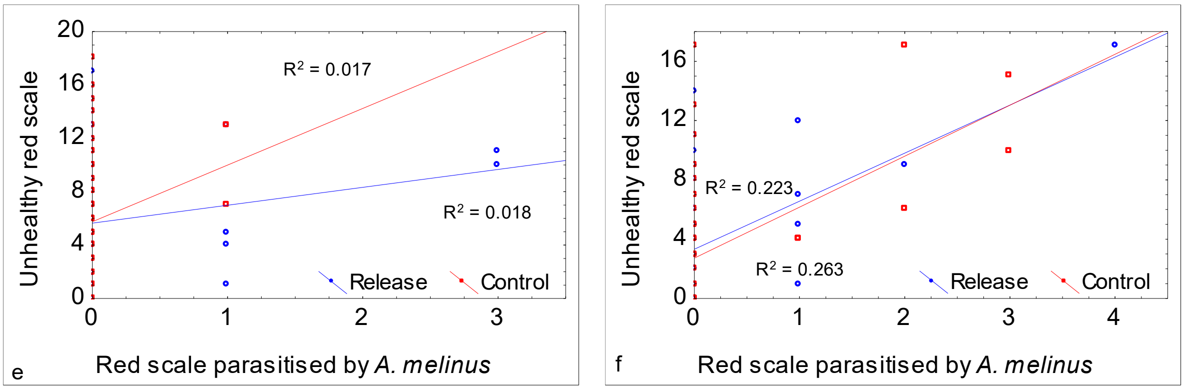
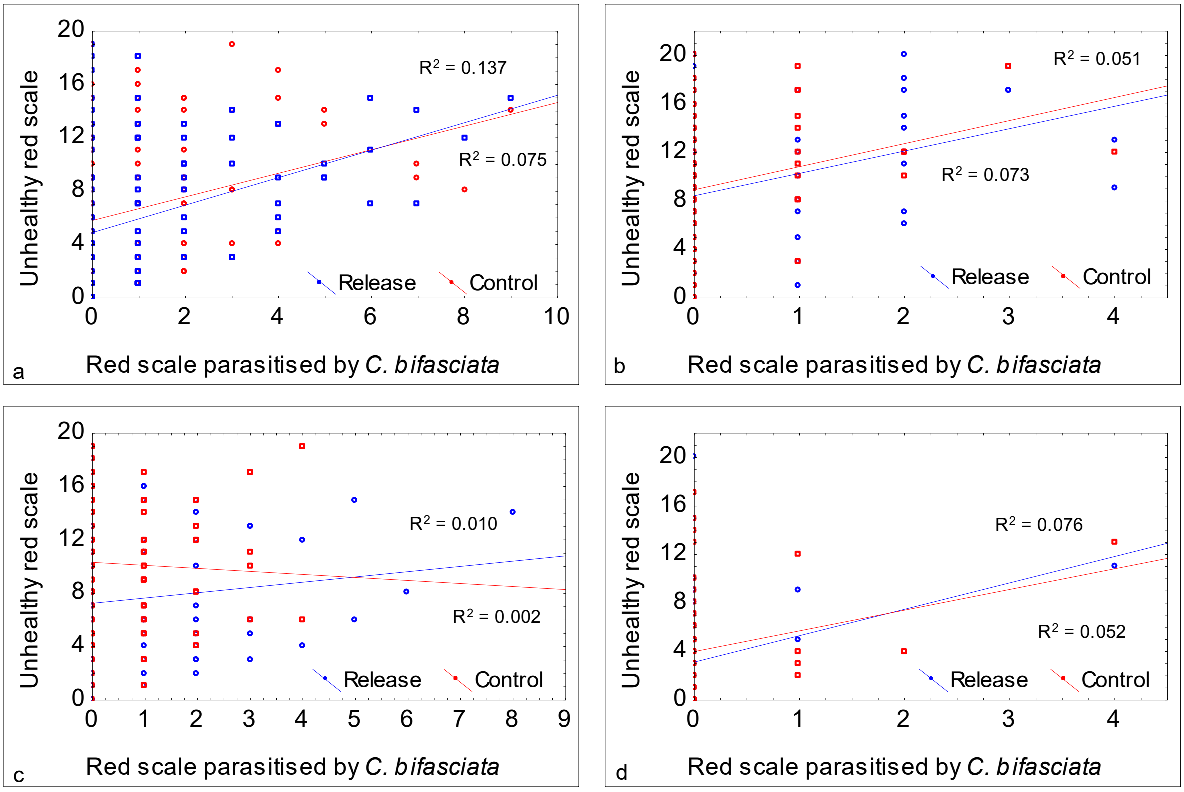


Figure 2.51. Number of unhealthy red scales per fruit correlated with red scales parasitised by *A. melinus* during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1731 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.38 for results of statistical analyses.



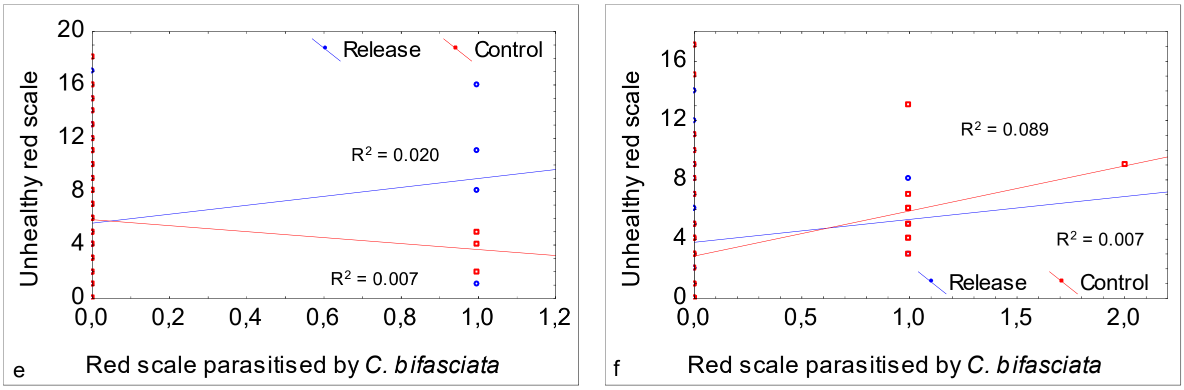


Figure 2.52. Number of unhealthy red scales per fruit correlated with red scales parasitised by *C. bifasciata* during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1731 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.38 for Results of statistical analyses.

Table 2.38. Univariate test of significance of the regressions between the number of unhealthy red scales per fruit and red scales parasitised by the various red scale parasitoid species during the 2021/2022 season.

Orchards	***A. africanus***	***A. lingnanensis***	***A. melinus***	***C. bifasciata***
Hexfarm 1350 T and B	F = 76.039 *P* = < 0.001*	F = 8.346 *P* = 0.004*	F = 14.023 *P* = < 0.001*	F = 0.334 *P* = 0.563
Hexfarm 1371 and 1272	F = 62.202 *P* = < 0.001*	F = 4.115 *P* = 0.043*	F = 16.497 *P* = < 0.001* Treatment × *A. melinus*: F = 8.409 *P* = 0.004*	F = 14.350 *P* = < 0.001*
Modderfontein 401 and 405	F = 120.449 *P* = < 0.001* Treatment: F = 4.797 *P* = 0.029*	F = 9.890 *P* = 0.002*	F = 33.301 *P* = < 0.001*	F = 0.072 *P* = 0.788
Mandaryn T and B	F = 1.634 *P* = 0.203	F = 3.967 *P* = 0.048*	-	F = 7.777 *P* = 0.006*
Normandale 33 and Step Aside 26	F = 52.084 *P* = < 0.001*	F = 13.627 *P* = < 0.001*	F = 2.591 *P* = 0.109	F = 0.094 *P* = 0.758
Step Aside 23 and 32	F = 21.60 *P* = < 0.001*	F = 8.442 *P* = 0.004*	F = 37.045 *P* = < 0.001*	F = 2.637 *P* = 0.107

		Treatment × *A. lingnanensis*: F = 6.007 *P* = 0.105		

* Result of ANCOVA indicating significant differences between the regression slopes at a 95% confidence level.

The number of unhealthy red scales correlated strongly with parasitism by all *Aphytis* spp., suggesting that *Aphytis* spp. is largely responsible for the number of unhealthy red scales (Fig. 2.53 and Table 2.39).

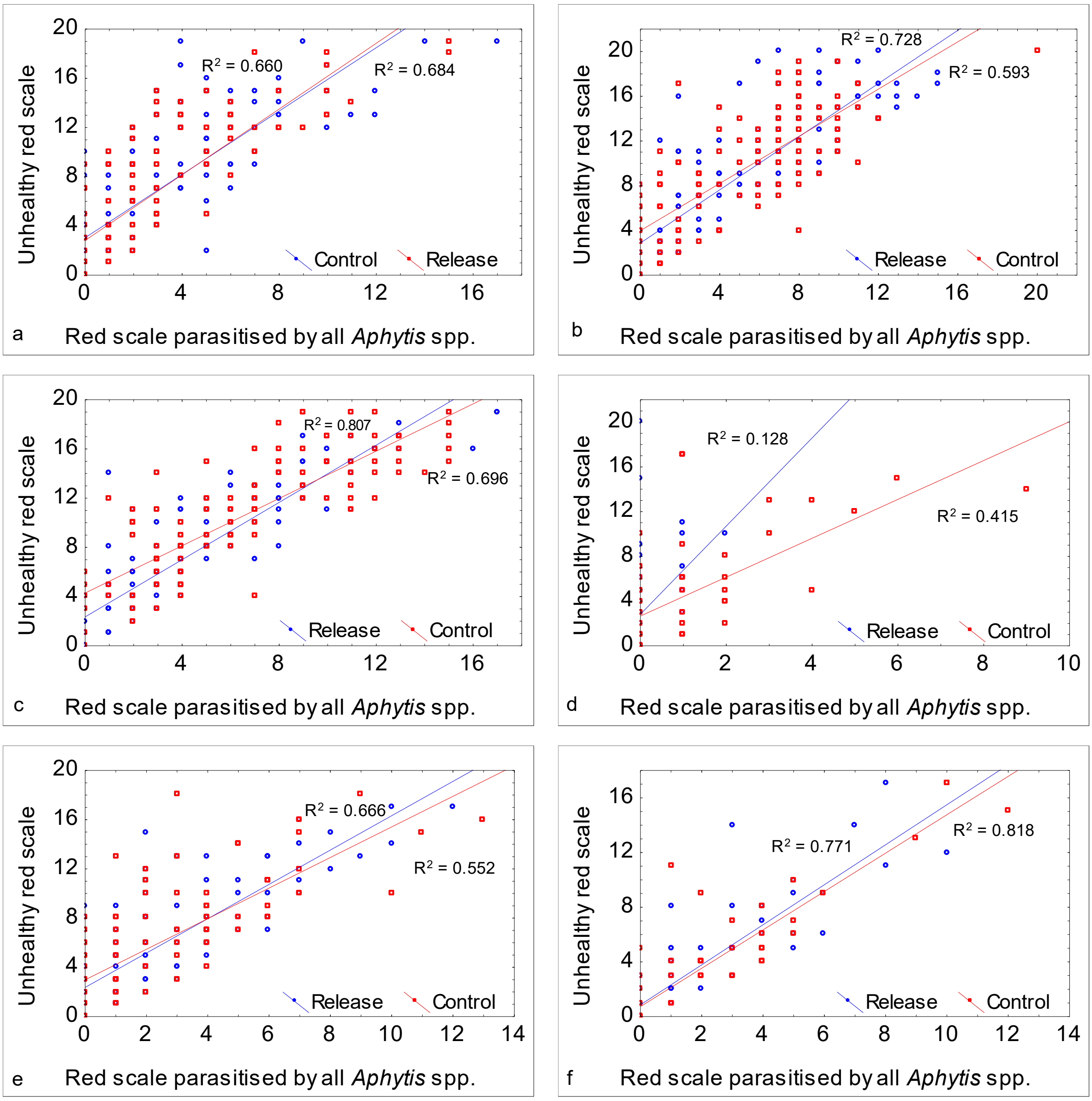


Figure 2.53. Number of unhealthy red scales per fruit correlated with red scales parasitised by all *Aphytis* spp. during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1731 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.39 for results of statistical analyses.

Table 2.39. Univariate test of significance of the regressions between the number of unhealthy red scales per fruit and red scales parasitised by all *Aphytis* spp. during the 2021/2022 season.

Orchard	**All *Aphytis* spp.**
Hexfarm 1350 T and B	F = 480.154 *P* = < 0.001*
Hexfarm 1371 and 1272	F = 452.214 *P* = < 0.001*
Modderfontein 401 and 405	F = 730.822 *P* = < 0.001* Treatment: F = 13.092 *P* = < 0.001* Treatment × All *Aphytis* spp: F = 6.626 *P* = 0.011*
Mandaryn T and B	F = 18.836 *P* = < 0.001*
Normandale 33 and Step Aside 26	F = 305.313 *P* = < 0.001*
Step Aside 23 and 32	F = 466.434 *P* = < 0.001*

* Result of ANCOVA indicating significant differences between the regression slopes at a 95% confidence level.

Parasitism by *A. africanus* showed the strongest correlation to red scale mutilation in all orchards, while there were no significant differences between release and control orchards during the 2021/2022 season (Figs. 2.54, 2.55, 2.56 and 2.57 and Table 2.40).

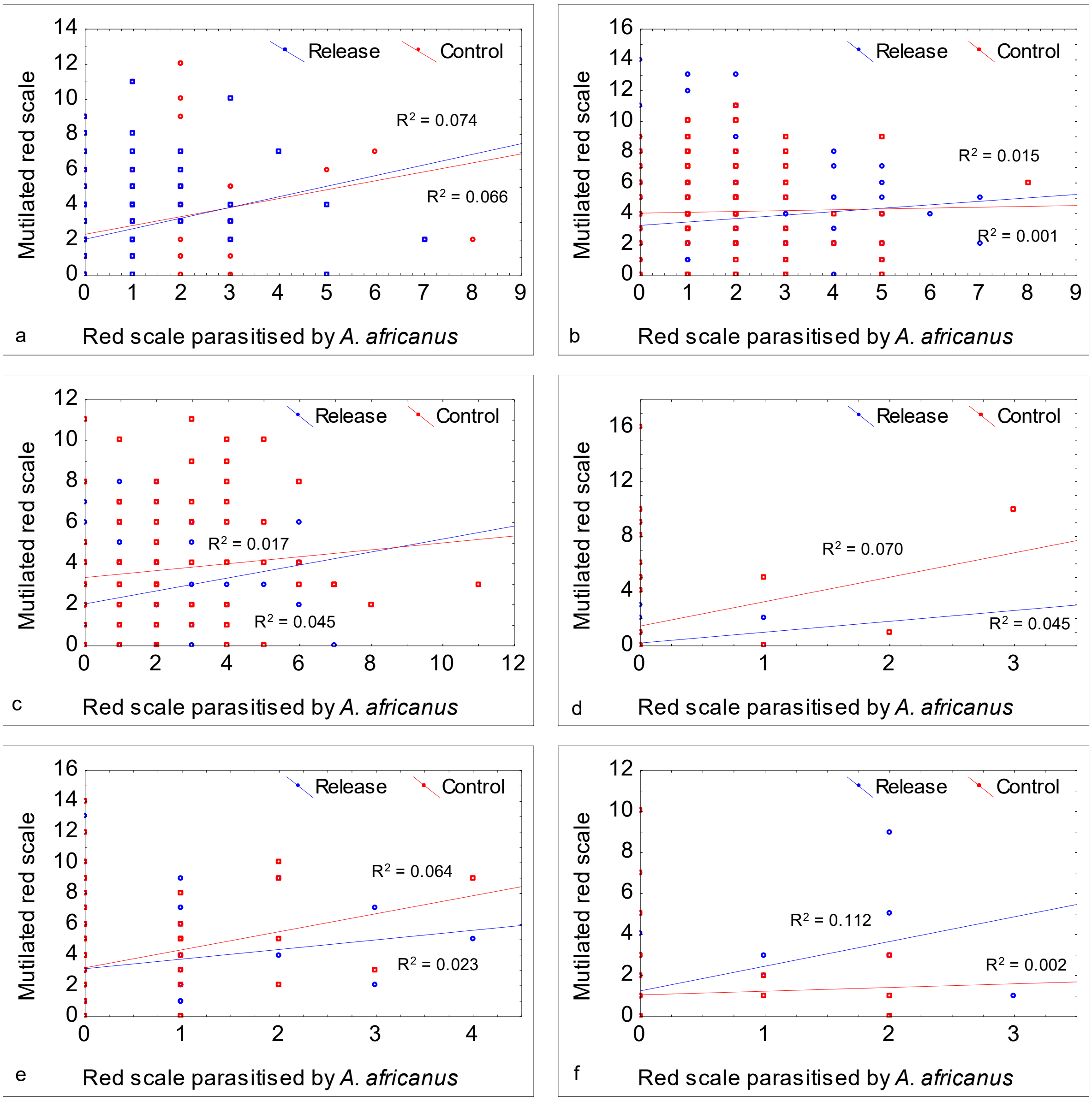
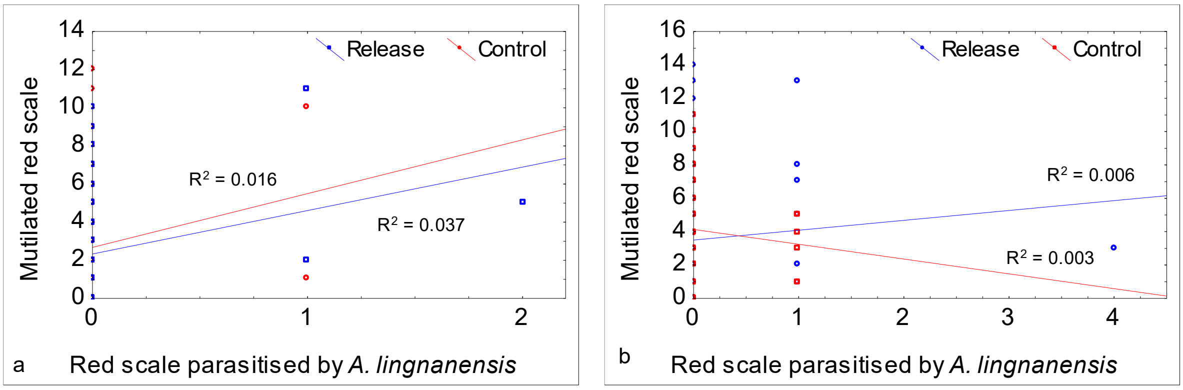


Figure 2.54. Number of mutilated red scales per fruit correlated with red scales parasitised by *A. africanus* during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1731 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.40 for results of statistical analyses.



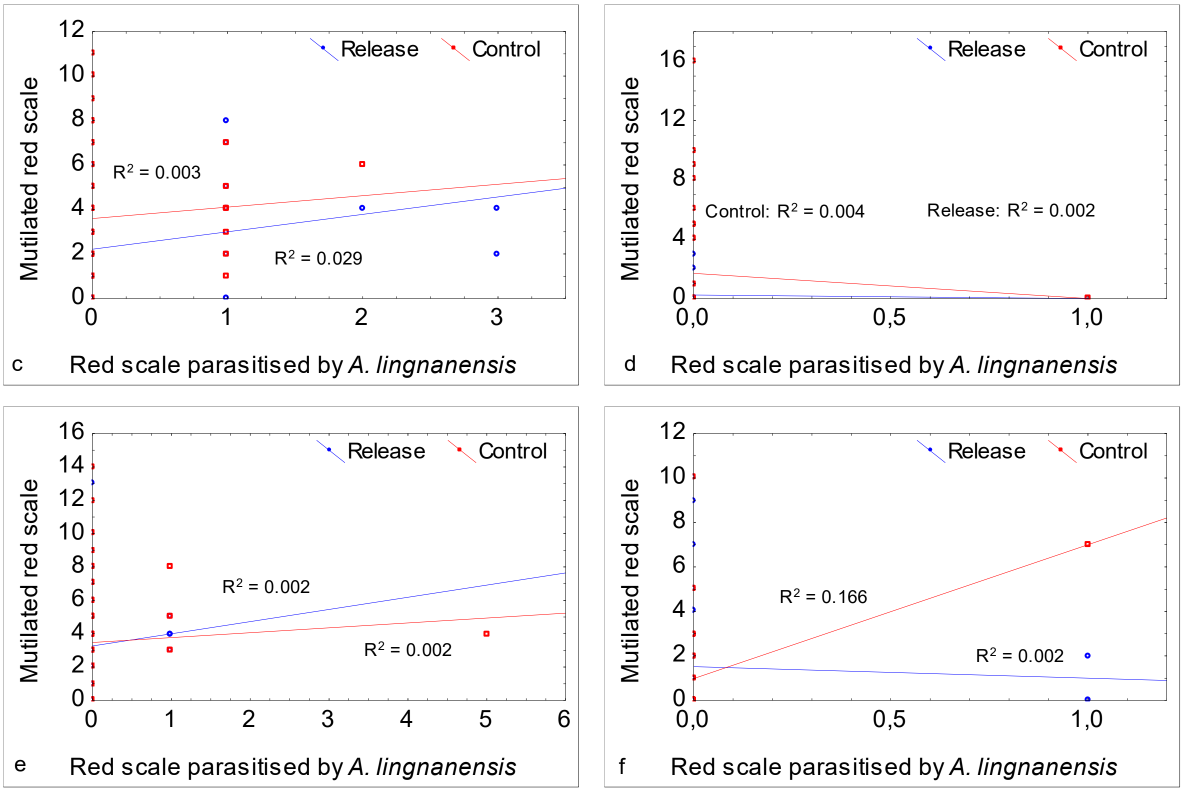
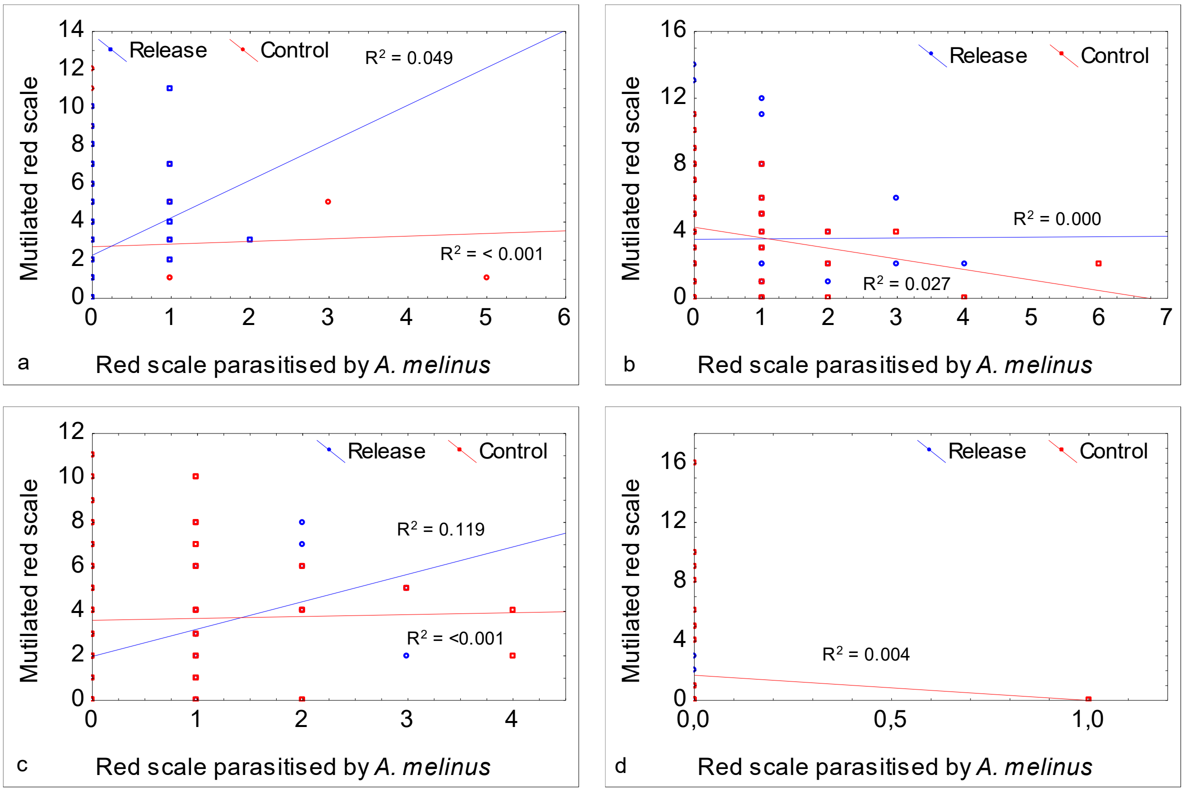


Figure 2.55. Number of mutilated red scales per fruit correlated with red scales parasitised by *A. lingnanensis* during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1731 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.40 for results of statistical analyses.



