Combining entomopathogenic fungi and parasitoids to control the green peach aphid

Myzus persicae

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ABSTRACT

The green peach aphid, *Myzus persicae*, is a major insect pest worldwide. The parasitoid *Aphidius colemani* and more recently, the entomopathogenic fungus *Lecanicillium muscarium* strain KV01 (Mycotal[®]), have been considered as potential biological control against *M. persicae*. However, no studies have been carried out on the interaction between *L. muscarium* and *A. colemani* against *M. persicae*. This study, therefore, was conducted to determine the most effective timing of application of this fungal strain in combination with either naturally occurring or introduced parasitoids to control *Myzus persicae* in the laboratory and controlled field environments. In the laboratory, mummification, emergence of parasitoid adults from mummified aphids and female sex ratio of the emerging adults, were not affected when the fungus was applied 6 or 7 days after the parasitoids were added, compared with treatment with *A. colemani* only. Although a 40 % reduction in the female sex ratio of emerging parasitoid adults was recorded when fungi applied 3 days after the parasitoids were added, fungal application had no significant effect on longevity and fecundity of the female *A. colemani* F1 generation that emerged from fungus-treated aphids. In the semi-field experiment, the mean number of aphids per leaf was significantly lower in the treatment involving *A. colemani* in combination with *L. muscarium* than those with *A. colemani* alone. However, aphid reduction in the treatments

involving naturally occurring parasitoids alone and those in combination with *L. muscarium* was not significantly different. This study suggests that it is possible to combine *L. muscarium* with *A. colemani* to increase the level of aphid control. A more detailed knowledge of the effects of naturally occurring parasitoids on pest control and their interaction with other biological control agents will help to develop environmentally sound crop management strategies with reduced insecticide applications.

Keywords: *Aphidius colemani*; *Lecanicillium muscarium*; Mycotal[®]; *Myzus persicae*

1. Introduction

Intraguild interactions are common among communities of biological control agents including interactions between parasitoids and entomopathogenic fungi (Mesquita and Lacey, 2000; Rashki et al., 2009). Ferguson and Stiling (1996) documented three possible outcomes of intraguild interactions in the context of biological control: (1) the natural enemies interact beneficially and their combined effects result in a higher level of control than the sum of their individual effects (a synergistic effect); (2) the natural enemies do not interact and their total effect is equivalent to the sum of their individual effects (an additive effect); or (3) the natural enemies may interact negatively, resulting in control either equivalent to or less than the control produced by one agent alone (an inhibitory effect).

Aphidius colemani (Hymenoptera: Braconidae) is a solitary koinobiont endoparasitoid of aphids and is one of the most successful commercial biological control agents used in greenhouse crops (Messing and Rabasse, 1995). It is mainly used to control the economically important aphids *Myzus persicae* and *Aphis gossypii* (Van Steenis and El-Khawass, 1995). In addition, naturally occurring parasitoid wasps (Hymenoptera: mainly Braconidae) are known to contribute to regulation of aphid populations in agroecosystems (Sigsgaard, 2002). Several species of aphidiine parasitoids such as *Diaeretiella rapae*, *Aphidius ervi*, *A. colemani* and *Praon volucre* have been reported parasitizing *M. persicae* in the field worldwide (Dixon,

2012). Another biological control agent, *Lecanicillium muscarium* (Hypocreales: Cordycipitaceae) is a well-known entomopathogenic fungus and possesses a wide insect host range that includes *Bemisia tabaci* (Cuthbertson and Walters, 2005), *Trialeurodes vaporariorum* (Pineda et al., 2007), and phytopathogenic fungi (Spencer, 1980). The strain KV01, commercially available as Mycotal[®] (Koppert Biological Systems, the Netherlands), has been reported as a promising biological control agent for use against *M. persicae* (Mohammed and Hatcher, 2016).

As *M. persicae* is a polyphagous insect with a high reproductive capacity, and short generation time, it can be difficult to achieve a high level of control using only a single biological control agent (Martins et al., 2014). In the recent years, there has been increasing interest in exploring the possibility of achieving greater control of *M. persicae* by using combinations of natural enemies such as parasitoids and entomopathogenic fungi (Rashki et al., 2009; Emami et al., 2013; Martins et al., 2014). However, the combined effects of *A. colemani* and *L. muscarium* (Mycotal[®]) on *M. persicae* populations under greenhouse or field conditions have been not investigated. Some laboratory studies have, however, shown that *A. colemani* and *L. muscarium* could be used as a part of integrated pest management (IPM) programmes against the cotton aphid, *Aphis gossypii* (Aiuchi et al., 2012) and cereal aphids (Aqueel and Leather, 2013). *L. muscarium* causes highest mortality in older aphids (Mohammed and Hatcher, 2016), while *A. colemani* causes highest mortality in young stages of *M. persicae* (Perdikis et al., 2004), thus we would hypothesize that the combined application of the parasitoids and entomopathogenic fungi would cause an additive mortality in the aphids

The introduction of parasitoids to control aphids on field crops is particularly problematic, not only do large numbers of parasitoids have to be introduced (Coll and Hopper, 2001), but there is no means of preventing them moving away from the crop.