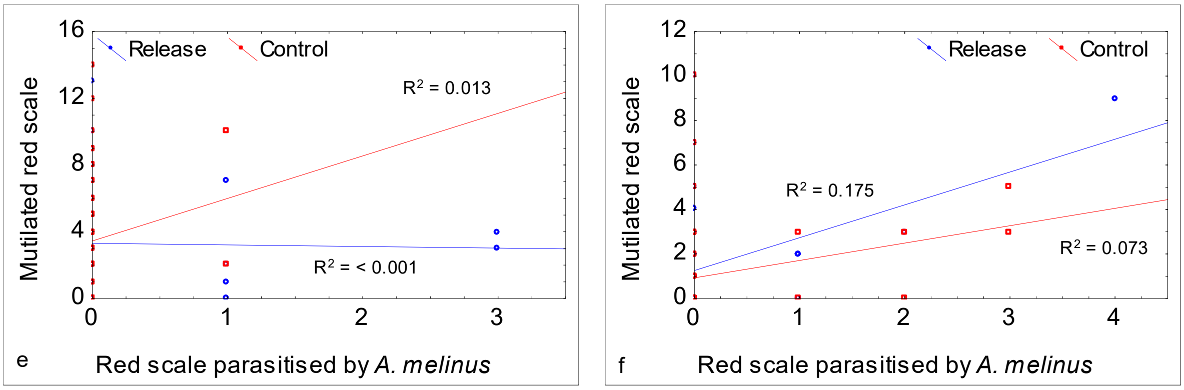


Figure 2.56. Number of mutilated red scales per fruit correlated with red scales parasitised by *A. melinus* during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1731 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.40 for results of statistical analyses.

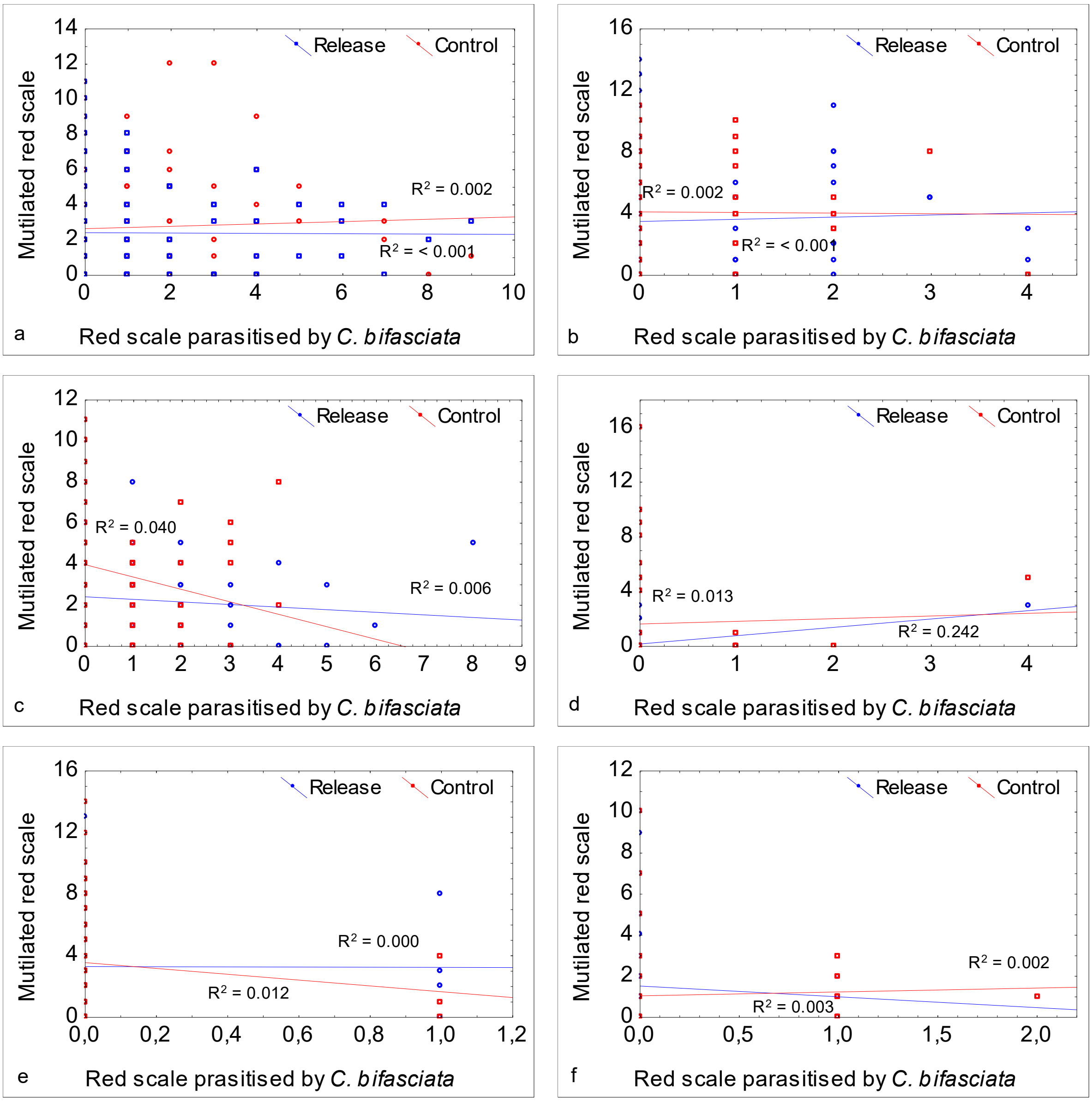


Figure 2.57. Number of mutilated per fruit red scales correlated with red scales parasitised by *C. bifasciata* during 2021/2022 season at Hexfarm 1350 T and B (a); Hexfarm 1731 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.40 for results of statistical analyses.

Table 2.40. Univariate test of significance of the regressions between the number of mutilated red scales per fruit and red scales parasitised by the various red scale parasitoid species during the 2021/2022 season.

Orchards	***A. africanus***	***A. lingnanensis***	***A. melinus***	***C. bifasciata***
Hexfarm 1350 T and B	F = 17.474 *P* = < 0001*	F = 5.178 *P* = 0.024*	F = 4.928 *P* = 0.0273*	-
Hexfarm 1371 and 1272	-	-	-	-
Modderfontein 401 and 405	F = 6.769 *P* = 0.010* Treatment: F = 10.654 *P* = 0.001*	Treatment: F = 17.298 *P* = < 0.001*	F = 7.982 *P* = 0.005* Treatment: F = 21.988 *P* = < 0.001* Treatment × *A. melinus*: F = 6.047 *P* = 0.015*	F = 6.007 *P* = 0.015* Treatment: F = 17.564 *P* = < 0.001*
Mandaryn T and B	Treatment: F = 8.301 *P* = 0.005*	Treatment: F = 10.832 *P* = 0.001*	Treatment: F = 11.077 *P* = 0.001*	Treatment: F = 10.578 *P* = 0.001*
Normandale 33 and Step Aside 26	F = 8.580 *P* = 0.004*	-	-	-
Step Aside 23 and 32	-	F = 5.000 *P* = 0.027* Treatment × *A. lingnanensis*: F = 7.056 *P* = 0.009*	F = 16.449 *P* = < 0.001*	-

* Result of ANCOVA indicating significant differences between the regression slopes at a 95% confidence level.

A significant correlation exists between the number of mutilated red scales per fruit and red scales parasitised by all the *Aphytis* spp. assessed in all release and control orchards (Fig. 2.58 and Table 2.41)

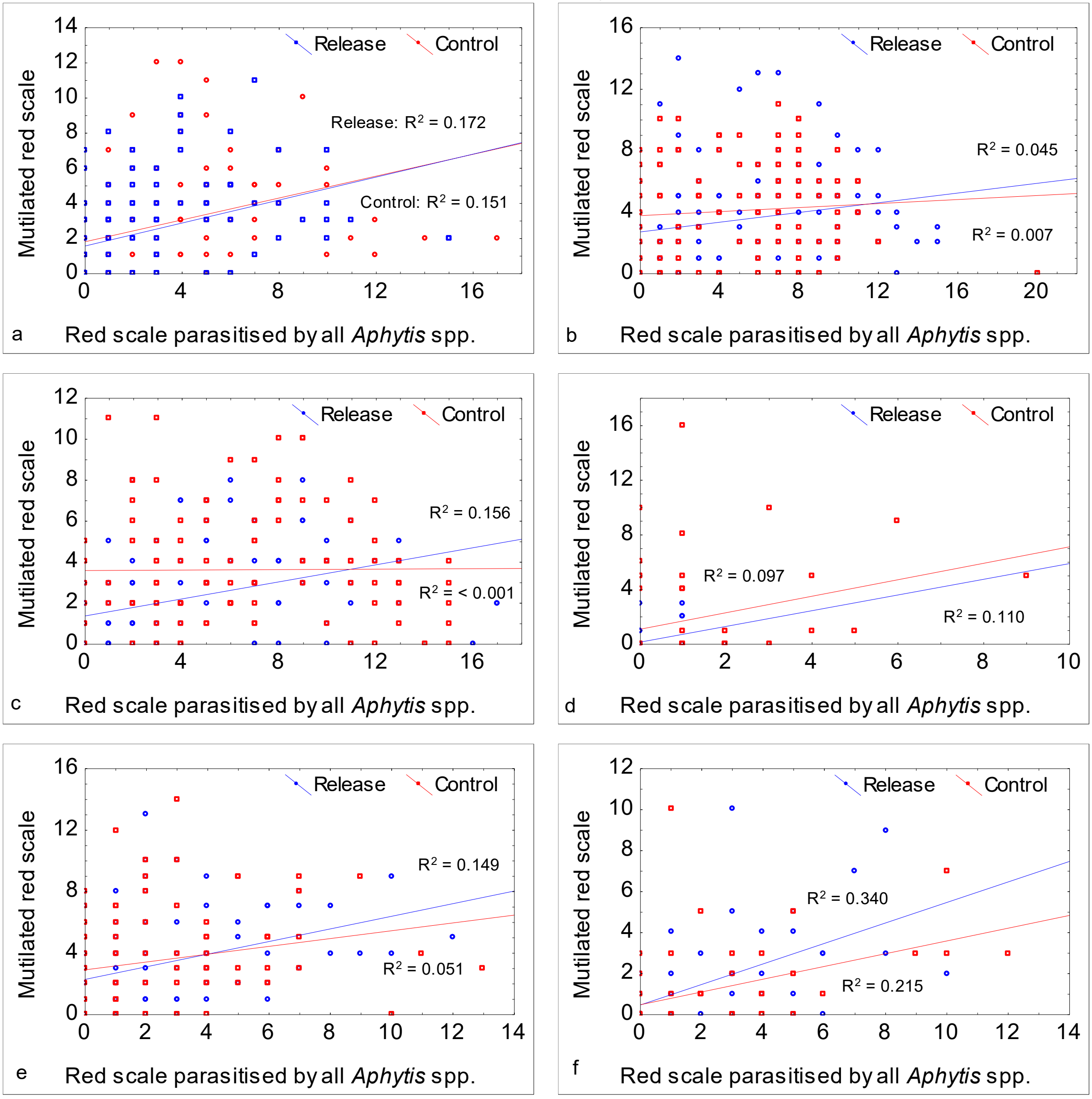


Figure 2.58. Number of mutilated red scales per fruit correlated with red scales parasitised by all *Aphytis* spp. during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Normandale 33 and 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.40 for results of statistical analyses.

Table 2.41. Univariate test of significance of the regressions between the number of mutilated red scales per fruit and red scales parasitised by all *Aphytis* spp. during the 2021/2022 season.

Orchard	**All *Aphytis* spp.**
Hexfarm 1350 T and B	F = 45.089; *P* = < 0.001*
Hexfarm 1371 and 1272	F = 5.045; *P* = < 0.026*
Modderfontein 401 and 405	F = 8.271; *P* = 0.004* Treatment: F = 19.113; *P* = < 0.001* Treatment × All *Aphytis* spp.: F = 7.410; *P* = 0.007*
Mandaryn T and B	- Treatment: F = 4.247; *P* = 0.042*
Normandale 33 and Step Aside 26	F = 19.907; *P* = < 0.001*
Step Aside 23 and 32	F = 45.743; *P* = < 0.001*

* Result of ANCOVA indicating significant differences between the regression slopes at a 95% confidence level.

2.3.3.3. Adult *Aphytis* spp. counts in traps during the 2021/2022 season

No significant differences were recorded in the number of adult *Aphytis* spp. caught in traps between any pairs of release and control orchards during the 2021/2022 season (Fig. 2.59).

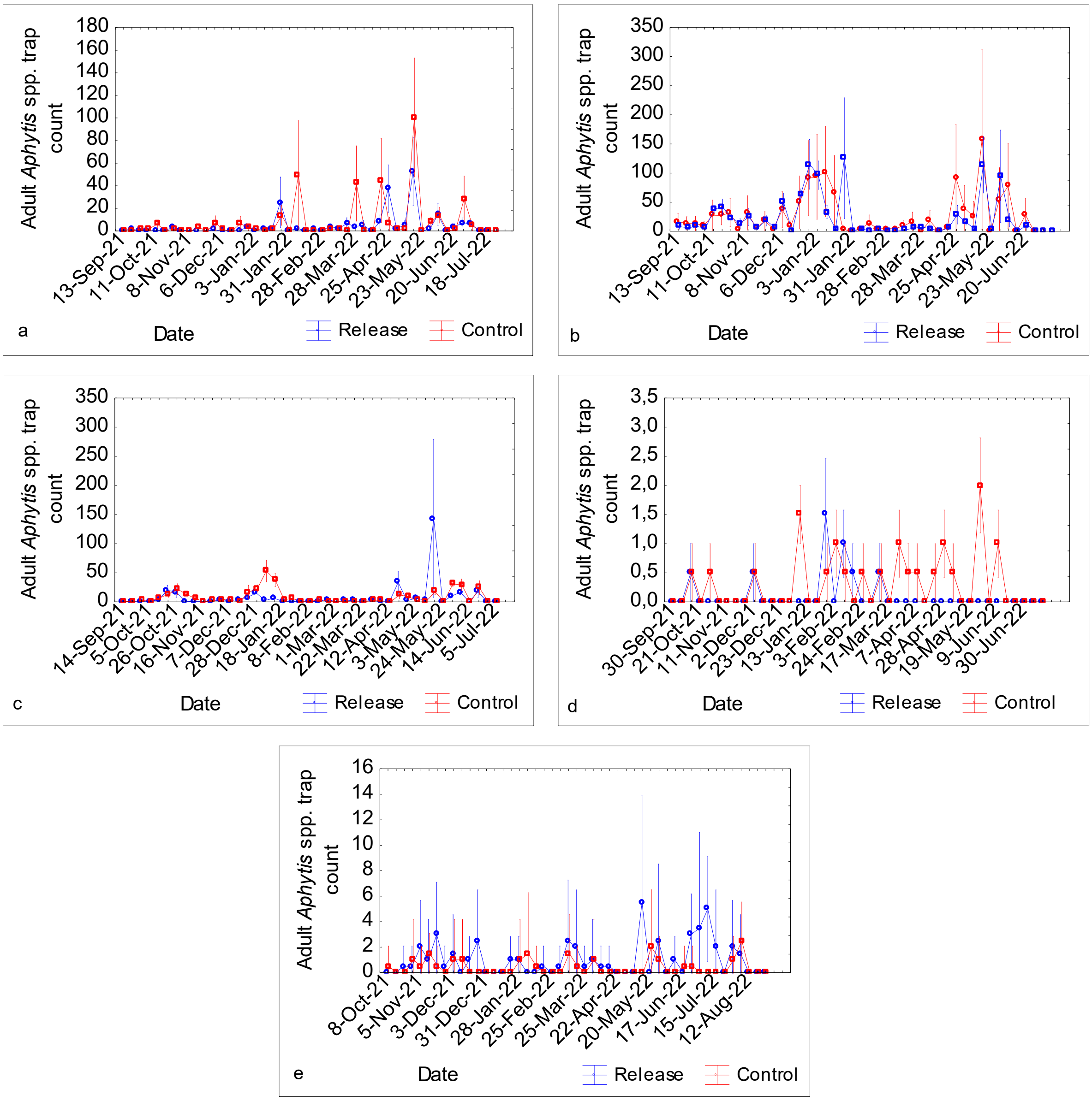


Figure 2.59. Mean counts of *Aphytis* spp. adult numbers during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and 1350 B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405 (c); Mandaryn T and B (d); Step Aside 23 and 32 (f). Error bars represent SE.

2.3.3.4. Fruit infestation during the 2021/2022 season

Red scale fruit infestation increased significantly over time through the season in all orchards (Effect of Date, Fig. 2.60, and Table 2.42). There was a significant difference in red scale infestation between the release and control orchards at Hexfarm 1371 and 1272 as well as Mandaryn T and B, but the release orchards had a lower red scale infestation at the start of the assessment periods. Although there were significant differences in red scale infestation between the release and control orchards at Normandale 33 and Step Aside 26, and Step Aside 32 and 23, it was only during parts of the season, and the red scale infestation was the same for the release and control orchards at harvest (Fig. 2.60 and Table 2.42).

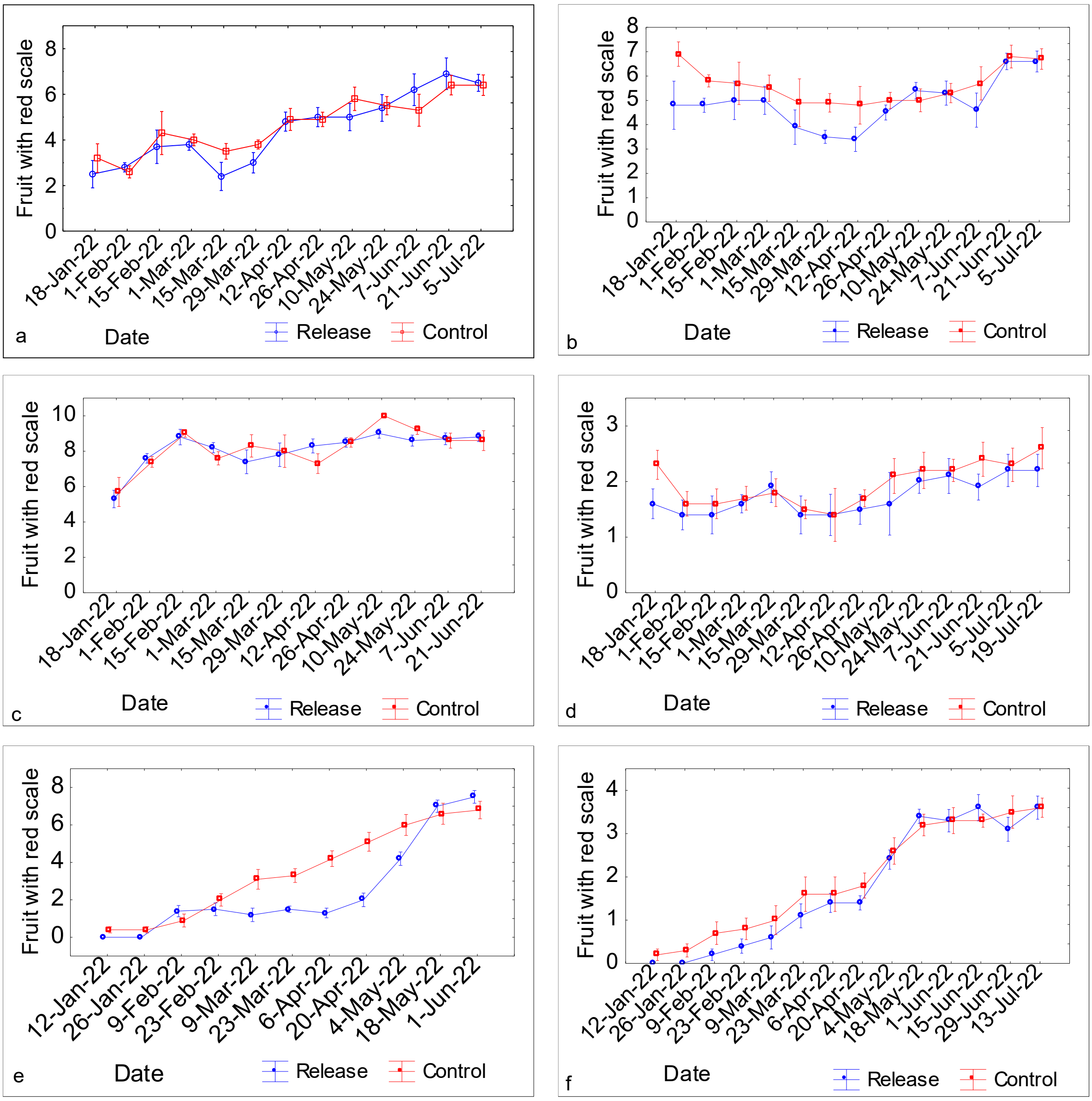


Figure 2.60. Mean number of fruit, out of 10 (10 fruit inspected on each of 10 trees), infested with red scale during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B (a); Hexfarm 1371 and 1272 (b); Modderfontein 401 and 405(c); Mandaryn T and B (d); Normandale 33 and Step Aside 26 (e); Step Aside 23 and 32 (f). Refer to Table 2.43 for results of statistical analyses. Error bars represent SE.

Table 2.42. Effect of date (assessment) and treatment (release or control) on red scale fruit infestation over time during the 2021/2022 season in release and control orchards at Hexfarm 1350 T and B orchards at Hexfarm 1371 and 1272; Modderfontein 401 and 405; Mandaryn T and B; Normandale 33 and Step Aside 26; Step Aside 23 and 32 (Wald test statistic).

Effect	**Df**	**F**	***P***
Hexfarm 1350 T and B			
Date	12, 234	13.846	< 0.001*
Treatment	1, 234	0.975	0.324
Date × Treatment	12, 234	0.625	0.820
Hexfarm 1371 and 1272			
Date	12, 234	3.997	< 0.001*
Treatment	1, 234	10.834	0.001*
Date × Treatment	12, 234	0.720	0.731
Modderfontein 401 and 405			
Date	11, 216	9.601	< 0.001*
Treatment	1, 216	0.278	0.598
Date × Treatment	11, 216	0.787	0.652
Mandaryn T and B			
Date	13, 252	2.445	0.003*
Treatment	1, 252	4.081	0.044*
Date × Treatment	13, 252	0.270	0.995
Normandale 33 and Step Aside 26			
Date	10, 198	88.479	< 0.001*
Treatment	1, 198	43.355	< 0.001*
Date × Treatment	10, 198	6.910	< 0.001*
Step Aside 23 and 32			
Date	13, 252	56.053	< 0.001*
Treatment	1, 252	5.012	0.026*
Date × Treatment	13, 252	0.502	0.922

* Denotes a significant interaction at a 95% confidence level.

2.4. Summary of results

No difference in red scale mortality, either by parasitism or host mutilation or both, above that of the untreated control orchards could be achieved in any of the 18 orchards where commercially available *A. melinus* were released in numbers of more than 200 000/ ha during three consecutive seasons (Table 2.43). However, in most orchards, red scale parasitism and mutilation by means of host feeding by *Aphytis* spp. were very effective in reducing red scale infestation. The indigenous, naturally occurring *A. africanus* contributed the most to red scale control.

Table 2.43. Summarised results of all three seasons.

Industry relevant remarks	**Season**		
	2019/2020	**2020/2021**	**2021/2022**
Number of trial orchards	14	10	12
Number of orchards in which *A. melinus* were released	7	5	6
Number of orchards where significant red scale control was achieved by releases	None	None	None
Orchards where established (non-released) *Aphytis* spp. (*A. africanus*, *A. lingnanensis* and *A. melinus*) occurred	All	All	All
Orchards where *A. melinus* was the most influential species	None	None	None
Orchards where there were the most significant relationships in red scale parameters* occurred	All	All	All

* Parameters = red scale mutilation by host feeding and parasitism.

2.5. Discussion

The augmentation of *A. melinus* did not increase the levels of red scale parasitism above that of the untreated control in the field trials over three seasons. This finding agrees well with what was found previously by Daneel and Ware (2000) (unpublished research) when *A. lingnanensis* was released and evaluated. Augmentation with *A. melinus* was tested in Sicily and also found ineffective in one of the two trial seasons (Zappalà et al., 2008).

However, naturally occurring (indigenous and established) *Aphytis* spp. as a complex of parasitoids was still very effective in red scale parasitism and host mutilation through each season. *Aphytis africanus* was responsible for most of the red scale parasitism and mutilation in both the release and control orchards during all three seasons. *Aphytis africanus* is indigenous, well-adapted to southern African climatic conditions and a highly effective parasitoid of red scale, which can maintain red scale at a very low population, on condition that orchards remain IPM-friendly (Bedford & Cilliers, 1994; Bedford, 1968; Rosen & DeBach, 1979; Samways, 1986a; Compere, 1961; Quednau, 1964a).

Although *A. melinus*, *A. lingnanensis* and *A. chrysomphali* established in most of the citrus-producing areas of southern Africa after introduction in the early 1960s, they did not displace *A. africanus* or surpass *A. africanus* in dominance in any of these areas (Daneel & Ware, 2000; Bedford, 1998; Bedford & Cilliers, 1994). Although *A. africanus* laid the fewest eggs in laboratory trials compared to all the imported *Aphytis* spp. (Quednau & Annecke, 1963; Quednau, 1964b; Bedford, 1998) and never has more than one parasitoid per host, as opposed to *A. melinus* and *A. lingnanensis*, which often have more than one, and sometimes up to three pupae per host (Bedford & Cilliers, 1994; Annecke & Moran, 1982; DeBach & Sundby, 1963), *A. africanus* still dominates red scale parasitism and mutilation in most, if not all, citrus-producing areas of South Africa (Bedford & Cilliers, 1994; Bedford, 1998; Daneel & Ware, 2000).

In experiments with *A. lingnanensis, A. fisheri* and *A. melinus* in confined environments with access to honey and excess host scales, DeBach and Sundby (1963) found that *A. melinus* females produced the most pupae per oleander scale (*Aspidiotis nerii*), which is the preferred host for mass-rearing of *A. melinus*. Since the scale body was mostly but not completely consumed by one larva, there would probably have been enough food for at least two larvae per scale. Also, when there were two larvae per host scale, the smaller one was always a male and the larger one a female. The fecundity of *A. lingnanensis* and *A. fisheri* females did not differ between females that emerged from scales that hosted either one or two parasitoids. These results suggest that a low level of host-sharing, which is two to three parasitoids per scale, should not be antagonistic to population growth when host scales are available in excess. However, it is doubtful that this would be true if red scales were used in the experiments instead of oleander scale, and in limited host numbers; red scales are smaller and a less favourable host than oleander scale for *A. lingnanensis* and *A. melinus*, as fewer females are produced per host scale and progeny are smaller than those of oleander scale (DeBach & White, 1960).

As *Aphytis* spp. female wasps host feed to satisfy their need for protein in order to lay eggs (Javier et al., 1991; Tena et al., 2016; DeBach & Sundby, 1963; Vanaclocha et al., 2014), it was determined that *A. lingnanensis*, which laid the most eggs per female in laboratory trials (Quednau, 1964b), requires a larger proportion of scales for host-feeding to lay its full determined number of eggs. In order to fulfil this need, it would be essential for such species to spend more time searching for hosts and to associate only with higher host densities (DeBach & Sundby, 1963; Collier, 1995). *Aphytis melinus* laid 51.5 eggs on average per female versus the 19.8 eggs of *A. africanus*. This might imply that *A. africanus* is less dependent on high host densities than *A. melinus* to acquire adequate protein. It was often found during the parasitism assessments that the number of hosts available for parasitism was limited and exhausted. This might suggest that *A. lingnanensis* and *A. melinus* which lay more eggs than *A. africanus*, and consequently require more host feeding, lean more towards being feeders in higher pest density environments.

According to both Quednau (1964b) and DeBach and Sundby (1963), in two isolated fecundity trials, *A. lingnanensis* laid more eggs than *A. melinus*, which was an average per female of 76.3 versus 51.5 and 31.8 versus 24.1 in the two studies, respectively. Despite this, progeny production trials done by DeBach and Sundby (1963) when limited hosts were offered, showed that *A. lingnanensis*, *A. melinus* and *A. fisheri* accumulated a total population of 6 379, 8 320 and 10 383 individual wasps respectively after eight consecutive generations, after starting with 50 mated females each. The reason for this may be assigned to the lower amount of host feeding executed by *A. melinus* and *A. fisheri* than that of *A. lingnanensis*, which leaves a bigger proportion of available host scale for oviposition and progeny production of *A. melinus* and *A. fisheri*. With *A. africanus*, which laid the fewest eggs, an average of only 19.8 per female during laboratory trials (Quednau, 1964b; Quednau & Annecke, 1963), one can surmise that a higher proportion of available host scale would be allocated for oviposition and less to host feeding. Furthermore, *A. lingnanensis* and *A. melinus* behave gregariously, as observed in laboratory studies, to lay an average of 1.7 and 2.7 eggs per female oleander scale respectively (DeBach & Sundby, 1963). Although *A. melinus* lays more eggs per female compared to *A. africanus,* but an average of 2.7 eggs per female oleander host scale (DeBach & Sundby, 1963) versus one egg per female of *A. africanus* (Annecke & Moran, 1982) about the same number of host scales will be parasitised by each species. Hence there is not a direct relationship between the number of parasitoid eggs oviposited and host scales parasitised for these imported *Aphytis* spp., which may be one of the reasons why *A. africanus* has not been displaced by the imported species. *Aphytis africanus* lays one egg per host, which has 100% survival rate compared to lower survival rates when more eggs are laid per host (Rosen & DeBach, 1979) and which may be accentuated under red scale hosts as these trials were done with the larger oleander scale. I further suggest that a study is carried out to determine whether the sex ratio of *A. africanus* is more female-biased than that of *A. melinus* when red scales are offered since a reduction in the female to male sex ratio was observed when oleander scales as hosts were compared to red scales by DeBach and Sisojevic (1960).

Aphytis africanus mostly prefer second instar female and male red scales for parasitism. Pupae of the larger *A. lingnanensis* and *A. melinus* were mostly found under second and third instar female red scales (Bedford & Cilliers, 1994). This arrangement of host sex and size preferences might provide another explanation why *A. africanus* is not displaced by the introduced species, as *A. africanus* occupies earlier or smaller life stages of host scales. Furthermore, since *A. africanus* is the indigenous species, it will almost certainly be better adapted to local climatic conditions.

Unfortunately, little research has been done on the biology and ecology of *A. africanus* and its hosts relative to that of *A. melinus*, probably because *A. africanus* is only in southern Africa, compared to the global distribution of *A. melinus*. Furthermore, *A. africanus* cannot be reared on oleander scale because oleander scale is not a host to *A. africanus*, which makes this species more difficult to study. This lack of research makes it difficult to compare all the well-defined aspects of *A. melinus* with those of *A. africanus*, which is simply not available for laboratory studies.

Chapter 3: Fitness assessment of commercially available *Aphytis melinus* wasps

3.1. Introduction

Numerous species of hymenopteran parasitoids are used widely in biological control systems as agents for suppression of pests, either in conservation agriculture or in the form of augmentative biological control practices (Luck et al., 1999; Moreno & Luck, 1992; Hare & Morgan, 1997; Van Lenteren, 2012). Augmentation becomes necessary in biological control systems, either when indigenous natural enemies of native pests or introduced natural enemies of invasive pests fail to achieve suppression, or for additional control after conservation practices have been implemented (Heimpel & Mills, 2017; Waage, 1997; Bedford & Cilliers, 1994; Moreno & Luck, 1992). Augmentation of natural enemies is a popular control method adopted by progressive farmers and stimulated by new and trending policies that endeavour to reduce the use of chemical pesticides (Van Lenteren, 2004; Grafton-Cardwell & Stewart-Leslie, 1998; Grové, 2022; Snyder, 2019). The augmentation of natural enemies requires mass-rearing of such agents in order to be released in pre-determined numbers (Luck et al., 1999; Leppla, 2013; Heimpel & Mills, 2017). However, the artificial conditions of mass production raise questions about quality control and the genetic stability of agents as their production focuses primarily on quantity rather than quality (Mackauer, 1972; Mackauer, 1976; Chambers, 1977; Heimpel & Mills, 2017; Hoy et al., 1991; Vasquez & Morse, 2012). Quality control in the production and use of mass-reared natural enemies is urgently needed as the deterioration of quality leads to failures in pest management (Bigler et al., 1994), and if the commercial application of biological control is notably inferior to conventional chemical insecticides in efficacy, the long-term credibility of this valuable technique will be put at risk (Van Lenteren, 2004; Hoy et al., 1991; O'Neil et al., 1998).

The methods used to mass rear the primary biological control agent of red scale, *A. melinus,* are currently based on techniques developed by DeBach and White (1960) more than 60 years ago and, apart from small adjustments, have changed little since then (Van Lenteren, 2012b; Hoy, 2000). Extensive research has shown that large hosts, such as oleander scale, *Aspidiotus nerii* (Hemiptera: Diaspididae) females, are needed to deliver female-biased sex ratios, and large, fit *A. melinus* wasps in insectaries (Luck et al., 1982; Opp & Luck, 1986; Luck & Podoler, 1985; Hare & Morgan, 1997; González-Zamora et al., 2012; Charnov et al., 1981). Furthermore, different growth and feeding protocols during mass-rearing of *A. melinus* influence their fitness, of which size and longevity are important components (Collier, 1995; Heimpel & Rosenheim, 1995; Heimpel, Rosenheim & Kattari, 1997; DeBach & White, 1960; Chambers, 1977). During certain periods of the year, insectaries often produce *A. melinus* wasps of reduced quality. Male-biased sex ratios of decreased longevity are delivered during the cooler season, and smaller females are produced during the warmer times of the year. Extended shipment dates can also affect longevity at release sites (Vasquez & Morse, 2012; Kfir & Luck, 1979). Despite all this, no clear criteria for compliance exist to ensure the delivery of *A. melinus* wasps according to pre-set quality guidelines.

The purpose of the trials presented in this chapter is to assess the fitness of mass-reared *A. melinus* wasps from two insectaries with test methods that are proxies for parasitoid performance in field situations, as biological control agents of red scale on citrus (Roitberg et al., 2001). Longevity and the ability to initiate flight can be regarded as key components of hymenopteran parasitoid fitness (Vasquez, 2010; Van Lenteren, 2004; Cerutti & Bigler, 1995; Dutton & Bigler, 1995). Although researchers and the producers of natural enemies have been working together to develop flight tests for hymenopteran parasitoids, they may not yet have been implemented as a minimum standard for *A. melinus* quality control (Van Lenteren, 2004). As longevity is important for released *Aphytis* spp. to attack red scale, the ability to initiate flight is also critical to execute basic actions, like finding hosts and mates, acquiring food sources, and escaping from predators (Zappalà et al., 2012; Bernal & Luck, 2007; Grafton-Cardwell & Stewart-Leslie, 1998; Moreno et al., 1984; Bedford & Cilliers, 1994; Tena et al., 2015; Rosen & DeBach, 1979). Maintaining genetic variation in insectary populations is important to uphold parasitoid fitness (DeBach & White, 1960; Van Lenteren, 2004; Hufbauer & Roderick, 2005). Flight tests hold potential as a proxy for asymmetry, which in turn is a valuable indicator of genetic variation and fitness (Leary & Allendorf, 1989; Van Lenteren, 2012a; Bennett & Hoffmann, 1998; Soares et al., 2012; Harvey & Walsh, 1993).

The aim of the study reported in this chapter was to evaluate wasps from two insectaries in terms of longevity and a short-range flight test, as they are relevant to this research in order to determine fitness of commercially available mass-reared *A. melinus* parasitoids (Van Lenteren, 2004; Posthuma-Doodeman et al., 1996; Van Lenteren, 2012a; Vasquez & Morse, 2012).

3.2. Materials and methods

Adult *A. melinus* wasps were obtained from two insectaries marketing beneficial insects in South Africa. An overseas insectary rears insects and exports them to South Africa, while a local insectary rears *A. melinus* in South Africa. Insectaries are referred to as A and B in no specific order. On specific dates (Tables 3.1 and 3.2), 20 000 wasps were shipped from each insectary to our laboratory in Gqeberha (Eastern Cape Province). Wasps from insectaries A and B reached the laboratory on days 4 and 2, respectively, after exiting their respective insectaries. Wasps were sent in paper cups containing sawdust and cotton wool swabs, as well as honey as a food source. The cups were packed in polystyrene boxes and kept cool with ice packs. The shipping methods were the same as those used for growers. Upon arrival at the laboratory, a small hole was made in the cap of each cup, through which carbon dioxide was applied for 30 seconds, as tested by Vasquez & Morse (2012). While the wasps were anesthetised, they were spooned out, together with the sawdust mixture, into 60 mm diameter petri dishes for the flight tests, and into 500 ml glass Consol® fruit jars for the longevity tests. Both the flight and longevity tests were repeated five times.

3.2.1. Flight tests

While they were anaesthetised, 1.5 ml and 1.125 ml of the wasp and sawdust mixture from insectaries A and B respectively, containing approximately 220 wasps each, were spooned out into each of the six petri dishes (60 mm diameter). Wasps from Insectary A had a higher ratio of sawdust to wasps than that of Insectary B. Flight initiation experiments were carried out inside flight chambers, comparable to those designed by Dutton and Bigler (1995) and Forsse et al. (1992), with minor modifications. Flight chambers were made of black PVC (Polyvinyl chloride) pipes with a diameter of 16 cm and cut into lengths of 30 cm each. The bottom of the chamber was closed by standing it on black cardboard and the top with clear Perspex®, fitting flush onto the chamber. A clear sticky card was placed between the top of the chamber and the clear Perspex®, to ensure that the entire surface at the ceiling of the chamber was sensitive to wasp landings and would also allow light to shine through into the chamber from above. A 1 cm-wide strip of sticky card was attached around the inside of the chamber at a height of 2 cm to prevent the crawling wasps that climb upwards from reaching the top of the chamber. The six petri dishes containing the wasp and sawdust mixture for each insectary were then carefully placed inside the six flight chambers (Table 3.1). The flight chambers were kept in a room at ~23 °C (±2 °C) and ~83% RH (±3%), with a light source at 2100 lux from above (Dutton & Bigler, 1995) for a light period of 24 h, after which, carbon dioxide was injected into each chamber to arrest all the wasps that remained on the chamber floor. Assessments were carried out to distinguish sexes of the following parameters: non-flyers non-crawlers (NFNC) that remained in the petri dish and on the chamber floor, crawlers (C) that were caught on the 1 cm sticky strip, and flyers (F) that were caught on the ceiling of the chamber.

Table 3.1. *Aphytis melinus* flight test event programme for wasps from two insectaries.

Replicate	Insectary	Start of test	End of test
1	A	01 Nov 2022; 09h00	02 Nov 2022; 09h00
1	B	01 Nov 2022; 12h00	02 Nov 2022; 12h00
2	A	15 Nov 2022; 09h30	16 Nov 2022: 09h30
2	B	22 Nov 2022; 12h00	23 Nov 2022; 12h00
3	A	29 Nov 2022; 11h30	30 Nov 2022; 11h30
3	B	30 Nov 2022; 15h00	01 Dec 2022; 15h00
4	A	14 Feb 2023; 10h00	15 Feb 2023; 10h00
4	B	11 Jan 2023; 15h00	12 Jan 2023; 15h00
5	A	24 Jan 2023; 10h00	25 Jan 2023; 10h00
5	B	25 Jan 2023; 12h00	26 Jan 2023; 12h00

All statistical analyses were conducted in Statistica Version 14.0.0.15. Differences between counts of NFNC-, C- and F-wasps from insectaries A and B, and males and females were analysed using a factorial ANOVA but expressed as a proportion of individuals.

3.2.2. Longevity tests

While anaesthetised, 1.5 ml and 1.125 ml of the wasp and sawdust mixture from Insectaries A and B, respectively, containing approximately 220 wasps, were spooned into each of the 18 Consol® fruit jars each per insectary. The tops of the open jars were covered with cotton cloth mesh onto which thin streaks of diluted (10% purified water) organic honey (Thompson, 1999; Tena et al., 2015; González-Zamora et al., 2015; DeBach & White, 1960) were applied every three days as a food source. The cloth was held in place by the original screw rings of the Consol® jars. These fruit jars were kept in a room at a constant temperature and humidity [22 °C (±0.9 °C) and 65% RH (±4%)] and 14L: 10D light regime. Destructive sampling was carried out on six jars at a time on days 1, 5 and 10. On the day of sampling, wasps were anesthetised with carbon dioxide for 30 seconds and transferred to a 90 mm diameter petri dish. The anesthetised wasps were then subjected to -25 °C for 10 minutes to kill them. Immediately after being killed, the wasps and sawdust mixture were scattered onto a sticky grid for counting. Live and dead wasps were then counted and sexed. If the eyes and abdomen of the wasps were still intact and not desiccated within four hours of killing, the wasp was considered to be alive at the time of destructive sampling (Vasquez & Morse, 2012). Refer to Table 3.2 for the event programme.

Table 3.2. *Aphytis melinus* longevity test event programme for wasps from two insectaries.

Replicate	Day	Date of assessment	
		Insectary A	**Insectary B**
1	1	01 Nov 2022	01 Nov 2022
1	5	05 Nov 2022	05 Nov 2022
1	10	10 Nov 2022	10 Nov 2022
2	1	15 Nov 2022	22 Nov 2022
2	5	19 Nov 2022	26 Nov 2022
2	10	23 Nov 2022	30 Nov 2022
3	1	29 Nov 2022	30 Nov 2022
3	5	03 Dec 2022	04 Dec 2022
3	10	08 Dec 2022	09 Dec 2022
4	1	14 Feb 2023	11 Jan 2023
4	5	18 Feb 2023	15 Jan 2023
4	10	23 Feb 2023	20 Jan 2023
5	1	24 Jan 2023	25 Jan 2023
5	5	28 Jan 2023	29 Jan 2023
5	10	02 Feb 2023	03 Feb 2023

Differences between counts of live and dead *A. melinus* wasps for Insectaries A and B and for males and females on day 1, 5 and 10 after arrival were compared using a repeated measures ANOVA ($P < 0.05$) for each replicate.

3.3. Results

3.3.1. Flight tests

There were significantly fewer C (crawlers) than both NFNC (non-flyers non-crawlers) and F (flyers) for all five replicates. (Fig. 3.1). In replicate 1, there were significantly more F from Insectary B than A, but no differences between insectaries for either NFNC or C (Fig. 3.1 a and Table 3.3).

In replicate 2 however, the proportion of NFNC from Insectary B was significantly higher than Insectary A. Insectary A had significantly more F while there was no difference in the number of C (Fig. 3.1 b).

In replicate 3, Insectary A had significantly more F than Insectary B, while there was no difference between insectaries for the NFNC and the C (Fig. 3.1 c).

Insectary B had significantly more F and significantly less NFNC in replicate 4 than Insectary A, while there was no difference in the number of C (Fig. 3.1 d).

In replicate 5, Insectary B had significantly more NFNC while there was no difference between the two insectaries for the number of C and F (Figure 3.1 e).

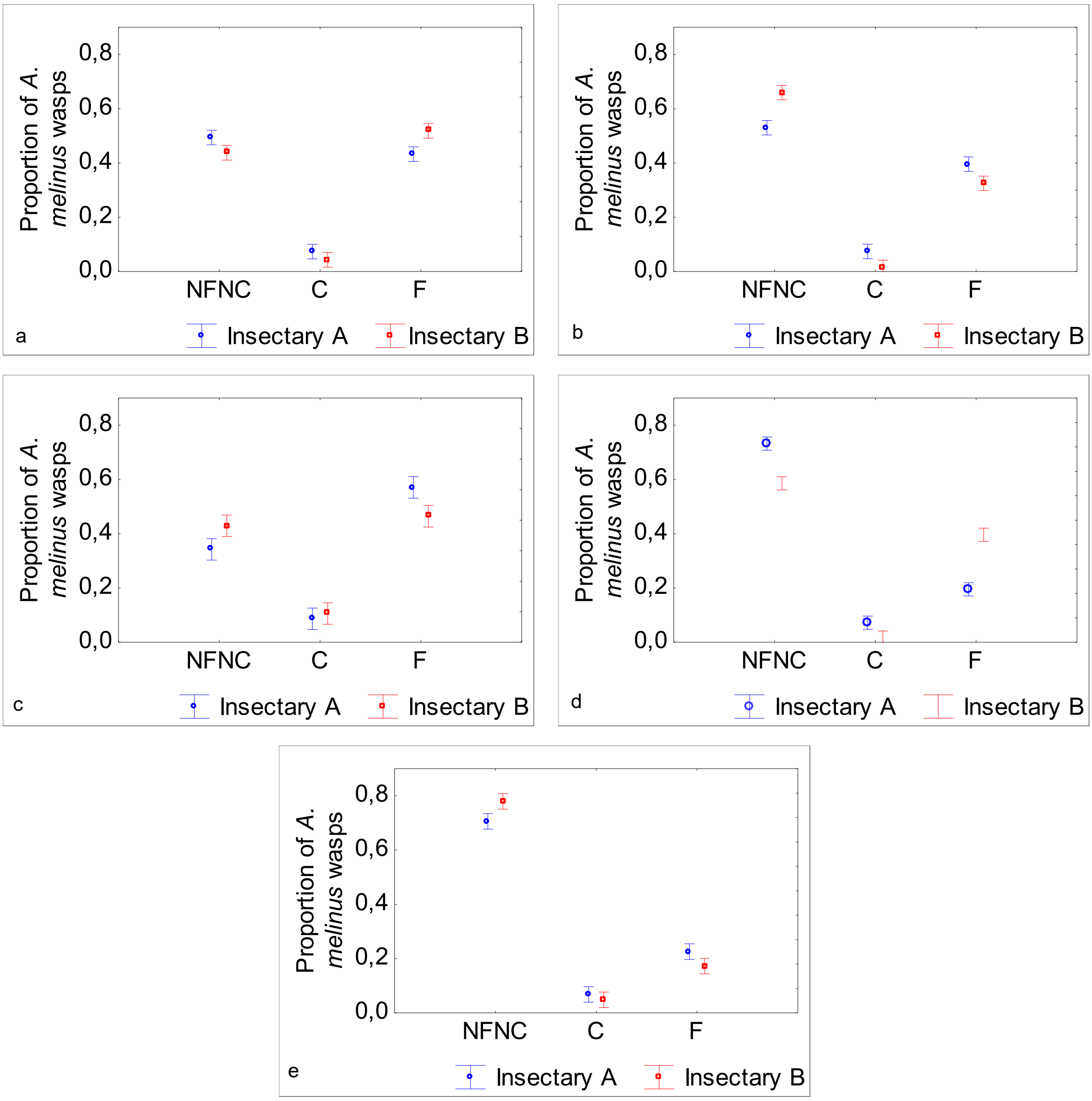


Figure 3.1. Proportion of *A. melinus* wasps allocated as non-flyers non-crawlers (NFNC), crawlers (C) and flyers (F) from Insectary A and B after 24 h in the flight chambers. Replicate 1 (a); 2 (b); 3 (c); 4 (d); 5 (e). Refer to Table 3.3 for results of statistical analysis. Error bars represent SE.

Table 3.3. The effects of insectary and parameter on the proportion of *A. melinus* wasps allocated as non-flyers non-crawlers (NFNC), crawlers (C) and flyers (F) from Insectary A and B after 24 h in the flight chamber.

Effect	**Df**	**F**	***P***
Replicate 1			
Insectary	1, 30	0.000	1.000
Parameter	2, 30	155.979	**< 0.001***
Insectary × Parameter	2, 30	3.932	**0.030***
Replicate 2			
Insectary	1, 30	0.000	1.000
Parameter	2, 30	**213.456**	**< 0.001***
Insectary × Parameter	2, 30	**8.858**	**< 0.001***
Replicate 3			
Insectary	1, 30	0.000	1.000
Parameter	2, 30	**59.318**	**< 0.001***
Insectary × Parameter	2, 30	3.074	0.061
Replicate 4			
Insectary	1, 30	0.000	1.000
Parameter	2, 30	**323.125**	**< 0.001***
Insectary × Parameter	2, 30	**27.496**	**< 0.001***
Replicate 5			
Insectary	1, 30	0.000	1.000
Parameter	2, 30	**319.014**	**< 0.001***
Insectary × Parameter	2, 30	2.657	0.086

* Denotes a significant interaction at a 95% confidence level.

The general trend for sex-ratio was consistent throughout all the replicates, except for Insectary A in the fourth replicate and Insectary B in the second replicate, where the proportion of females was notably lower (Table 3.4). There were no significant differences between flight performance parameters between the two insectaries in this trial (Table 3.5). Significant differences between the insectaries for Sex and Parameter × Sex were evident in all the replicates. In all the replicates except replicate 3, Insectary × Sex had no significant interaction. The interaction between Insectary × Parameter × Sex was significant in all the replicates except for replicate 2 (Table 3.5).

In replicate 1, where the female to male ratio was 1.70 and 1.82 for Insectary A and B respectively, the same sex ratio was not represented in the NFNC. Insectary A had significantly more C than Insectary B, while the F was the same for both insectaries (Figure 3.2 a).

In replicate 2 the sex ratio of Insectary A was 1.75 for females to males while that of Insectary B was 0.88 which explains the difference in proportion of the NFNC of the two insectaries. However, even from Insectary A, the sex ratio is not represented in the NFNC proportion (Fig. 3.2 b).

The sex ratio of Insectary B in the third replicate was 1.26 for females to males, which explains the high male proportion in the NFNC (Fig. 3.2 c). Insectary A had an ideal male proportion of 37%, however, 64% of the NFNC was males (Table 3.4).

In replicate 4, the sex ratio of Insectary A was 0.97 for females to males and the males were overrepresented in terms of C and F performance (Fig. 3.2 d). Insectary B had a sex ratio of 2.22 for females to males, which is ideal and was better represented in all the parameters than Insectary A (Fig. 3.2 and Table 3.4). Female-biased sex ratios are preferable because the females does the host feeding and scale parasitism which is the cause of scale death, while just enough males are necessary to ensure fertilisation of females (DeBach & White, 1960).

The sex ratio for both Insectaries A and B in the 5th replicate was 1.7 and 1.74 respectively for females to males (Table 3.4), which is closer to ideal as a greater number of females is preferable. This ratio was well represented in the C and F parameters but not in the NFNC parameter (Fig. 3.2 e and Table 3.4).

Table 3.4. The sex ratios of *A. melinus* wasps from Insectary A and B of the various shipment dates.

Shipment date	Replicate	Insectary	% Female	% Male	Female: Male ratio
2022/11/02	1	Insectary A	63.0%	37.0%	1.70
2022/11/16	2	Insectary A	63.6%	36.4%	1.75
2022/11/30	3	Insectary A	63.1%	36.9%	1.71
2023/02/15	4	Insectary A	49.3%	50.7%	0.97
2023/01/25	5	Insectary A	63.0%	37.0%	1.70
2022/11/02	1	Insectary B	64.5%	35.5%	1.82
2022/11/23	2	Insectary B	46.9%	53.1%	0.88
2022/12/01	3	Insectary B	55.7%	44.3%	1.26
2023/01/12	4	Insectary B	68.9%	31.1%	2.22
2023/01/26	5	Insectary B	63.5%	36.5%	1.74

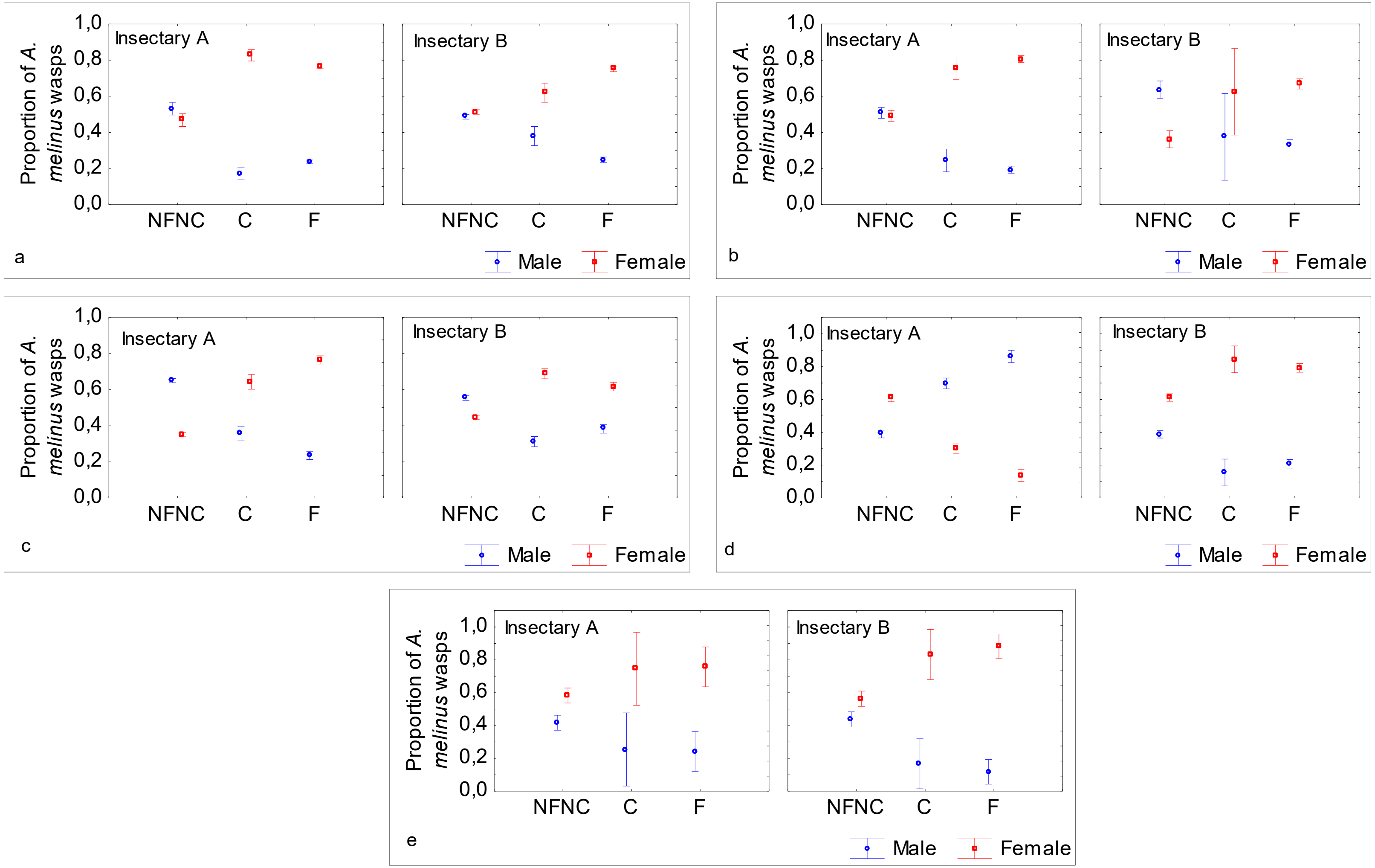


Figure 3.2. Proportion of *A. melinus* male and female wasps allocated as non-flyers non-crawlers (NFNC), crawlers (C) and flyers (F) from Insectary A and B according to sex and compared between Insectary A and B after 24 h in the flight chambers. Replicate 1 (a); 2 (b); 3 (c); 4 (d); 5 (e). Refer to Table 3.5 for results of statistical analyses. Error bars represent SE.

Table 3.5. Effect of parameter and sex on the proportion of *A. melinus* male and female wasps allocated as non-flyers non-crawlers (NFNC), crawlers (C) and flyers (F) from Insectaries A and B according to sex and compared between Insectary A and B after 24 h in the flight chambers.

Effect	**Df**	**F**	***P***
Replicate 1			
Insectary	1, 60	0.000	1.000
Parameter	2, 60	0.000	1.000
Sex	1, 60	**320.982**	**< 0.001***
Insectary × Parameter	2, 60	0.000	1.000
Insectary × Sex	1, 60	**10.736**	**0.002***
Parameter × Sex	2, 60	**91.288**	**< 0.001***
Insectary × Parameter × Sex	2, 60	**18.904**	**< 0.001***
Replicate 2			
Insectary	1, 60	0.000	1.000
Parameter	2, 60	0.000	1.000
Sex	1, 60	**27.709**	**< 0.001***
Insectary × Parameter	2, 60	0.000	1.000
Insectary × Sex	1, 60	**8.667**	**< 0.001***
Parameter × Sex	2, 60	**19.621**	**< 0.001***
Insectary × Parameter × Sex	2, 60	0.004	0.996
Replicate 3			
Insectary	1, 60	0.000	1.000
Parameter	2, 60	0.000	1.000
Sex	1, 60	**134.857**	**0.000***
Insectary × Parameter	2, 60	0.000	1.000
Insectary × Sex	1, 60	0.027	0.869
Parameter × Sex	2, 60	**164.365**	**0.000***
Insectary × Parameter × Sex	2, 60	**25.835**	**0.000***

Replicate 4			
Insectary	1, 60	0.000	1.000
Parameter	2, 60	0.000	1.000
Sex	1, 60	**16.292**	**< 0.001***
Insectary × Parameter	2, 60	0.000	1.000
Insectary × Sex	1, 60	**262.888**	**< 0.001***
Parameter × Sex	2, 60	**12.623**	**< 0.001***
Insectary × Parameter × Sex	2, 60	**66.955**	**< 0.001***
Replicate 5			
Insectary	1, 60	0.000	1.000
Parameter	2, 60	0.000	1.000
Sex	1, 60	**252.446**	**< 0.001***
Insectary × Parameter	2, 60	0.000	1.000
Insectary × Sex	1, 60	**5.001**	**0.029***
Parameter × Sex	2, 60	**29.492**	**< 0.001***
Insectary × Parameter × Sex	2, 60	2.286	0.110

* Denotes as significant interaction at a 95% confidence level.

While there were significantly more NFNC than F and C, and more F than C for all the replicates combined, there was no significant difference between the two insectaries for each of these parameter categories (Fig. 3.3 and Table 3.6).

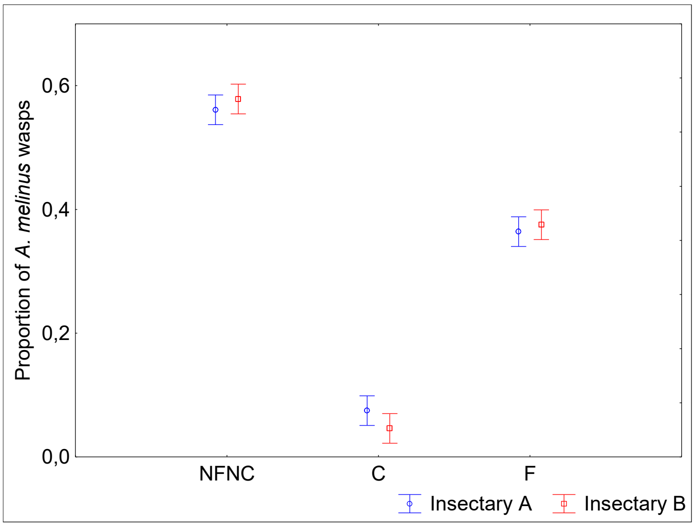


Figure 3.3. Proportion of *A. melinus* wasps allocated as non-flyers non-crawlers (NFNC), crawlers (C) and flyers (F) from Insectaries A and B after 24 h in the flight chambers for all replicates combined. Refer to Table 3.6 for results of statistical analyses. Error bars represent SE.

Table 3.6. The effects of insectary and parameter on the proportion of *A. melinus* wasps allocated as non-flyers non-crawlers (NFNC), crawlers (C) and flyers (F) from Insectaries A and B after 24 h in the flight chamber.

Effect	Df	F	*P*
Parameter	2, 174	**229.289**	**< 0.001***
Insectary	1, 174	0.000	1.000
Parameter × Insectary	2, 174	0.549	0.578

* Denotes a significant interaction at a 95% confidence level.

Overall, there was no significant difference in the proportion of male and female wasps in the NFNC parameter between insectaries, but there were significantly more females for the C and F parameters for both insectaries, with significantly more females from Insectary B (Fig. 3.4 and Table 3.7).

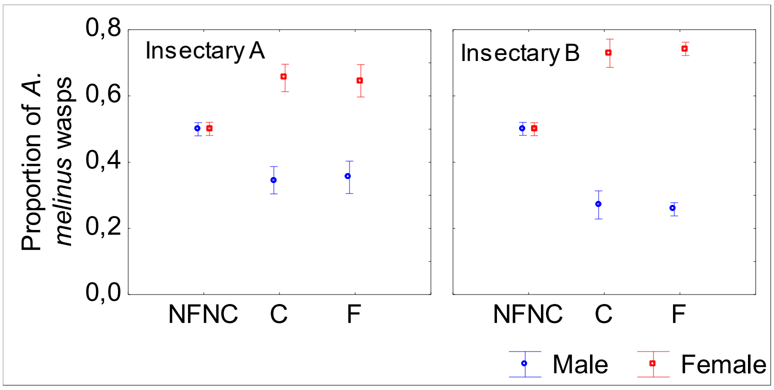


Figure 3.4. Proportion of *A. melinus* male and female wasps allocated as non-flyers non-crawlers (NFNC), crawlers (C) and flyers (F) from Insectaries A and B according to sex and compared between Insectary A and B after 24 h in the flight chambers for all replicates combined. Refer to Table 3.7 for results of statistical analyses. Error bars represent SE.

Table 3.7. Effect of parameter and sex on the proportion of *A. melinus* male and female wasps allocated as non-flyers non-crawlers (NFNC), crawlers (C) and flyers (F) from Insectaries A and B after 24 h in the flight chambers according to sex and compared between Insectary A and B for all replicates combined.

Effect	Df	F	*P*
Parameter	2, 344	0.000	1.000
Sex	1, 344	**169.109***	**< 0.001**
Insectary	1, 344	0.000	1.000
Parameter × Sex	2, 344	**42.557***	**< 0.001**
Parameter × Insectary	2, 344	0.000	1.000
Sex × Insectary	1, 344	**8.190***	**0.004**
Parameter × Sex × Insectary	2, 344	2.264	0.105

* Denotes a significant interaction at a 95% confidence level.

3.3.2. Longevity tests

Wasps from both insectaries died in significant proportions from Day 1 to Day 5 and to Day 10 ((Fig. 3.5, 3.6 and 3.7; Table 3.8: R1 (repeated measures) × Status)). Over time there was not a significant difference between the two insectaries, apart from replicate 1 (R1 × Insectary; F = 0.000: *P* = < 0.001). The proportion of live wasps over time was consistently higher for Insectary B than Insectary A (Fig. 3.5). At Day 1, Insectary A had more live wasps than Insectary B for replicates 1 and 3, while there were no differences for the other 3 replicates. Almost all the wasps from Insectary A were dead at the end of the trial, while an average of 71.2% from Insectary B were dead (Fig. 3.5).

For the females, significantly more wasps were alive at Day 10 for Insectary B than for Insectary A, except for replicates 4 and 5, where there was no difference (Fig. 3.6).

Significantly more male wasps were dead from Insectary A than Insectary B in replicates 1, 2 and 3 (Fig. 3.7).

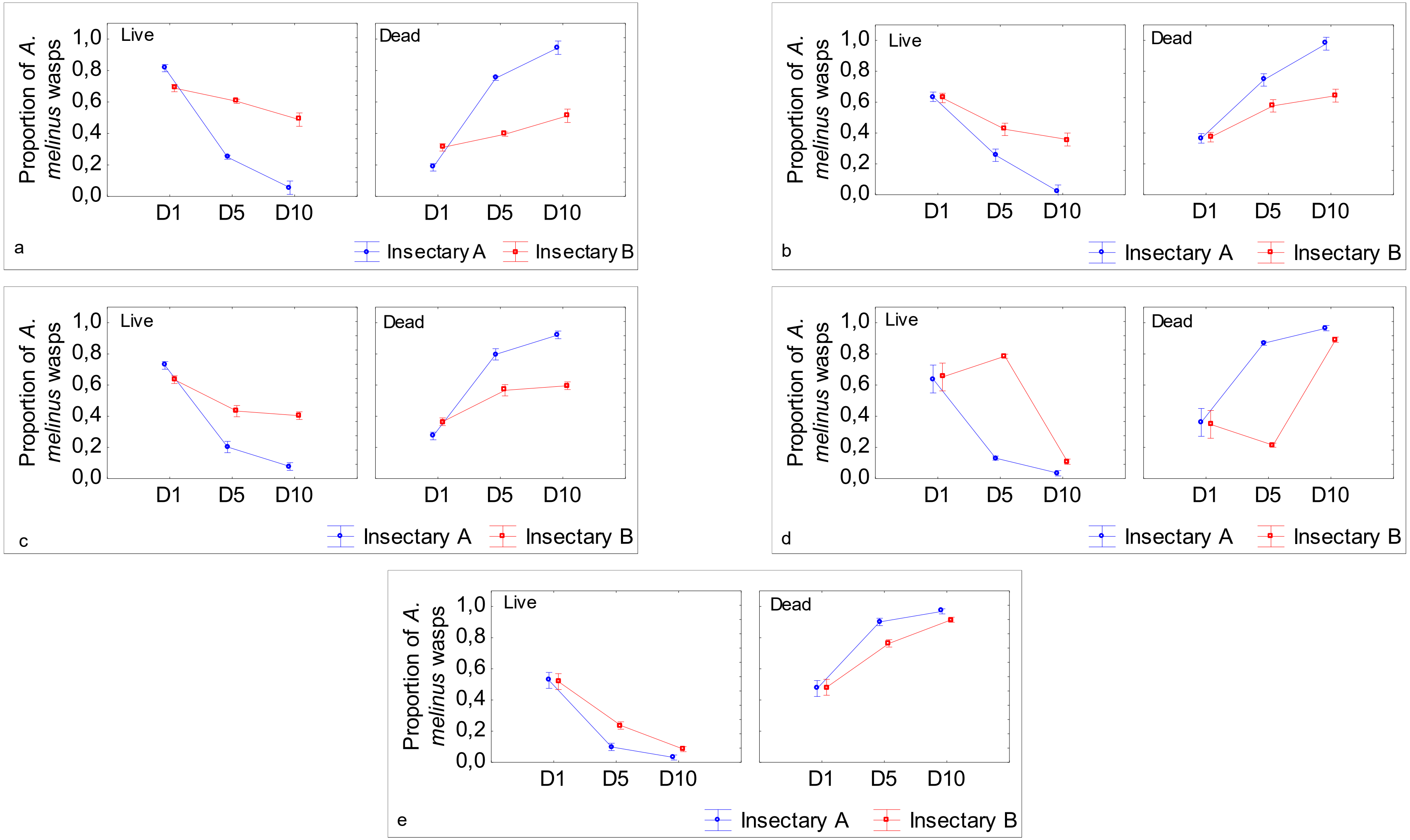


Figure 3.5. Proportion of live and dead *A. melinus* wasps (males and females combined) from Insectaries A and B, recorded in replicate 1 (a); 2 (b); 3 (c); 4 (d); 5 (e) after 1 day (D 1), 5 days (D 5) and 10 days (D 10). Refer to Table 3.8 for results of statistical analyses. Error bars represent SE.

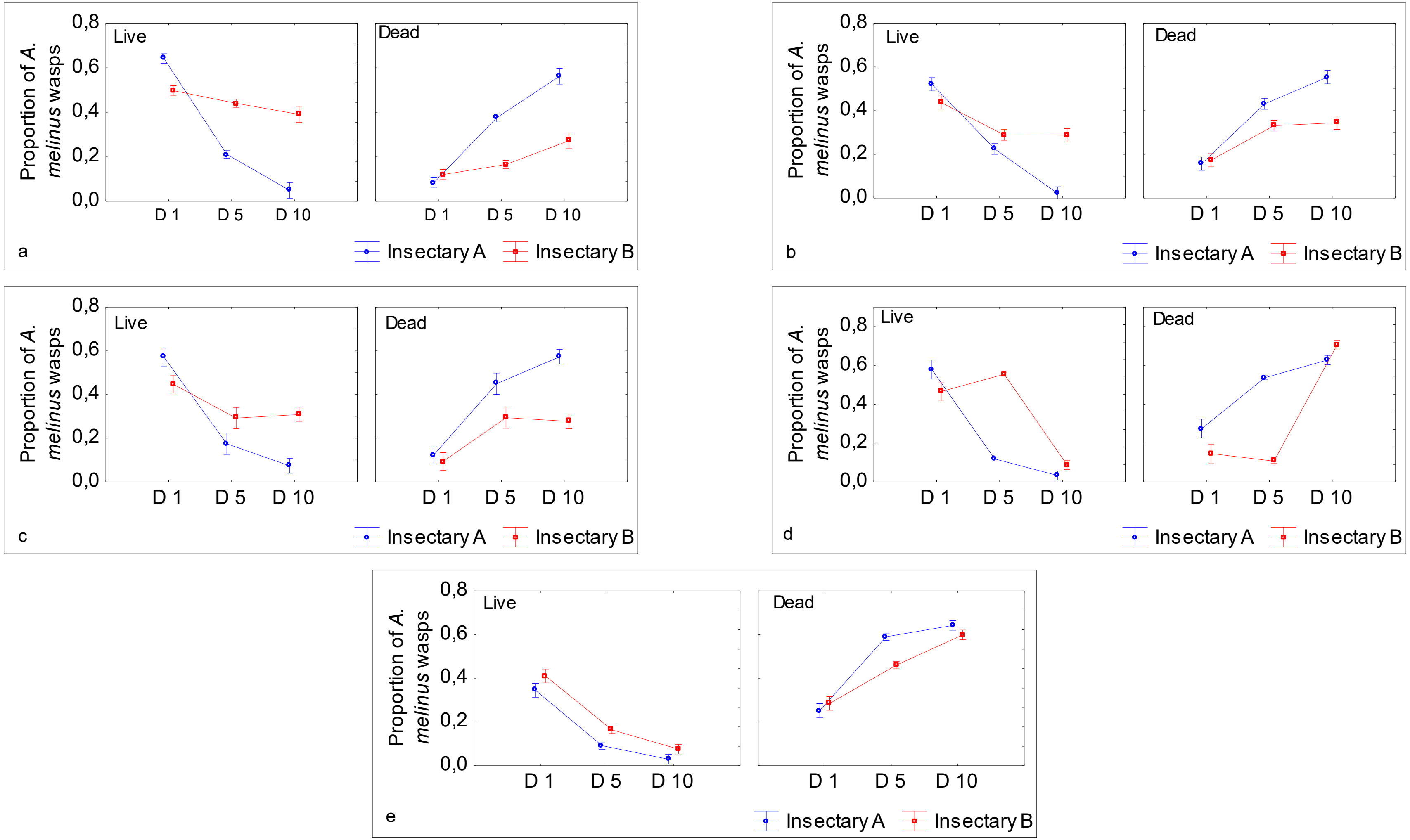


Figure 3.6. Proportion of live and dead *A. melinus* female wasps from Insectaries A and B, recorded in replicates 1 (a); 2 (b); 3 (c); 4 (d); 5 (e) after 1 day (D 1), 5 days (D 5) and 10 days (D 10). Refer to Table 3.8 for results of statistical analyses. Error bars represent SE.

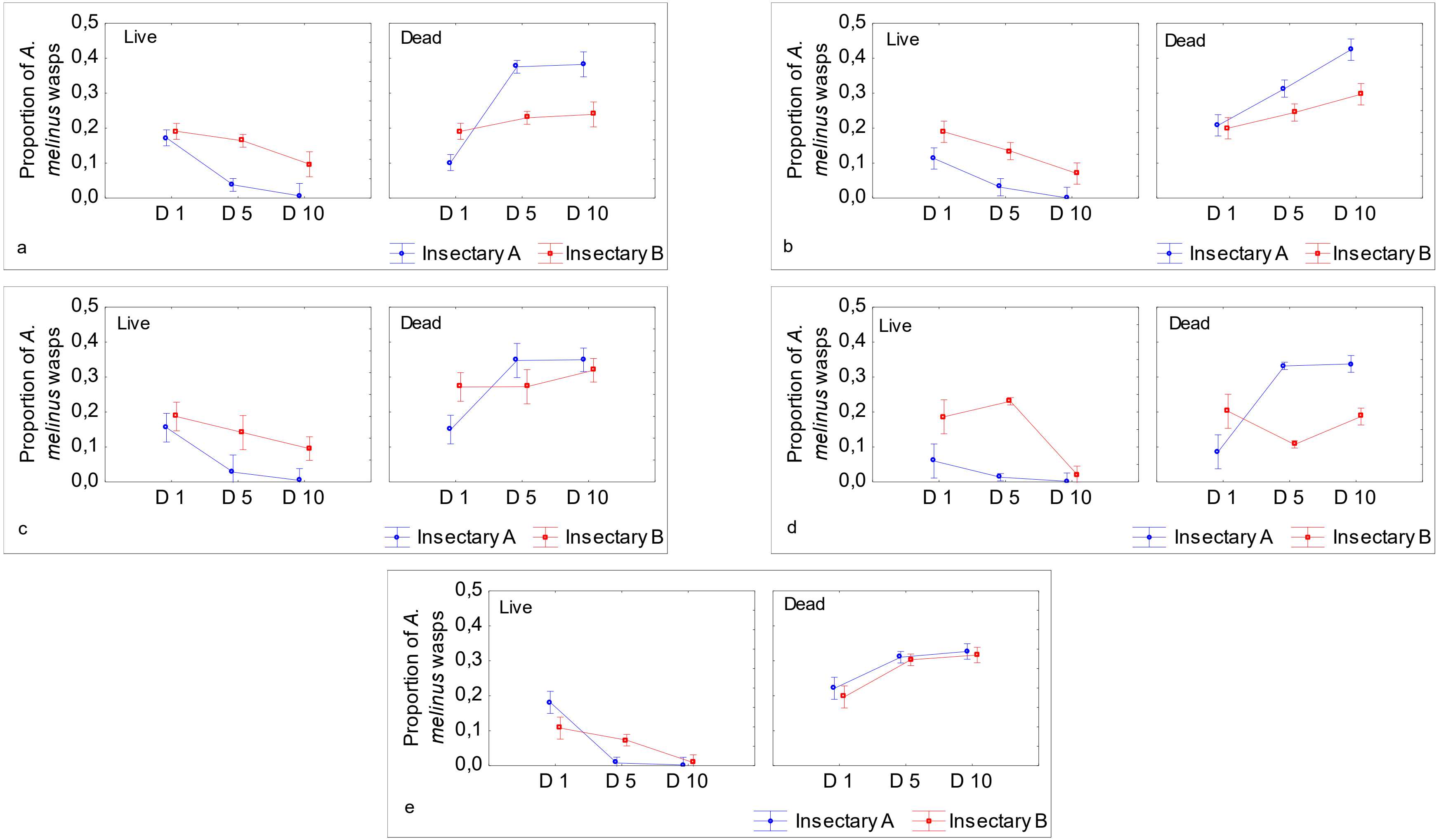


Figure 3.7. Proportion of live and dead *A. melinus* male wasps from Insectaries A and B recorded in replicates 1 (a); 2 (b); 3 (c); 4 (d); 5 (e) after 1 day (D1), 5 days (D5) and 10 days (D10). Refer to Table 3.8 for results of statistical analyses. Error bars represent the SE.

Table 3.8. Effect of insectary and sex on the proportion of *A. melinus* wasps recorded as live or dead from Insectaries A and B.

Effect	**Df**	**F**	***P***
Replicate 1			
R1 × Sex	2, 80	6.298	1.000
R1 × Status	2, 80	**265.745**	**0.002***
R1 × Insectary	2, 80	**0.000**	**< 0.001***
R1 × Sex × Status	2, 80	38.075	1.000
R1 × Sex × Insectary	2, 80	**8.003**	**< 0.001***
R1 × Status × Insectary	2, 80	**101.696**	**< 0.001***
R1 × Sex × Status × Insectary	2, 80	**18.030**	**< 0.001***
Replicate 2			
R1 × Sex	2, 80	0.970	0.383
R1 × Status	2, 80	**100.435**	**< 0.001***
R1 × Insectary	2, 80	0.000	1.000
R1 × Sex × Status	2, 80	**16.739**	**< 0.001***
R1 × Sex × Insectary	2, 80	2.141	0.124
R1 × Status × Insectary	2, 80	**14.815**	**< 0.001***
R1 × Sex × Status × Insectary	2, 80	**6.815**	**0.002***
Replicate 3			
R1 × Sex	2, 80	0.201	0.817
R1 × Status	2, 80	**243.307**	**< 0.001***
R1 × Insectary	2, 80	0.000	1.000
R1 × Sex × Status	2, 80	**51.177**	**< 0.001***
R1 × Sex × Insectary	2, 80	**4.155**	**0.019***
R1 × Status × Insectary	2, 80	**52.799**	**< 0.001***
R1 × Sex × Status × Insectary	2, 80	**13.066**	**< 0.001***
Replicate 4			
R1 × Sex	2, 80	**4.073**	**0.020***
R1 × Status	2, 80	**204.892**	**< 0.001***
R1 × Insectary	2, 80	0.000	1.000
R1 × Sex × Status	2, 80	**73.755**	**< 0.001***
R1 × Sex × Insectary	2, 80	**21.659**	**< 0.001***
R1 × Status × Insectary	2, 80	**74.817**	**< 0.001***
R1 × Sex × Status × Insectary	2, 80	**14.805**	**< 0.001***
Replicate 5			
R1 × Sex	2, 80	0.705	0.497
R1 × Status	2, 80	**222.723**	**< 0.001***
R1 × Insectary	2, 80	0.000	1.000
R1 × Sex × Status	2, 80	**45.573**	**< 0.001***
R1 × Sex × Insectary	2, 80	**5.859**	**0.004***
R1 × Status × Insectary	2, 80	**5.044**	**0.008***
R1 × Sex × Status × Insectary	2, 80	0.223	0.800

* Denotes as significant interaction at a 95% confidence level.

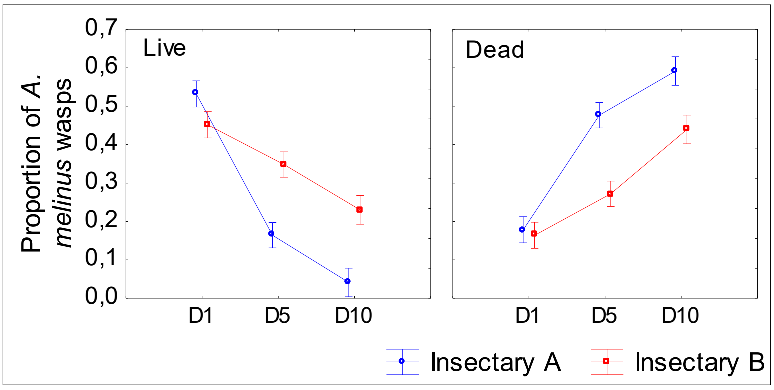


Figure 3.8. Proportion of live and dead *A. melinus* female wasps from Insectaries A and B, recorded in all replicates combined after 1 day (D1), 5 days (D5) and 10 days (D10). Refer to Table 3.9 for results of statistical analyses. Error bars represent SE.

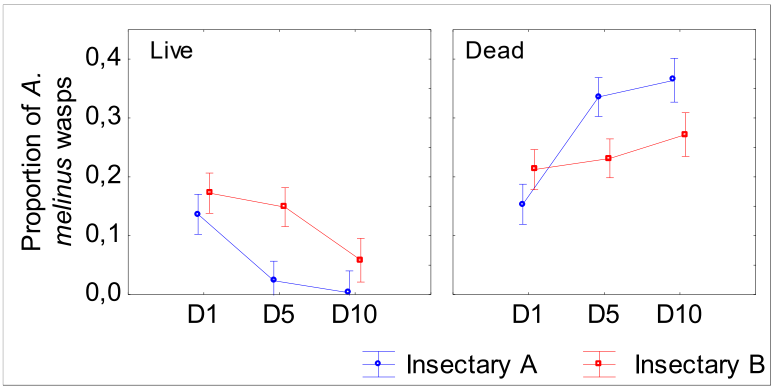


Figure 3.9. Proportion of live and dead *A. melinus* male wasps from Insectaries A and B recorded in all replicates combined after 1 day (D1), 5 days (D5) and 10 days (D10). Refer to Table 3.9 for results of statistical analyses. Error bars represent SE.

Significantly fewer female wasps were alive at Day 10 for Insectary A (4.12%) than Insectary B (23.01%) (Fig. 3.8). There was no significant difference between the number of live males from Insectary A and B at Day 10 (Fig. 3.9). There was no significant interaction between sex and status as live or dead during the trials (R1 × Sex, F = 2.186 and *P* = 0.113) and overall, there was no significant difference between the two insectaries for both sexes combined (R1 × Insectary, F = 0.000 and *P* = 1.000) (Table 3.9).

Table 3.9. Effect of insectary and sex on the proportion of *A. melinus* wasps allocated as live or dead recorded in all replicates combined from Insectaries A and B according to sex and compared between Insectary A and B.

Effect	**Df**	**F**	***P***
R1 × Sex	2, 464	2.186	0.113
R1 × Status	2, 464	**474.887**	**< 0.001***
R1 × Insectary	2, 464	0.000	1.000
R1 × Sex × Status	2, 464	**100.043**	**< 0.001***

R1 × Sex × Insectary	2, 464	**8.781**	**< 0.001***
R1 × Status × Insectary	2, 464	**71.320**	**< 0.001***
R1 × Sex × Status × Insectary	2, 464	**8.216**	**< 0.001***

* Denotes as significant interaction at a 95% confidence level.

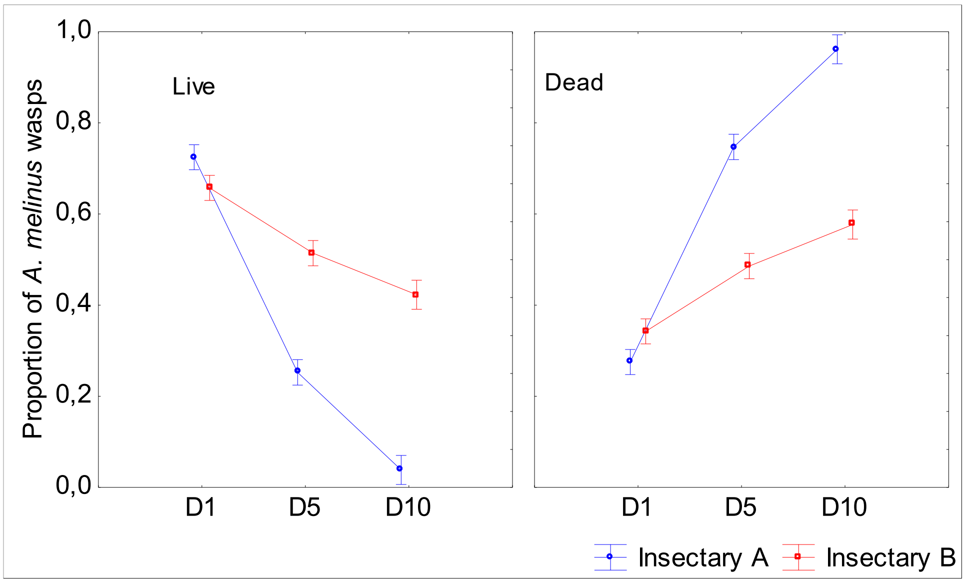


Figure 3.10. Proportion of live and dead *A. melinus* wasps (males and females combined) from Insectaries A and B recorded in all replicates combined after 1 day (D1), 5 days (D5) and 10 days (D10). Refer to Table 3.10 for results of statistical analyses. Error bars represent SE.

Although Insectary A had proportionally more live wasps on Day 1, over time significantly more wasps from Insectary A died from Day 1 to Day 5 and to Day 10 than from Insectary B (R1 × Status × Insectary, F = 75.566 and *P* = < 0.001) (Fig. 3.10 and Table 3.10).

Table 3.10. Effect of insectary on the proportion of *A. melinus* wasps allocated as live or dead recorded in all replicates combined from Insectaries A and B according to sex and compared between Insectary A and B.

Effect	**Df**	**F**	***P***
R1 × Status	2, 88	**304.699**	**< 0.001***
R1 × Insectary	2, 88	0.000	1.000
R1 × Status × Insectary	2, 88	**75.566**	**< 0.001***

* Denotes a significant interaction at a 95% confidence level.

3.4. Summary of flight and longevity test results

Wasps from Insectary A that were reared overseas had to travel longer than locally reared wasps and consequently lived for a shorter period during the longevity tests (Fig. 3.10). However, overall, 72.47% and 65.75% of wasps from Insectary A and B were alive on Day 1, respectively (Table 3.11).

Only 36.41% and 37.53% of wasps from Insectary A and B, respectively, could initiate flight which was tested on Day 1 (Fig. 3.3 and Table 3.11).

Table 3.11. Summary of status of *A. melinus* wasps in percentage (%) as derived from flight and longevity test data from Fig. 3.3 and Fig. 3.10 of all replicates combined for parasitoids from Insectaries A and B.

Flight tests (Day 1 - Fig. 3.3)				**Longevity tests** (Day 1 – Fig. 3.10)	
Source of wasps	**Alive**		**Non-flyer non-crawler**	**Alive**	**Dead**
	Flyer	**Crawler**			
Insectary A	36.41%	7.49%	56.09%	72.47%	27.53%
Insectary B	37.53%	4.62%	57.83%	65.75%	34.25%

The NFNC proportion of the wasps that remained on the flight test tube after 24 h can be divided into portions of live and dead wasps based on longevity data from Fig. 3.10. Live wasps can further be allocated as either F, C or live NFNC (L-NFNC) as in Table 3.12 based on data from Figs. 3.3, 3.10, and Table 3.11.

Table 3.12. Summary of status of live and dead *A. melinus* wasps on Day 1 in percentage (%) as Flyers (F), crawlers (C) and live non-flyers non-crawlers (L-NFNC) as derived from Table 3.11 for parasitoids from Insectaries A and B.

Source of wasps	**Status Day 1: alive or dead**	**Portions of live and dead wasps**
Insectary A	72.47% live	36.41% F 7.49% C 28.57% L-NFNC
	27.53% dead	27.53% dead
Total	100%	100%
Insectary B	65.75% live	37.53% F 4.62% C 23.60% L-NFNC
	34.25% dead	34.25% dead
Total	100%	100%

3.5. Discussion

Since 27.53% and 34.25% of these wasps from Insectaries A and B, respectively, were dead on Day 1, and 56.09% and 57.83%, respectively, could not initiate flight, it can be calculated that 36.06% (L-

NFNC + C) and 28.22% (L-NFNC + C), respectively, of the wasps in the flight tests were alive in the flight chambers but not flying. The fitness of these wasps was impaired, which may have included, but not be limited to, genetic stagnation in the cultures of both insectaries. It was shown that wing asymmetry, which compromises flight in *Trichogramma* spp. and other Hymenoptera, is a result of poor genetic variation (Parsons, 1992; Bennett & Hoffmann, 1998; Van Lenteren, 2004; Posthuma-Doodeman et al., 1996; Zappalà et al., 2012; Vasquez & Morse, 2012; Van Lenteren, 2012a; Leary & Allendorf, 1989; Salmanova, 1991; Harvey & Walsh, 1993).

As no honey was provided to the wasps in the flight test tubes, and the flight test assessment was done after 24 h, some wasps may have starved as the wasps from the same batches destined for the longevity tests rushed to feed when honey was provided upon arrival. Although honey was provided by the insectaries for consumption during transit, it is dark inside the paper cups which would have impaired feeding (DeBach & White, 1960; Rosen & DeBach, 1979). The implication of this would be that wasps should be able to have the choice to feed on an energy source prior to field releases after transit as tests conducted by Vasquez (2010) showed that wasps can survive for only two days without honey.

Since there was no difference in the flight performance of the wasps from Insectaries A and B when all the replicates were combined, it can be deducted that flight ability is to a certain extent not sensitive to travel time. Since the flight performance parameters from these insectaries compared so similarly, it must be further investigated to what level genetics influences flight performance. Based on this data, only *Aphytis* spp. populations collected from natural habitats should be used to establish populations in newly founded insectaries.

Since these tests did not start with only live wasps, many of the dead wasps found on Day 1 might have died during transit. If longevity tests were to be done by experimenting on the direct environmental factors potentially affecting them, it would be suggested to start with only live wasps. The total number of wasps, live and dead, was never counted when the shipments arrived at our laboratory.

There was no significant difference in longevity between the wasps from the two insectaries, where the wasps that travelled longer before the tests lived shorter during the tests, which means that travel time impacts the available period for field parasitism and host feeding. It is considered unlikely that wasps which are released later would compensate with a higher rate of parasitism immediately after release. Field parasitism would be further reduced, as females that have their highest parasitism rate per day during the first few days after eclosing and mating (Quednau, 1964b; Vanaclocha et al., 2014; Hopper et al., 2013) were in transit during the 4 and 2 days, respectively, for Insectaries A and B before being released.

A useful addition to this study would have been the measurement of the wasps' wings to investigate the actual degree of asymmetry. This would assist in understanding if there was another factor resulting in the live wasps not being able to initiate flight towards the light source. As only a low proportion of wasps from both insectaries could initiate flight, release numbers should probably be increased to compensate for this reduction in potentially effective wasps that were eventually available immediately after releases. This factor may also have a cost implication for growers. Insectaries should investigate this and determine if the low proportion of F is caused by inbreeding.

Sex ratios varied between insectaries. Insectary A had a sex ratio of 49% females and 51% males in the 4th replicate, while the sex ratios of the wasps in the rest of the replicates was better, that is, more female-biased (DeBach & White, 1960; Vasquez & Morse, 2012). The wasps used in this replicate, from Insectary A, were produced in the winter months of the northern hemisphere and imported to South Africa; the poor female ratio and longevity issues in replicates 4 and 5 could be ascribed to this, despite insectaries being climatically controlled. This is in line with what Vasquez and Morse (2012) found previously.

Mechanical handling, transportation and releasing *A. melinus* poses a risk to their fitness, as the adult life stage is very delicate. A reduction of fertility was previously recorded in such adults of other parasitoids species, when compared to the fertility of parasitoids that were released as immatures (Van Lenteren & Tommasini, 2003). These conditions may explain the high mortality on Day 1 and the low percentage of F amongst the wasps from both insectaries. However, it would be impractical to release *A. melinus* during other life stages.

Another component of fitness that should be tested for when quality of commercially reared *Aphytis* spp. is assessed, is the number of eggs laid by females after releasing the wasps. As eggs are resorbed during periods of stress and protein starvation (Vanaclocha et al., 2014; Hopper et al., 2013) and given that wasps spend a period of time in transit between the insectary and destination orchards, this would be relevant.

Wolbachia are a common and widespread group of bacteria found in the reproductive tissues of arthropods (Werren, 1997; Jeyaprakash & Hoy, 2000). Commercially reared and field collected *A. melinus* was found to be susceptible by Wolbachia and that longevity and fecundity was negatively affected. No female progeny was produced due to cytoplasmic incompatibility when a Wolbachia infected *A. melinus* male mated with an uninfected female. Furthermore, fecundity was also impaired because of Wolbachia infection as significantly less eggs were laid by Wolbachia infected females. *Aphytis melinus* adults lived significantly longer than adults infected with Wolbachia (Vasquez, 2010). With this background it is suggested that screening for Wolbachia infection as a component of fitness assessments be tested for regularly at insectaries.

Chapter 4: Molecular identification of *Aphytis* species

4.1. Introduction

The importance of accurate species identification for biological control has been highlighted repeatedly over the years (Rosen & DeBach, 1973; Compere, 1961; Compere, 1969; DeBach, 1969; Debach, 1960). In this study there was a need for dependable methods for species identification in mixed *Aphytis* populations. Since *A. africanus* is naturally present in most of the orchards infested with red scale in South Africa, it would be beneficial to be able to distinguish introduced species from *A. africanus* (Quednau, 1965). Different methods are currently used to identify and differentiate between *Aphytis* spp. The method mostly used to identify *Aphytis* spp. is by means of morphological differences, according to pigmentation patterns of the pupa from a ventral view, using a light microscope of between 40 and 60 × magnification. This, however can only be accomplished with increased certainty when the red and green-eyed pupal stages are advanced and final pupal colouration occurs, which leaves a small window of opportunity for reliable identification (Prinsloo, 1984; Rosen & DeBach, 1979; Bedford & Cilliers, 1994; Taylor, 1935; Muma & Selhime, 1966). However, only 13% and 7% respectively of the total life cycle is comprised of these two distinct stages (Yu & Luck, 1988; Rosen & DeBach, 1979; Quednau, 1965). Although pupal pigmentation pattern is traditionally considered as a stable method for identification (DeBach, 1964; Rosen & DeBach, 1976; Rosen & DeBach, 1979), it is not completely dependable in all cases, as climatic factors may play a role in altering pigmentation patterns (Pina et al., 2012). Morphological identification has numerous drawbacks; while taxonomic expertise is diminishing, additional challenges include phenotypic plasticity, the high level of taxonomic knowledge that is required, and key morphological characteristics may be influenced during preservation (Mani et al., 2022; Hebert et al., 2003).

Specimens from various populations that are morphologically indistinguishable are often regarded as the same species by taxonomists (Mayr, 1948; Mayr, 2005; Noor, 2002; Hebert et al., 2003). Cryptic species present themselves, for this very reason, mostly unsuspected because initially they appear similar but might later be confirmed as a different species (Paterson, 1991). Cryptic or sibling species play an important role and the recognition of their existence and ability to identify them is important to accurately pursue effective assembling of host parasitoid interactions (Debach, 1960). One such example out of many is the successful biological control of the olive scale, *Parlatoria oleae* Colvée (Hemiptera: Diaspididae) in California. An apparent biparental strain of *Aphytis maculicornis* Masi, a sibling species introduced from Iran, was conceded as biologically distinct from various other morphologically indistinguishable strains introduced from other countries. After the introduction of this species into California, it soon became a major factor in the control of olive scale on various fruit tree crops (DeBach et al., 1971).

Because it is difficult to separate species of *Aphytis* during the adult, egg, larval and prepupal stages, behavioural tests can be used, which will add certainty to morphological identification (Mayr, 1948; Quednau & Annecke, 1963; Pina et al., 2012; Rosen & DeBach, 1979; Paterson et al., 2016), as each species has its own distinct biological traits. The acceptance or refusal of certain host scales or sexes of these host scales by *Aphytis* parasitoids as hosts to produce progeny, and the length of the life cycle at 26.6 °C and 75% RH are reliable species differentiation characteristics. Quednau (1965) developed a differentiation scheme to investigate parasitoid and host relationships, as field collected material can be placed in emergence boxes to eventually collect eclosed adults of *Aphytis* spp. These wasps can then be subjected to a feeding preference trial on Ivy scale (*Aspidiotus hederae* Valot) and circular purple scale (*Chrysomphalus aonidum* Linnaeus), both on Tsamma melon (*Citrullus lanatus* Matsum), and red scale on lemons, as the different species of wasps show significant preferences for different hosts (Quednau, 1965).

Life cycle duration can also be used for species discrimination, using the clockwork-trap method. After suitable host scales have been exposed to *Aphytis* spp. wasps for two to four hours, pupae are removed 10 days later and placed in the clockwork-trap. Emerging *Aphytis* are separated by being caught automatically at two-hourly periodic increments, after which this information can be compared to existing *Aphytis* species life table data (Quednau, 1965).

Apart from morphological and behaviour approaches, molecular identification has been shown to be a stable method of identification for most insects (Mani et al., 2022; Pina et al., 2012; Gariepy et al., 2014; González-Chang et al., 2016; Hebert et al., 2003). Molecular identification has numerous advantages over morphological identification. Insect species can potentially be identified from body fragments, which were initially difficult to relate to known species. Deoxyribonucleic acid (DNA) barcoding is based on DNA sequencing of a standard gene region (Hebert et al., 2003). The 5’ region of the mitochondrial cytochrome c oxidase subunit I (mtCO-I) was selected for this purpose, because its mutation rate is often fast enough to differentiate between closely related species and its sequence is maintained between conspecifics, while exhibiting a lack of recombination (Mani et al., 2022). Unfortunately, only species for which a collection of datasets exists in Genbank as a result of sound research can be identified (Hebert et al., 2003; Mani et al., 2022). A weakness of DNA barcoding as an identification instrument, is that it requires better quality control of the reference libraries and requires curated open access databases (Grant et al., 2021).

Given the challenges in the identification of *Aphytis* spp., the objective of this study was to collect *Aphytis* spp. from different field populations and compare morphological and molecular identification methods for these wasps.

4.2. Materials and methods

4.2.1. Sample collection

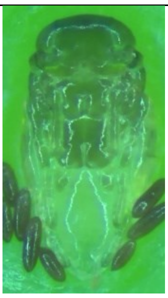
Red scale infested fruit were collected from various locations across the Eastern -, Western – and Northern Cape provinces from April to July in 2022 (Table 4.1).

Table 4.1. Different localities where *Aphytis* spp. pupae were collected for morphological and molecular identification.

Farm, coordinates, elevation, and variety (cultivar)	**Closest town, province**	**Climatic region (SANS 204-2) and annual rainfall** (Conradie, 2012)**.**
Normandale -33.403386, 25.455324 95 m, Lemon (Eureka)	Kirkwood, Eastern Cape	Temperate interior 450 mm
Step aside -33.404491, 25.451817 95 m, Valencia (Midknight)	Kirkwood, Eastern Cape	Temperate interior 450 mm
Hexfarm -32.432759, 18.963260 400 m, Lemon (Eureka)	Citrusdal, Western Cape	Temperate interior 300 mm
Modderfontein -32.610690, 18.999011 600 m, mon (Eureka)	Citrusdal, Western Cape	Temperate interior 300 mm
Melon -33.887322, 24.980658 30 m, Lemon (Eureka)	Loerie, Eastern Cape	Temperate coastal 500 mm
Vrugbaar -28.761249, 19.376483 400 m, Valencia (Benny)	Kakamas, Northern Cape	Arid interior 120 mm

Red scale individuals were inspected and inverted to determine whether they were parasitised. When an *Aphytis* sp. pupa was found, it was identified according to morphological keys based on the pigmentation patterns of the *Aphytis* spp. during the various phases of the pupal stage, according to the keys provided by Debach (1959) Prinsloo (1984) Wang et al. (2021) and Bedford (1998) (Table 4.2).

Table 4.2. Different pigmentation patterns of relevant *Aphytis* spp. during the phases of the pupal stage (Quednau, 1964a; Debach, 1959; Wang et al., 2021; Bedford, 1998). Pictures aligned according to literature descriptions.

Different pupa stages	***A. melinus***	***A. africanus***	***A. lingnanensis***	***A. chrysomphali***
Pigmentation pattern of the colourless-eyed stage.	 Thorax slightly darkened. Head and abdomen clear to pale.	 Forehead darkened early in pupal stage. Slightly darkened on the thorax, clear abdomen.	 Pale head. Infuscate thorax and abdomen.	 Pale pupa. No mark on the mesosternum.
Pigmentation pattern during red-eyed stage.	 Dark thorax. Head and abdomen clear.	 Dark forehead and thorax. Infuscate abdomen.	 Thorax and abdomen dark. Clear head.	 Pale yellow pupa. Mark on the mesosternum.
Pigmentation pattern during the green-eyed stage.	 Dark thorax. Head and abdomen clear.	 Abdomen becomes infuscate towards the end of the pupal stage. Older pupae may be completely dark.	 Head becomes lightly infuscate towards the end of the pupal stage.	 Pale to bright yellow. Mark on the mesosternum.

Each specimen was then individually collected and preserved in a 2 ml Eppendorf tube with a 70% ethanol solution and then stored at -18 °C.

4.2.2. DNA extraction

DNA was extracted from specimens in Table 4.1, using the salting-out method with an adapted protocol by Sunnucks and Hales (1996), with TNES buffer (50 mM Tris, pH 7.5, 400 mM NaCl, 20 mM EDTA, 0.5% SDS) substituted for 180 µl ATL buffer (Qiagen). Incubation time at 56 °C was lengthened to overnight. The second precipitation was performed with isopropanol overnight at -20 °C.

4.2.3. Polymerase chain reaction (PCR)

Primer set LCO1498/HCO2198 (Folmer et al., 1994) was used to amplify the barcoding region of the mitochondrial cytochrome oxidase subunit I (mtCO-I). Each reaction contained KAPA (1 ×) Taq buffer A (Roche, Basel, Switzerland), 0.2 mM dNTP mix (Thermo Scientific, Waltham, MA, USA), 0.4 µM forward and reverse primers (IDT, Coralville, IA, USA), and 1.25 U/µl KAPA Taq DNA polymerase (Roche, Basel, Switzerland). All the DNA extracted was used in the reaction. Cycle conditions included an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and elongation at 72 °C for 30 s, and a final extension of 72 °C for 7 min. Amplicons were visualised using gel electrophoresis of ethidium bromide stained 1% agarose Tris Acetate-EDTA (TAE) gels which were used to confirm that the amplicons were successful and that the band length of 710 bp was correct (Folmer et al., 1994). The PCR products were bidirectionally Sanger sequenced (Central analytical facility (CAF), Stellenbosch University) with the primers used for generating the amplicon. CLC main workbench 7 (Qiagen) was used for data quality control, assembly, trimming and pairwise sequence comparisons. The sequence was annotated by finding the closest match in NCBI GenBank using BLASTn analyses.

4.2.4. Phylogenic analyses

Representative sequences of *Aphytis* spp. were obtained from GenBank and aligned with all the amplicon sequences using MAFFT version 7. Using the 500 nt alignment, Maximum Likelihood (ML) trees were inferred using IQ-Tree (IQ-TREE multicore version 1.6.12). Model selection was automatically determined, and 1000 replicates were performed for Ultra-Fast Bootstrap support. The final tree was drawn using FigTree v1.4.4

4.3. Results

Out of the 143 extractions performed, 16 samples failed to extract, 26 DNA extracts failed to amplify, and seven PCR reactions resulted in unreliable sequence data, and were therefore excluded (Table 4.3.). Of the 101 sequences generated, 95 were more like *Aphytis* spp. accessions on GenBank than any other genus. According to BLASTn analyses and phylogenetic clustering, only *A. melinus* and *A. chrysomphali* were identified. The same morphological and molecular identification to species level were assigned to 46 pupae. Only *A. melinus* and *A. chrysomphali* were identified according to BLASTn analyses and phylogenetic clustering (Figs. 4.1, 4.2, and 4.6).

The morphological identification of *A. melinus* and *A. chrysomphali* was mostly successful, as 80.95% and 90.09% of the specimens respectively, were confirmed by molecular identification as the same species. Specimen no. 238, which was morphologically identified as *A. melinus*, was molecularly identified as "Hymenoptera" (Table 4.3 and Fig. 4.3). This general identification might be because of the vast species diversity in *Aphytis* genus not represented by enough accessions on GenBank.

The morphological identifications of *A. africanus* and *A. lingnanensis* were not confirmed by the molecular identifications (Table 4.3, Fig. 4.4, and Fig. 4.5). The specimens identified morphologically as *A. lingnanensis* did not cluster with the two accessions JQ083705.1 and JQ083707.1 on GenBank, which were separated from the *A. melinus* and *A. chrysomphali* clusters with a 98% bootstrap support. It can therefore be concluded that the specimens that were morphologically identified as *A. lingnanensis* were not confirmed as same by the molecular identification.

Specimens that were in the colourless-eyed stage of all the *Aphytis* spp. were also included in this study and were morphologically identified according to the traits described for colourless-eyed pupae as in Table 4.2. The purpose of this was to explore whether these *Aphytis* spp. could possibly be identified morphologically at an earlier stage than the red- and green-eyed stages. The result of this approach was successful for *A. melinus* and *A. chrysomphali* (Table 4.3).

Both colony specimens from the overseas and the local insectary, received as adults, were molecularly identified as *A. melinus*. The bootstrap support for the separation of the reference sequences of *A. lingnanensis* from other *Aphytis* spp. was much higher at 98% compared to the bootstrap support for the separation of *A. melinus* from *A. chrysomphali* which was 74%.

There are only two sequences annotated as *A. africanus* in GenBank (Fig. 4.1). These sequences clustered separately from all other *Aphytis* sequences with a strong bootstrap support at 98%. None of the amplicon sequences clustered with these two reference sequences, none of the sampled specimens were molecularly identified as *A. africanus* (See Figs. 4.2 to 4.7).

Specimens 249 and 311 which was morphologically identified as *A. melinus* but morphologically identified as "mites" (Fig. 4.4). Specimens 266 and 526 which was morphologically identified as *A. melinus* and *A. africanus* respectively was morphologically identified as "fungus" (Fig. 4.3 and Fig. 4.4).

Table 4.3. Summarised results of morphological and molecular identifications of samples.

Results of identification	***A. melinus***	***A. africanus***	***A. lingnanensis***	***A. chrysomphali***	**Total**
Morphological identification of the samples from which sequences were generated	42	30	18	11	101
Number (and %) of samples confirmed as same species by molecular identification according to BLASTn analyses and phylogenetic clustering	36 (85.71%)	0 (0.00%)	0 (0.00%)	10 (90.09%)	46 (45.54%)
Samples failed to extract	5	5	4	2	16
No amplification yielded	3	17	2	4	26
No sequence yielded	3	1	3	0	7
Other results of molecular identification	*A. chrysomphali* = 6 Hymenoptera = 1 Fungus = 1	*A. melinus* = 9 *A. chrysomphali* = 17 Mites = 2 Fungus = 2	*A. melinus* = 17 Red scale = 1	*A. melinus* = 1	*A. melinus* = 27 *A. chrysomphali* = 23 Hymenoptera = 1 Mites =

The phylogenetic analysis of the COI barcoding region in Fig. 4.1 for *A. melinus* is presented in Fig. 4.2, listing, and showing all the pupae that clustered with the *A. melinus* JQ083689.1 Isolate_Asp1 and KX065208.1 *A. melinus* 16407 accessions. Both batches of *A. melinus* wasps obtained from the overseas insectary (Amel1 *A. melinus*) and the local insectary (BBA *A. melinus*) also clustered with these two accessions, but only at a 56% bootstrap support.

The phylogenetic analysis of the COI barcoding region in Fig. 4.1 for *A. chrysomphali* is presented in Fig. 4.6. All the specimens that clustered with the *A. chrysomphali* KX065204.1 voucher 16502 and *A. chrysomphali* JQ083695.1 isolate Ach 25 with a bootstrap support of 54% were listed here, regardless of any morphological characteristics.

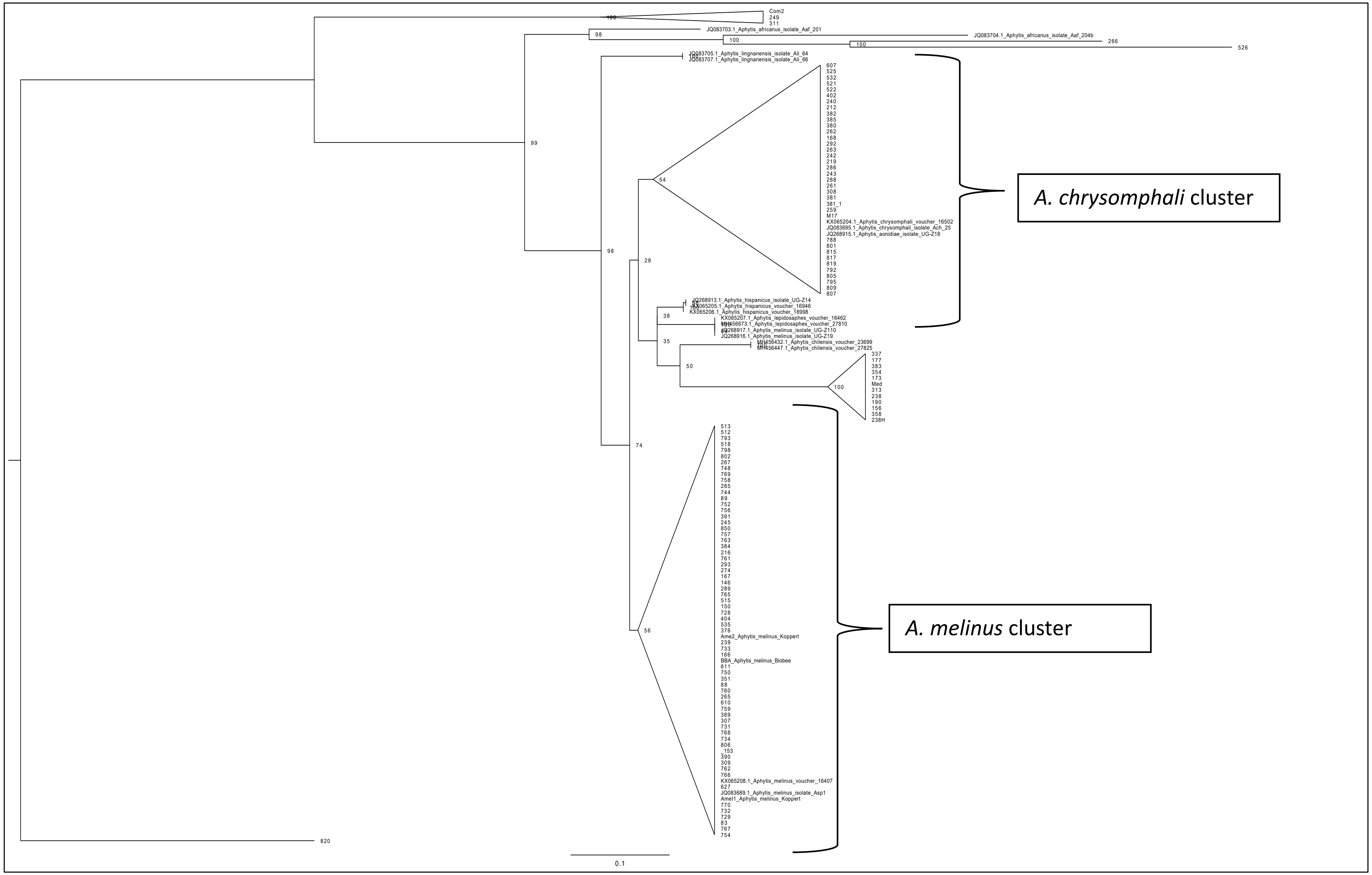
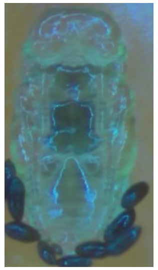
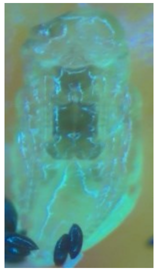
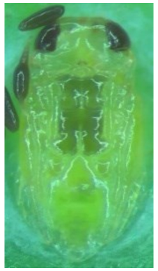
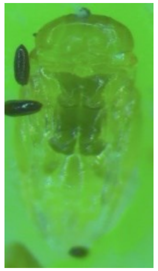
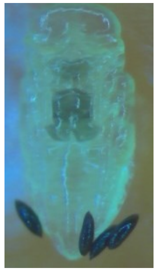
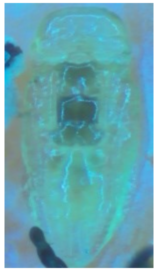
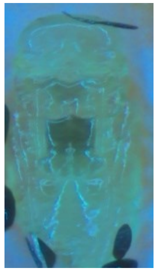
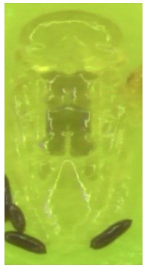
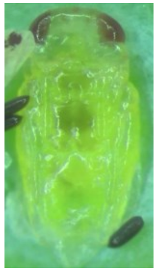
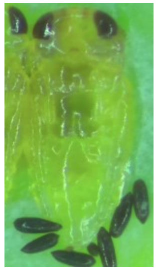
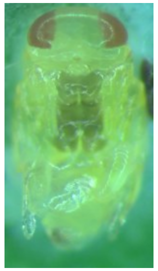
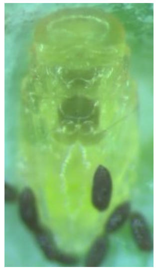
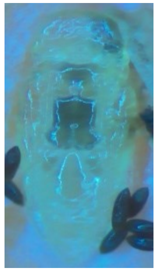
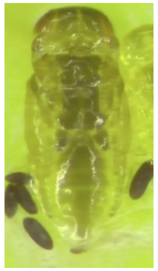
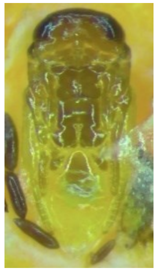
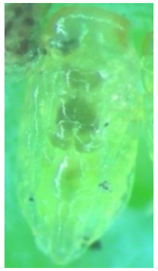
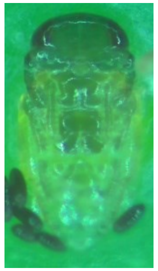
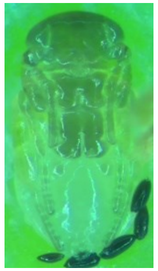
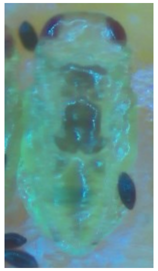
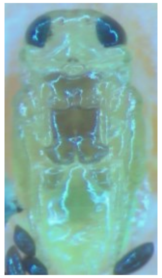
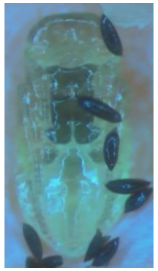
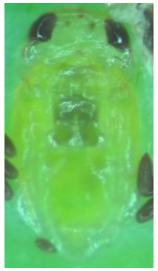


Figure 4.1. Phylogenetic analyses of the COI barcoding region of field collected *Aphytis* specimens and GenBank *Aphytis* reference sequences. A Maximum Likelihood (ML) tree was inferred using 1000 replicates for Ultra-Fast Bootstrap support. The tree was rooted using a red scale amplicon sequence generated from a field collected specimen. The different groupings used for the molecular identification are indicated by using the cartoon option on the branch.

Picture										
Morph. ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Molecular ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Source	Hexfarm 1371	Vrugbaar	Hexfarm 1272	Vrugbaar	Modderfontein 405	Vrugbaar	Hexfarm 1272	Modderfontein 401	Hexfarm 1371	Vrugbaar
Reference	274	761	239	770	351	769	245	389	265	759
Picture										
Morph. ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Molecular ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Source	Normandale 33	Vrugbaar	Vrugbaar	Vrugbaar	Modderfontein 405	Step aside 26	Normandale 33	Modderfontein 405	Vrugbaar	Vrugbaar
Reference	535	763	765	767	376	515	850	150	752	754

Picture										
Morph. ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Molecular ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Source	Vrugbaar	Vrugbaar	Vrugbaar	Hexfarm 1272	Modderfontein 405	Modderfontein 405	Hexfarm 1371	Vrugbaar	Modderfontein 405	Vrugbaar
Reference	756	757	758	307	166	167	216	744	153	768
Picture										
Morph. ID	*A. africanus*	*A. africanus*	*A. lingnanensis*	*A. africanus*	*A. lingnanensis*	*A. lingnanensis*	*A. africanus*	*A. lingnanensis*	*A. melinus*	*A. lingnanensis*
Molecular ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Source	Step aside 26	Step aside 26	Step aside 26	Melon	Melon	Melon	Hexfarm 1371	Vrugbaar	Modderfontein 405	Hexfarm 1272
Reference	513	512	518	793	798	802	267	748	89	285

Picture										
Morph. ID	*A. africanus*	*A. lingnanensis*	*A. melinus*	*A. lingnanensis*	*A. africanus*	*A. lingnanensis*	*A. africanus*	*A. lingnanensis*	*A. melinus*	*A. lingnanensis*
Molecular ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Source	Modderfontein 401	Modderfontein 401	Hexfarm 1272	Vrugbaar	Hexfarm 1272	Vrugbaar	Modderfontein 405	Vrugbaar	Vrugbaar	Vrugbaar
Reference	391	384	293	762	289	728	404	766	732	733
Picture										
Morph. ID	*A. lingnanensis*	*A. melinus*	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*	*A. chrysomphali*	*A. africanus*	*A. africanus*	*A. lingnanensis*	*A. melinus*
Molecular ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Source	Vrugbaar	Modderfontein 405	Vrugbaar	Vrugbaar	Vrugbaar	Melon	Modderfontein 401	Hexfarm 1272	Vrugbaar	Modderfontein 401
Reference	750	88	760	731	734	806	390	309	729	83

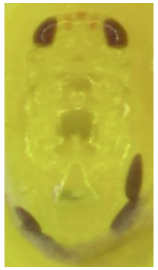
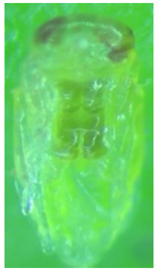
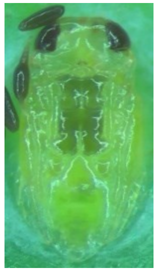
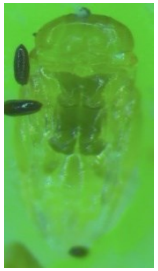
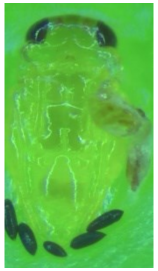
Picture				No picture available	No picture available	No picture available	No picture available
Morph. ID	*A. lingnanensis*	*A. melinus*	*A. lingnanensis*	N. a.	N. a.	N. a.	N. a.
Molecular ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Source	Modderfontein 405	Modderfontein 405	Hexfarm 1371	JQ083689.1 *A. melinus*	Amel1 *A. melinus*	BBA *A. melinus*	KX065208.1 *A. melinus*
Reference	610	611	627	Isolate_Asp1	Overseas	Local insectary	16407

Figure 4.2. A comparison between morphological and molecular identification listed as *A. melinus*, according to phylogenetic clustering with the *A. melinus* JQ083689.1 Isolate_Asp1 and KX065208.1 *A. melinus* 16407 accessions at a 56% bootstrap support.

It is difficult to find common morphological characteristics amongst all the pupae that clustered together with *A. melinus* (Fig. 4.2). Specimen no. 802 morphologically resembles *A. lingnanensis* but clustered with *A. melinus*, however at a bootstrap support of only 56% (Fig. 4.2). Other specimens from the *A. melinus* phylogenetic cluster being morphologically typically *A. africanus* are 513, 512, 793, 267, 391, 289, 404, 390, and 309 as examples.

Picture										
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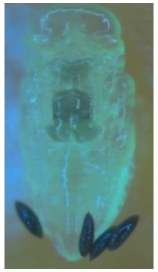
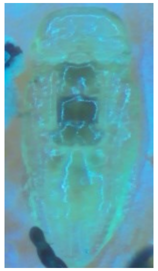
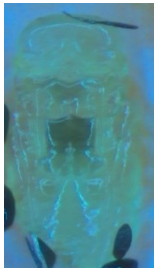
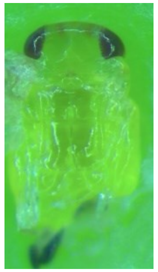
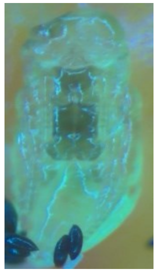
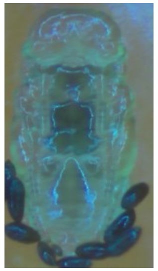
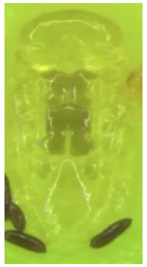
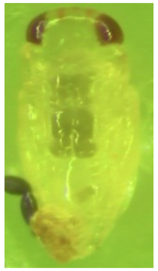
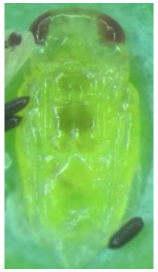
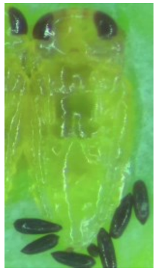
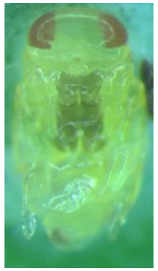
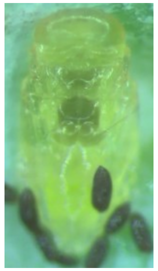
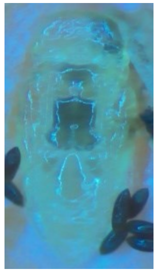
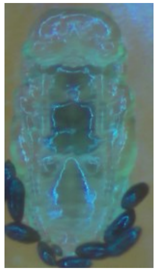
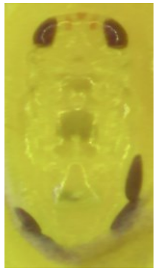
Molecular ID	*A. melinus*	Hymenoptera	*A. melinus*	*A. chrysomphali*	*A. melinus*	*A. chrysomphali*	*A. melinus*	*A. chrysomphali*	*A. melinus*	Fungus
Source	Hexfarm 1371	Hexfarm 1272	Hexfarm 1272	Hexfarm 1272	Modderfontein 405	Hexfarm 1272	Hexfarm 1272	Hexfarm 1371	Hexfarm 1371	Hexfarm 1371
Reference	274	238	239	240	351	242	245	259	265	266
Picture										
Morph. ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Molecular ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. chrysomphali*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Source	Vrugbaar	Vrugbaar	Vrugbaar	Vrugbaar	Modderfontein 405	Modderfontein 401	Modderfontein 401	Vrugbaar	Vrugbaar	Vrugbaar
Reference	759	763	765	767	376	380	389	769	770	761

Picture										
Morph. ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Molecular ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Source	Step aside 26	Normandale 33	Normandale 33	Modderfontein 405	Vrugbaar	Vrugbaar	Vrugbaar	Vrugbaar	Vrugbaar	Hexfarm 1272
Reference	515	535	850	150	752	754	756	757	758	307
Picture taken										
Morph. ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Molecular ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Source	Modderfontein 405	Modderfontein 405	Hexfarm 1371	Vrugbaar	Modderfontein 405	Vrugbaar	Vrugbaar	Vrugbaar	Modderfontein 405	Modderfontein 405
Reference	166	167	216	744	153	768	770	761	89	611

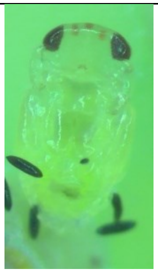
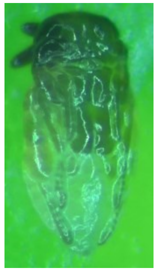
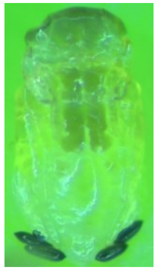
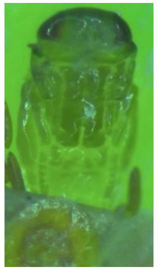
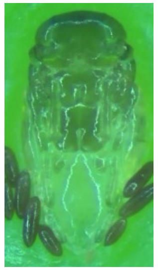
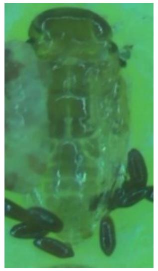
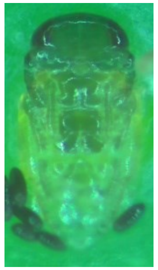
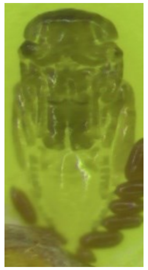
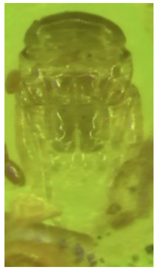
Picture		
Morph. ID	*A. melinus*	*A. melinus*
Molecular ID	*A. chrysomphali*	*A. chrysomphali*
Source	Hexfarm 1272	Hexfarm 1272
Reference	292	243

Figure 4.3. A comparison between morphological and molecular identification listed as *A. melinus*, according to morphological identification.

Picture										
Morph. ID	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*
Molecular ID	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. melinus*	*A. chrysomphali*	*A. melinus*	*A. melinus*
Source	Hexfarm 1371	Hexfarm 1371	Hexfarm 1371	Hexfarm 1371	Hexfarm 1272	Hexfarm 1272	Hexfarm 1272	Hexfarm 1272	Hexfarm 1272	Hexfarm 1371
Reference	219	261	262	263	286	288	289	308	309	267
Picture										
Morph. ID	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*
Molecular ID	Mite	Mite	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. melinus*	*A. melinus*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*
Source	Hexfarm 1371	Hexfarm 1272	Modderfontein 401	Modderfontein 401	Modderfontein 401	Modderfontein 401	Modderfontein 401	Modderfontein 405	Normandale 33	Normandale 33
Reference	249	311	381	382	385	390	391	402	521	522

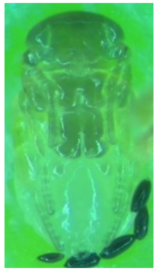
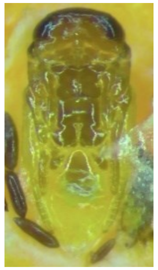
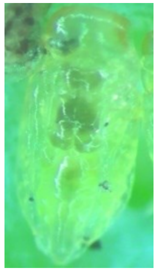
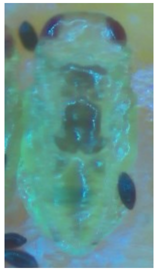
Picture taken										
Morph. ID	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*
Molecular ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. chrysomphali*	Fungus	*A. chrysomphali*	*A. melinus*	*A. chrysomphali*	*A. africanus*	*A. chrysomphali*
Source	Modderfontein 405	Step aside 26	Step aside 26	Normandale 33	Normandale 33	Normandale 33	Melon	Modderfontein 405	Normandale 33	Step Aside 23 T
Reference	404	512	513	525	526	532	793	168	526	607

Figure 4.4. A comparison between morphological and molecular identification listed as *A. africanus*, according to morphological identification.

Specimens 308 and 381 appear morphologically to be *A. africanus*, rather than *A. chrysomphali*, and specimen 259 resembles *A. melinus* more closely than it does *A. chrysomphali* (Fig. 4.6).

Picture										
Morph. ID	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*
Molecular ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*
Source	Vrugbaar	Hexfarm 1272	Modderfontein 401	Step aside 26	Vrugbaar	Vrugbaar	Vrugbaar	Vrugbaar	Vrugbaar	Vrugbaar
Reference	748	285	384	518	728	729	731	733	734	766

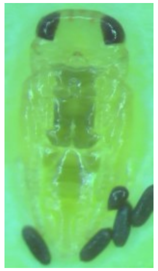
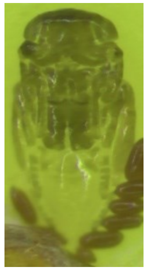
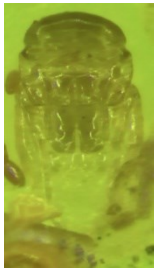
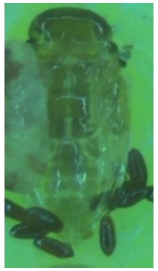
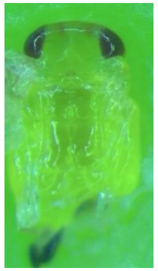
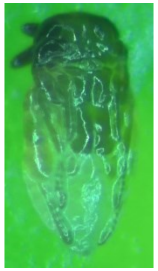
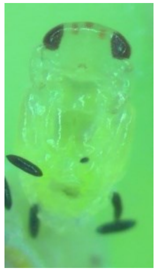
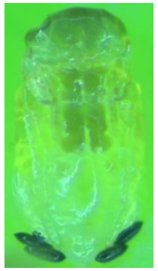
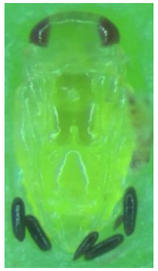
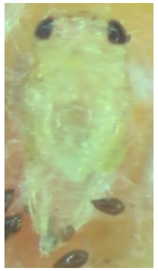
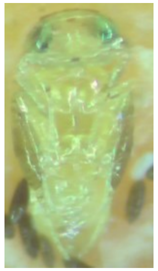
Picture								
Morph. ID	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*	*A. lingnanensis*
Molecular ID	*A. melinus*	*A. melinus*	*A. melinus*	*A. melinus*	Red scale	*A. melinus*	*A. melinus*	*A. melinus*
Source	Vrugbaar	Vrugbaar	Vrugbaar	Melon	Melon	Melon	Modderfontein 405	Hexfarm 1371
Reference	750	760	762	798	820	802	610	627

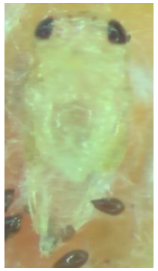
Figure 4.5. A comparison between morphological and molecular identification listed as *A. lingnanensis*, according to morphological identification.

Picture										
Morph. ID	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. africanus*	*A. melinus*	*A. africanus*	*A. africanus*	*A. melinus*
Molecular ID	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*
Source	Step Aside 23 T	Normandale 33	Normandale 33	Normandale 33	Normandale 33	Modderfontein 405	Hexfarm 1272	Modderfontein 401	Modderfontein 401	Modderfontein 401
Reference	607	525	532	521	522	402	240	382	385	380
Picture										
Morph. ID	*A. africanus*	*A. africanus*	*A. melinus*	*A. africanus*	*A. melinus*	*A. africanus*	*A. africanus*	*A. melinus*	*A. africanus*	*A. africanus*
Molecular ID	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*
Source	Hexfarm 1371	Modderfontein 405	Hexfarm 1272	Hexfarm 1371	Hexfarm 1272	Hexfarm 1371	Hexfarm 1272	Hexfarm 1272	Hexfarm 1272	Hexfarm 1371
Reference	262	168	292	263	242	219	286	243	288	261

Picture										
Morph. ID	*A. africanus*	*A. africanus*	*A. melinus*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*
Molecular ID	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*
Source	Hexfarm 1272	Modderfontein 401	Hexfarm 1371	Melon	Melon	Melon	Melon	Melon	Melon	Melon
Reference	308	381	259	788	801	815	817	819	792	805

Picture				No picture available	No picture available	No picture available
Morph. ID	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	N. a.	N. a.	N. a.
Molecular ID	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. aonidiae*
Source	Melon	Melon	Melon	KX065204.1 *A. chrysomphali* voucher	JQ083695.1 *A. chrysomphali* isolate	JQ268915.1 Isolate
Reference	795	809	807	16502	Ach 25	UG-Z18

Figure 4.6. A comparison between morphological and molecular identification listed as *A. chrysomphali*, according to phylogenetic clustering with the *A. chrysomphali* KX065204.1 voucher 16502 and *A. chrysomphali* JQ083695.1 isolate Ach 25 at a 54% bootstrap support.

Picture										
Morph. ID	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*
Molecular ID	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. melinus*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*	*A. chrysomphali*
Source	Melon	Melon	Melon	Melon	Melon	Melon	Melon	Melon	Melon	Melon
Reference	788	819	792	795	801	806	807	809	815	817

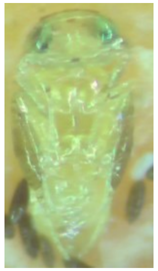
Picture	
Morph. ID	*A. chrysomphali*
Molecular ID	*A. chrysomphali*
Source	Melon
Reference	805

Figure 4.7. A comparison between morphological and molecular identification listed as *A. chrysomphali*, according to morphological identification.

4.4. Discussion

In this chapter both morphological and molecular tools was utilised to identify *Aphytis* spp. in field populations present in South Africa. The correlation between morphological and molecular identification was assessed to glean a better understanding of the diversity of *Aphytis* populations in South Africa.

The probability of failed extractions like when no amplification is yielded, is high because of the low amount of DNA material of suboptimal quality available for the PCR test. Since a single pupa is individually sampled from natural field populations, pupae from different host scales cannot be combined to create a larger sample with more DNA to be tested. The low amount of DNA extracted from each specimen and the short 500 nucleotide COI region (insect barcoding region) used in the current analyses limit the analyses that can be performed. The molecular identification of all *Aphytis* spp. will benefit greatly from the generation of more sequence information, either complete mitochondrial genomes or other genome segments that can be utilised for identification. This will only be possible from colony specimens, as a high DNA concentration is required for high-throughput sequencing. A high DNA concentration can only be obtained from an extraction from multiple specimens. Field collected specimens do not represent a homogenous population and therefore multiple specimens cannot be pooled, even if the morphological identification can confirm that all samples are the same species.

The morphological identification of *A. melinus* was 85.71% accurate against the molecular identification. However, there were only 22 data sets presenting *A. melinus* in the NCBI GenBank. The few inputs for *A. africanus* and *A. lingnanensis* in the nucleotide database of the National Centre of Biotechnology Information (NCBI) GenBank, limits the accurate and trustworthy molecular identification of these mentioned species. It seems that the two and six sets, which are available for *A. africanus* and *A. lingnanensis*, respectively, is not adequate to provide a reliable resolution. It is also a possibility that some annotations in the database have been wrongly executed (Buckner et al., 2021).

Although morphological identification for *A. africanus* pupae was done strictly and consistently using all available identification keys, the results were largely inconsistent with molecular identification. To support the result of the morphological identification, pupae that were identified as *A. africanus*, were always found singly and never more than one or two per host scale, as with *A. melinus* and *A. lingnanensis* (DeBach & Sundby, 1963; Annecke & Moran, 1982; Bedford & Cilliers, 1994).

There are still more morphological similarities amongst specimens when grouped according to pupal pigmentation patterns, as opposed to phylogenetic clustering based on molecular identification. This seems to be logical, but to develop identification keys one would expect more morphological similarities amongst the grouping of phylogenetic clusters.

The poor correlation between the morphological and molecular identification of *A. lingnanensis* could be explained by the small data base in the NCBI GenBank and the possible rich and diverse species complex existing in South Africa, of which *A. coheni* is a part. Also, there are no known morphological differences between *A. lingnanensis* and *A. coheni* (Debach, 1959). *Aphytis lingnanensis* and *A. coheni* are sibling or cryptic species and morphologically identical. For this reason, the name *A. lingnanensis-coheni* has been used in South African literature (Prinsloo, 1984).

Cryptic species resembling *A. africanus* morphologically may also exist, as all the *A. africanus* specimens clustered with either *A. melinus* or *A. chrysomphali*, while only two accessions of *A. africanus* are present on GenBank. The accessions JQ083703 and JQ083704 were submitted by Pina et al. (2012) based on specimens provided by Grout (2020) from Mpumalanga in South Africa. The two sequences annotated as *A. africanus* are only 72% identical on nucleotides sequence level and 68-81% and 62-72% identical to the other *Aphytis* sequences, respectively. The nucleotide variation between the sequences identified as *A. melinus* are only 8.3% and between the *A. chrysomphali* sequences it is only 7.3%. This indicates that more *A. africanus* sequences are needed to understand the diversity observed between the sequences annotated as *A. africanus*.

Red scale, fungus, and mite identifications as outcomes of the PCR test can be a result of contamination when *Aphytis* spp. pupae were collected. Since DNA was extracted from all the posing material in the ethanol solution, the presence of any other organism could have interfered with the aim here.

The COI region used is potentially not diverse enough to differentiate between closely related species, it is therefore suggested that sequencing of the 28s ribosomal RNA be added in future studies.

Chapter 5: General discussion

5.1. Introduction

Red scale is one of the most important pests of citrus grown in South Africa. As a phytosanitary pest, it may render fruit exported to Japan and South Korea unmarketable, and it has an effect on tree health (Bedford, 1998; DALRRD, 2005; Forster et al., 1995; Samways, 1986b). *Aphytis* spp., as natural enemies, is acknowledged worldwide as the primary biological control agents for this pest (Grout & Richards, 1992; Bedford, 1998; Forster et al., 1995; Quednau & Annecke, 1963; Bedford & Cilliers, 1994; Samways, 1986a).

Since *A. melinus* has recently become commercially available for augmentation in South Africa, trials on its efficacy on red scale were conducted in orchards across the Eastern and Western Cape provinces during the 2019/2020, 2020/2021 and 2021/2022 citrus production seasons. Surprisingly, releases of *A. melinus* from a local and an overseas insectary did not improve red scale control above that of the untreated control in any of the trial orchards. However, red scale was successfully controlled by naturally occurring *Aphytis* spp. in all the orchards, except for two sites, where parasitism was negligible, possibly due to the negative effect of kaolin sprays in these two orchards. In all the other orchards, *A. africanus*, which is indigenous to Southern Africa, dominated red scale attacks.

There are various reasons why *A. africanus* is more effective than naturally occurring *A. lingnanensis* and *A. melinus*, which was introduced in the 1960s. Since *A. africanus* lays significantly fewer eggs than the introduced species and lays its eggs singly per red scale host, it is better adapted to be a low-density parasitoid and therefore has a higher chance of surviving (Quednau, 1964b; DeBach & Sundby, 1963). Furthermore *A. africanus,* being indigenous, is better adapted to the local climatic conditions. *Aphytis africanus* also attacks mostly second instar male and female red scales, while *A. melinus* favours second and third instar female red scales, and *A. lingnanensis,* mostly the third instar female red scales (Bedford, 1998; Forster et al., 1995; Luck & Podoler, 1985). The behaviour of *A. africanus* enables the parasitoid to exhaust available host scales first before other *Aphytis* spp.

Augmentation of commercially produced natural enemies is widely employed in agriculture and considered one of the solutions to control pests, which in turn, will reduce dependency on potentially dangerous pesticides that don’t lend themselves to sustainable control (Grafton-Cardwell & Stewart-Leslie, 1998; Van Lenteren, 2004; Snyder, 2019). As artificial rearing conditions face challenges with regard to the quality of such beneficial agents, it is necessary to rear in accordance with best practices (Bigler et al., 1994; Dutton & Bigler, 1995; Van Lenteren et al., 2003). Flight and longevity tests on hymenopteran parasitoids are both appropriate proxies for field survival and performance after release (Vasquez & Morse, 2012; Cerutti & Bigler, 1995; Dutton & Bigler, 1995). Here, *A. melinus* from two insectaries, local and abroad, was subjected to flight and longevity tests. There were significant differences in the longevity between the wasps from the two insectaries. However, the time that the imported wasps spent in transit was not included in the longevity calculations, clearly highlighting the importance of swift logistics. There were no significant differences between wasps from the two insectaries when the results of the flight tests of all the replicates were combined. However, only a low number of wasps was able to initiate flight. Since so few of the wasps could initiate flight, wing asymmetry, possibly due to genetic bottlenecks, cannot be ruled out in commercially reared parasitoids. Unfortunately, the quality of wasps from both insectaries was unknown before augmentation in the orchards, and this could have affected some of the results, as we might have been able to adjust the number of wasps released to compensate for the poor quality.

There are currently four approaches that are used to differentiate the *Aphytis* spp. from one another i.e. morphological, molecular, geographical, and biological. The integration of these approaches is often necessary for comprehensive and therefore accurate identification (Pina et al., 2012).

Morphological identification is used to separate species from one another, according to physical differences, but it is often very difficult, such as when only certain life stages are available or when cryptic species exist (Syromyatnikov et al., 2017; Compere, 1961; Bickford et al., 2007). Because of the small size of *Aphytis* spp. and the existence of only a few reliable distinguishing characteristics, the morphological identification of *Aphytis* spp. is complex. Furthermore, the existence of cryptic species, and some of the species being uniparental (no mating required for reproduction), species separation is often near impossible (Rosen & DeBach, 1979). With *Aphytis* spp., morphological identification is mainly based on distinct pigmentation patterns during the pupal life stage and the physical characteristics of the adults (Debach, 1959).

Molecular identification, which has recently become very popular (Hebert et al., 2003; Barrett & Hebert, 2005; Gariepy et al., 2007), entails DNA barcoding and phylogenetic analysis to map out taxonomic association of relevant insect species. This technique includes sequencing the mitochondrial cytochrome C oxidase 1 profile, which has been shown to be an effective method of identifying and differentiating various insect species from one another. Species identification is normally very difficult to accomplish by means of the morphological identification process, which requires scarce taxonomical expertise (Rugman-Jones et al., 2011; Barrett & Hebert, 2005; Tahir et al., 2018; Ball & Armstrong, 2006; Sigut et al., 2017). This study showed that the morphological approach was not satisfactory for *Aphytis* identification, and that more molecular techniques, such as DNA barcoding, are necessary to determine accurate identification. However, as amplification and sequencing products of gene fragments are compared to relevant sequences in existing databases, such as in GenBank, an adequate number of reliable accessions should be present in such a database to provide a clear resolution on the submitted sample (Syromyatnikov et al., 2017; Rugman-Jones et al., 2011), which is not the case for the genus *Aphytis*.

Biological separation of individuals or populations can be based on distinct differences in their behaviour (Quednau, 1965; Mayr, 1948; Rosen & DeBach, 1976). For instance, the ability of different parasitoid populations to reproduce or not will explain species reproduction isolation. A disadvantage of the method is highlighted by uniparental species, when no mating is required to produce offspring, as with *A. chrysomphali* (Rosen & DeBach, 1979). Host acceptance tests with mixed populations of parasitoids can be applied usefully to biologically separate parasitoids that appear morphologically identical, but this is time consuming and often impractical (Quednau, 1965).

5.2. Considerations on the control of red scale with *Aphytis* spp.

5.2.1. Species identification

The correct identification of biological control agents is critical in compiling sustainable IPM systems (Rosen & DeBach, 1973; Compere, 1961; DeBach, 1969; Debach, 1960). Four *Aphytis* spp. were morphologically separated and identified when samples were collected from trial and other orchards (see Chapter 4). It would be important to be able to promptly identify the specimens with more certainty for future research. Because of the need to evaluate the success of augmentation of *A. melinus*, coupled with the existence of cryptic species in this genus, dependable methods for species identification must be explored. Although there are many benefits to using molecular identification to identify cryptic species, it relies on an adequate number of correct accessions on GenBank, which, as we showed in this study, are lacking for certain groups. Integrative taxonomy (biological, molecular, geographical and morphological) (Pina et al., 2012; Quednau, 1965; Mayr, 1948) should rather be applied to cryptic species to serve as an accurate identification method.

It would, however, be a major benefit to the South African citrus industry if revised morphological keys could be developed with the backstop of molecular identification for *A. africanus*, *A. melinus*, *A. lingnanensis* and *A. chrysomphali*. It is important that these identification aids are simple and practical, to ensure broader participation in their use and application. This study has succeeded in providing examples of pupal pigmentation patterns from the colourless-eyed stage, to the red- and green-eyed stages, which agreed well with graphics provided by Debach (1959, Prinsloo (1984), and Wang et al. (2021). Pupal pigmentation patterns are still the most useful stage for morphological identification because the pest is immobile and easy to find when red scale parasitism assessments are done. A graphic illustration of the pupal life stage progressing from colourless to final pupal pigmentation and the green-eyed stage would be of assistance to growers, technical personnel on farms, and extension officers. However, monitoring adult *Aphytis* spp. in this study with yellow sticky cards has been shown to be an effective method for monitoring wasp presence and activity, but the ability to morphologically identify the adults caught on the sticky traps would be very useful. Unravelling the composition of the *Aphytis* spp. complex at an orchard level would aid in constructing intervention thresholds, which is critically needed, as late-corrective sprays are expensive, not very effective, increase chemical residue levels on fruit, and increase chances of insect resistance development.

The poor and inconsistent molecular identification of *A. africanus* either points to the small number of nucleotide sequence accessions presently available on GenBank, or the vast genetic variation within the species, or maybe both. *Aphytis africanus* has been found only in Southern Africa to date, which has led to very little research being done on it, relative to other *Aphytis* spp. occurring elsewhere in the world, which have drawn broader research interest. Although Annecke and Moran (1982) reported that *A. africanus* is not attacked by the hyperparasitoid, *Marietta javensis*, this study found many pupae that resembled the appearance of *A. africanus* seen with either an *M. javensis* egg or larva. Also, the typical *A. africanus* exuvia, with the dark forehead, was observed at the scene of *M. javensis* parasitism*.* This is a clear case where biological traits and behaviour during interactions with hyperparasitoids might help to distinguish between cryptic species that should be investigated further.

Aphytis coheni was introduced into South Africa in the 1960s, together with *A. lingnanensis* (Bedford, 1998). It is very difficult to distinguish between these two species, as they appear morphologically identical, and they were never taxonomically separated in South Africa. Consequently, the name *A. lingnanensis-coheni* was assigned. In Israel, *A. coheni* has not been displaced by *A. lingnanensis* from the warmer inland valleys where it plays a major role in red scale control. This shows that *A. coheni* possesses the ability to contribute uniquely to red scale control if properly identified and acknowledged (Avidov et al., 1970; Rosen, 1965; Ben-Dov & Rosen, 1969). Judging by the inaccurate results of the molecular identification outcome of "*A. lingnanensis*" in Chapter 4, the possibility that there are other cryptic species within our morphological version of *A. lingnanensis* must be further researched.

5.2.2. Evaluation of red scale attacks by *Aphytis* spp.

The assessment of red scale parasitism and mutilation started in January each year, with one or two exceptions where red scale infestation was very low. In these orchards, it was necessary to wait for the red scale infestation to increase. In hindsight, future research should be initiated in December so that evaluations could more likely start with 100% healthy red scales in each orchard, that is, before the first signs of parasitism and parasitoid mutilation of scales. Furthermore, it would be insightful to shorten the assessment intervals from four weeks to two weeks, which would double the number of samples taken. Better trendlines which reveal even more accurate information would be available since the life cycle of *A. melinus* can be as short a 24.83 days at 20 °C during the summer months (Yu & Luck, 1988).

5.2.3. Augmentation with *A. melinus*

Aphytis spp., as a group, is recognised as the main biological control agents for red scale on citrus (Forster et al., 1995; Rosen & DeBach, 1979; Bedford & Cilliers, 1994). Various *Aphytis* spp. are present in all the citrus-producing regions of South Africa, either by virtue of natural presence, as is the case with *A. africanus,* or because of the introduction of exotic species. *Aphytis lingnanensis* and *A. melinus* were intentionally introduced into South Africa early in the 1960s, while *A. chrysomphali* was probably introduced with infested plant material at about the same time (Bedford & Grobler, 1981; Quednau & Annecke, 1963; Annecke, 1969; Bedford & Cilliers, 1994). All these introduced *Aphytis* spp. have established and now occur naturally in South African citrus orchards, alongside the indigenous *A. africanus* (Bedford, 1998).

When red scale numbers are high in the orchards, it is assumed that parasitoid numbers are low, and the convention is for growers to resort to the release of commercially reared *A. melinus*. However, this is invariably based on little scouting information with regard to parasitoid numbers, and the perception that there is little to no natural occurrence of *Aphytis* spp. parasitoids in the orchard. Further, these *A. melinus* releases are often made during the second half of the season and too close to harvest, when there is not enough time left for the released *A. melinus* to accumulate to an effective parasitoid population. In addition, the current recommendation by biological control agent suppliers, where only 40 000 to 60 000 *A. melinus* wasps are released per ha, comes from a mindset that the cost thereof should be comparable to chemical options against red scale, while the number of wasps should rather be based on the population dynamics of the pest and the parasitoid. This approach is problematic as biological control then suffers a negative reputation (Van Lenteren, 2012b; Van Lenteren, 2004) and growers return to pesticide applications.

Aphytis africanus is very effective in controlling red scale in citrus in South Africa when compared to other red scale parasitoids (Bedford, 1998; Daneel & Ware, 2000; Samways, 1986a); it is indigenous, adapted to local climatic conditions, and prefers the second instar of red scale females and males. *Aphytis melinus* and *A. lingnanensis*, which were introduced into South Africa with the aim of achieving improved parasitism, and are possibly not as well adapted as *A. africanus,* attack later (larger) life stages of red scale females and do not favour males (Bedford & Cilliers, 1994). This study showed that *A. africanus* was responsible for the greatest suppression of red scale populations, and not the augmented, or naturally occurring *A. melinus* and *A. lingnanensis*. In this study, augmented

A. melinus did not improve the control of red scale above that of any of the untreated control orchards because of the dominating presence of *A. africanus* in all the trial orchards and the poor fitness of commercially available *A. melinus* wasps. It is therefore not considered worthwhile to release *A. melinus* where *A. africanus* is present. *Aphytis melinus* might only be effective when released where *A. africanus* is not present, which would be an anomalous situation. This may only be possible if climatic conditions are not suitable for *A. africanus*, as *A. melinus* is better adapted to high temperatures than *A. africanus* (Samways, 1985; Atkinson, 1977; Bedford, 1998). If *A. africanus* is not present due to poor IPM practices, augmentation with *A. melinus* will then also not be effective. If no or very low numbers of *A. africanus* are present while accommodating IPM practices were followed, the augmentation of *A. melinus* might be considered, but only if the following criteria are met:

- Wasps should be delivered from the insectary to destination orchards as soon after their eclosion as possible, so that the full oviposition (parasitism) potential of *A. melinus* can be realised. *Aphytis melinus* lays most of its eggs during the first four to seven days after eclosing (Quednau, 1964b). If wasps spend time in transit, fewer total eggs will be laid per female after release, since females cannot compensate by delaying oviposition and increasing their rate of egg-laying later (Vanaclocha et al., 2014). Since stressed and starved Hymenoptera also lay fewer eggs, due to egg resorption (Collier, 1995; Hopper et al., 2013), the number of eggs laid by commercially available *A. melinus* should also be tested after an extended transit period, as part of a comprehensive fitness assessment procedure, as such transit could be a meaningful stress factor.
- The sex ratios of *A. melinus* wasps must be female-biased and a ratio of at least 1.2 to 1.5 females to males should be achieved throughout the year. Lower female to male ratios would require each female to lay more eggs to attain the same rate of parasitism as *A. melinus* populations with higher female sex ratios (DeBach & White, 1960).
- Wasps should be fed with honey during transit and before release. Although protein acquired by means of host feeding is necessary for egg production, nectar, honeydew or honey as a sugar source is required for energy to forage for suitable hosts and for ovipositing (Tena et al., 2015; Lenaerts et al., 2016; Calabuig et al., 2015). Females seldom live longer than two days without honey in laboratory conditions (Vasquez, 2010). Although current packaging of *A. melinus* wasps includes honey applied to cottonwool swabs as a food source during transit, it is ineffective as it is dark inside the paper cups and wasps need light to be able to find the honey (Rosen & DeBach, 1979; DeBach & White, 1960).

Inoculative augmentation should be conducted early in the season, preferably during spring (September). This extensive period before fruit harvest will enable augmented *A. melinus* to increase to large effective populations. Larger populations of *A. melinus,* in turn, have a higher chance of controlling red scale down to acceptable levels of infestation. Also, nectar from citrus and other blossoms from a diverse plant community are in abundance in spring to serve as an energy source to *A. melinus* wasps. Another benefit of releasing parasitoids early in the season is that the crop protection spray programmes are then adjusted to accommodate such parasitoids, that is, by not using pesticides that are harmful to specific parasitoids. This window that is created in the crop protection spray programme, often unintentionally, facilitates the buildup of naturally occurring *A. africanus* to a level that effectively controls red scale.

Inundative augmentation of *A. melinus* has not been tested in South Africa. This entails augmenting very large numbers of wasps per ha during spring. Even if the released *A. melinus* does establish in the target orchards, the released generation itself is supposed to have an immediate effect on the red scale populations. Since not all the life stages of red scale are susceptible to attack by *Aphytis* spp., a second release might be considered. Red scales that were in the moulting and adult stages (not susceptible to *Aphytis* spp. attack) during the first release are later in the susceptible (instar) stages. However, it is doubtful that such an approach would ever be followed because of the high cost of commercially available *A. melinus* wasps.

Ultimately, when there is no biological control of red scale in an orchard, it is very likely that the released *A. melinus* will also not be effective.

5.2.4. Biological control of red scale by *A. africanus*

As *A. africanus* is indigenous, well adapted and the most abundant and effective red scale parasitoid in South Africa, it has not been displaced by *A. melinus*, *A. lingnanensis* or *A. chrysomphali*, as shown by this study, even though these other *Aphytis* spp. were introduced as long ago as the early 1960s. Hence, *A. africanus* appears to be a very attractive option for mass-rearing for augmentation to control red scale in citrus orchards. Although the uniparental strain of oleander scale (*Aspidiotus nerii*) is itself easy to breed on banana squash, and the best host scale to rear large and fecund *A. melinus* and *A. lingnanensis* at a suitable sex ratio, oleander scale is not a host to *A. africanus*. Since *A. africanus* is indigenous to southern Africa, its natural host scale species must be found which will hold answers to which host plants can be used in insectaries to rear *A. africanus.* Currently, insectaries wanting to rear *A. africanus* need to initiate and maintain a red scale population on citrus fruit. This is not currently feasible, as no practical method for sustainable rearing of red scale on citrus fruit in an insectary has been developed (Daneel & Ware, 2000) (unpublished data). Even if it was practical to have *A. africanus* commercially available to growers, it would not be worthwhile to pursue releases, as this species is ever-present if IPM-friendly practices are followed, which is, in any case, a prerequisite for releases of any *Aphytis* spp.

5.2.5. Conservation agriculture and IPM

Currently in the citrus industry, releases are made mostly into typical agro-ecosystems that lack biodiversity. Citrus orchards without adequate biodiversity, like flowering plants that provide nectar to hymenopteran wasps, would most likely not sustain released parasitoids. Also, deleterious pesticides are applied, especially during the first half of the fruiting growing season, exactly when *Aphytis* spp. are supposed to establish. There are many aspects that should be investigated to determine whether an orchard is a suitable environment to receive reared *A. melinus* or not. Although a healthy and diverse community of plants and arthropods is not a guarantee of successful biological control, it is a definite prerequisite thereof (Tscharntke et al., 2016; Jonsson et al., 2017). The availability of nectar or honeydew is important for adult *A. melinus* parasitoids (Tena et al., 2015; Tena et al., 2013). Furthermore, no dust in and around orchards should be tolerated, because of its negative effect on hymenopteran parasitoids. Dust particles are very fine, and if deposited regularly, the effect can be the same as deleterious chemicals to beneficial arthropods (Rosen & DeBach, 1979). (Hulley, 1962; Compere, 1961; Rosen & DeBach, 1979; Debach, 1965). All orchards should be free of ants that attend honeydew-producing hemipteran pests in trees. Ant activity disrupts *Aphytis* spp. activity, and certain ant species cause concomitant infestations of red scale, among other non-honeydew-producing insect pests (Rosen & DeBach, 1979; DeBach et al., 1951; Steyn, 1954b; Compere, 1961; Annecke, 1958; Bedford, 1968; Steyn, 1954a; Pekas et al., 2011; Samways et al., 1982; Samways, 1983; Samways et al., 1998; Flanders, 1945). It has also been demonstrated that ants compete with *Aphytis* spp. for honeydew as a source of carbohydrates produced by non-*Aphytis* hosts (Calabuig et al., 2015; Tena et al., 2013). Ants also prey on *Aphytis* wasps (Heimpel, Rosenheim & Mangel, 1997).

A general awareness of the benefits and potential of *Aphytis* spp. should be advocated in the citrus industry. Growers, technical managers and distributors of agricultural remedies and biological control agents especially should be fully aware of how effective naturally occurring *Aphytis* spp. can be to resolve red scale problems. Growers and technical personnel on farms should be convinced to invest in the necessary equipment, like microscopes, and the training to be able to use them. This would enable them to explore and experience first-hand how effective *Aphytis* spp. host parasitism and mutilation is, and how it progresses through the season in IPM-friendly environments.

Because red scales do not immediately fall off fruit when they are killed by parasitoids, their extended presence, even when dead, creates an exaggerated impression of the severity of infestation. This might lead the uninformed grower to apply unnecessary chemical remedies to control an infestation that is already under control. The installation of high-pressure spray systems in packhouses to remove dead red scales would be beneficial to growers and will become a necessity as we progress into an era when the use of chemical pesticides will be limited. During this study it was evident that a high red scale infestation was recorded in many orchards, but that most of the red scales were dead and easily removable by such a fixture in a packhouse.

Research is needed in South Africa to develop intervention thresholds, based not only on the level of red scale infestation, but also on levels of parasitism and host mutilation, taking into consideration the cultivar and projected harvest time. Work in this field was conducted by Moore and Richards (2002, unpublished data) in the early 2000s on which further research can be based. Hopefully, this should then enable decision making on whether corrective chemical applications are necessary or not.

Artificially providing sugar formulations to enhance resident *Aphytis* spp. has been shown to increase parasitism and host mutilation (Tena et al., 2015). This is currently an unutilised practice in South Africa, which should be a research priority, aimed at determining if *A. africanus* populations present in orchards can also be enhanced when sugar is artificially provided in local citrus ecosystems. As citrus ecosystems lack biodiversity, flowering plants providing nectar are generally not present, while honeydew-producing hemipteran pests are mostly controlled preventatively. This leads to a situation where hymenopteran parasitoids, like *Aphytis* spp., might starve, because no sugar is present to meet energy needs (Tena et al., 2015; Lenaerts et al., 2016; Calabuig et al., 2015; Benelli et al., 2017).

The relationship between red scale and its natural enemies is self-regulating and in a stable interaction, when in an IPM-friendly environment (Hassell & Comins, 1978; Huffaker & Messenger, 1964; Eilenberg et al., 2001). This means that neither the host (red scale) nor the natural enemies (parasitoids or predators) of red scale ever become extinct in such orchards. Thus, in orchards that are under full biological control, fruit infested with red scale will present itself occasionally at low levels (Bedford & Cilliers, 1994; Compere, 1961; Grafton-Cardwell & Stewart-Leslie, 1998; Pekas, 2010). Therefore on-farm tolerance levels towards red scale should be relaxed, while fruit infested with red scale can be sorted from those free of red scale, as it is unlikely that export market standards will be eased to allow red scale on fruit, even for markets for which red scale is not regarded as a phytosanitary organism.

Horticultural mineral oil is available as an IPM-friendly remedy to address scale accumulating on the main scaffold branches of citrus trees where *Aphytis* spp. are reluctant to attack red scale (Grout & Hattingh, 2019; Grout & Richards, 1989b; Grout, 2020; Murdoch et al., 1989; Walde et al., 1989; Reeve, 1987).

Mating disruption of red scale may form another valuable tool in red scale IPM, leading to a delay in the fertilisation of third instar red scale females, or even no fertilisation occurring at all. Consequently, red scale females will remain virginal for an extended period. As virgin red scale females during the third instar are susceptible to attacks by *Aphytis* spp. until fertilisation takes place, red scale parasitism and host feeding will increase. In addition to the enhanced parasitism and host mutilation, the red scale population will, by virtue of impaired reproduction, be affected.

5.3. Implications for the biological control of red scale in South Africa

The sustainability of citrus production depends how successfully high-quality fruit can be grown and protected. Pests and diseases should therefore be managed in an environmentally friendly manner to the satisfaction of a very demanding market. Since residues of chemical remedies used to control red scale are the active ingredients most commonly found in residue analyses conducted on fruit destined for export markets (Fourie, 2020), their continued acceptance by discerning markets may be under threat. Consequently, it is of high priority to explore non-chemical alternatives which will not leave detectable residues on fruit.

This study has unfortunately shown that the augmentation of commercially reared *A. melinus* is not a viable option to consider currently as a control measure against red scale on citrus. Further research with inoculative and inundative augmentative releases should only be conducted with wasps with an improved fitness profile and shortened transit time between eclosion and release. However, this study did reveal how effective the indigenous and naturally occurring *A. africanus* is when conserved through facilitating a favourable agricultural ecosystem, by implementing IPM.

A shift in mindset is needed for most of the stakeholders in the citrus industry to be able to embrace and capitalise on the benefits of biological control of red scale. Attention must be given to factors affecting biological control. All the stakeholders must learn what to look for and must be able to observe positive and negative progression in biological control of red scale in orchards. Technical advisers and officers in the citrus industry have become too complacent, relying on standard chemical remedies for red scale control and thereby not acquainting themselves with the biology and ecology of red scale and its natural enemies.

Although it is difficult to comprehend and quantify the services offered by *A. africanus* present in IPM-friendly orchards, the benefits of such free ecosystem services to control red scale is by far the most economical option to growers. As the presence of *A. africanus* and other *Aphytis* spp. holds an economic value for growers, it should be monitored regularly because it must be regarded as natural capital (Bedford & Cilliers, 1994; Quednau & Annecke, 1963; Costanza et al., 1997). It would be important for growers and other stakeholders to explore and accept biological control of red scale. Parasitism-related intervention thresholds would be an important method in the future of red scale control but will most probably only gain traction in the industry if growers start to explore and accept conservation agriculture and the myriad of benefits it offers.

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