Therefore, it is not surprising that there has been a low success rate of this biocontrol method in the past (Van Driesche et al., 2008; Nagasaka et al., 2010). Although *M. persicae* is often attacked by a large number of naturally occurring parasitoids, their efficacy in controlling *M. persicae* is not sufficiently understood (Sunderland et al., 1997). Most of the studies investigated the abundance, seasonal occurrence and distribution of aphidiine parasitoids of *M. persicae* through modification of the environment or existing practices. This known as conservation biological control (Barbosa 1998), which is appropriate in organic agriculture because there is minimal use of disruptive broad-spectrum pesticides and other biological control agents that otherwise may constrain the action of natural enemies (Barry et al., 2005). Some field studies have, however, shown that the efficacy of several naturally occurring aphid parasitoids in reducing aphid populations was low ($\leq 5\%$) (Feng et al., 1991; Wraight et al., 1993; Tòth et al., 2009). Therefore, increasing the efficacy of naturally occurring parasitoids by combining them with other biological control agents such entomopathogenic fungi could provide a cost-effective, safe and sustainable method for aphid control, which could be the central component of an integrated aphid control program. This is the first study on using a combination of naturally occurring parasitoids and *L. muscarium* in biological control programs of *M. persicae*.

Entomopathogenic fungi, especially Hypocreales fungi with a generalist nature, can infect non-target insects, including parasitoids, which can result in direct deleterious effects in the parasitoids (Brooks, 1993). As the intra-host development time for Hypocreales fungi is generally shorter than that of parasitoids, if a entomopathogenic fungus infects a host insect before a parasitoid, then the fungus will usually exclude the parasitoids because the competition is skewed in favor of the fungus (Furlong and Pell, 2005). Thus, one important way avoid inhibition between these biological control agents is to apply the entomopathogenic fungus when the parasitoid is less susceptible to it (van Lenteren and

Fransen 1994). For example, Kim et al. (2005) found that the percentages of mummification and *A. colemani* emergence were not affected when *Verticillium lecanii* was applied 5 days after parasitization. Aqueel and Leather (2013) showed that the percentage of aphids mummified was not significantly affected when aphids exposed to *V. lecanii* 3 days after parasitization.

The objectives of the current study were, therefore, to determine the optimal time of fungal application that will reduce competition between *L. muscarium* and *A. colemani* and thus enhance their additive interactions against *M. persicae*, and to evaluate the effects of *L. muscarium* on the percentage of aphids mummified, emergence rate, sex ratio, and fecundity and longevity of F1-generation parasitoids under laboratory conditions. A choice experiment evaluated mummification rates when *A. colemani* was offered both fungus-treated and untreated aphids. We also investigated the effect of the separate and combined efficacy of *L. muscarium* and either added *A. colemani* or natural parasitoid populations on a population of *M. persicae* in a controlled field experiment. This is the first study on the combined use of these biological control agents for the control of *M. persicae* under field conditions.

2. Materials and methods

2.1. Laboratory experiments

2.1.1 Plant material

Brussels sprout, *Brassica oleracea* var. *gemmifera* L. cultivar Bedford-Winter Harvest (Suttons Seeds, Paignton, UK) were established for the *M. persicae* cultures and for the experimental work. This cultivar has been identified as *M. persicae* susceptible (Qayyum, 1999), and ensured a regular supply of large numbers of aphids throughout the year. Loam-based potting compost (John Innes No. 2: Roffey Brothers, Bournemouth, UK) was used.

2.1.2 Host insect

The green peach aphid, *M. persicae*, was collected initially from cabbage plants in the experimental garden at the University of Reading (Reading, UK, 51°N / 1° W), in 2013. The aphids were identified under a light compound microscope, using both cabbage aphid and polyphagous aphid keys (Blackman and Eastop, 2000). *Myzus persicae* was reared on Brussels sprout plants 5-6-weeks old in 45 x 45 x 45 cm cages at 21± 2°C and with 16:8 h daily photoperiod for several generations. Plants were replaced every 2 weeks with healthy 5-6 week-old plants.

2.1.3 Parasitoid

The *Aphidius colemani* colony was initiated from stock material provided by Syngenta Bioline (Little Clacton, Essex, UK) and reared on *M. persicae* feeding on Brussels sprout plants in 45 x 45 x 45 cm cages at 21± 2°C and with a photoperiod of 16: 8 (L: D) h. Brussels sprout plants infested with *M. persicae* were transferred from the stock aphid culture above (2.1.2) to the parasitoid cages every two weeks.

2.1.4 Entomopathogenic fungus

A formulation of *Lecanicillium muscarium* (Mycotal® by Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands) was used. The product is based on conidia of the KV01 strain isolated from a dead *T. vaporariorum* adult from a glasshouse in the UK (Fargues et al., 2003). This mycoinsecticide contains 10¹⁰ spores g⁻¹ and was kept at 4°C until use. A stock suspension was prepared by mixing 1 g of wettable Mycotal® powder in 1000 ml of sterile water and 0.02% Tween 80 (BDH Chemicals Ltd., Poole, UK) in 2000 ml beakers then agitating for 30 minutes with a magnetic stirrer. The resulting concentration was either used as prepared or diluted 100-fold to produce a fungal concentration of 1×10⁸ spores ml⁻¹. The viability of spores was determined by spraying 0.1 ml of 1×10⁸ spores ml⁻¹ on a sterile Petri dish with 1.5% Sabouraud dextrose agar (SDA). The dishes were sealed with parafilm

and incubated at 20°C, 90 ± 2% RH and a photoperiod of 16:8 (L:D) h. After 24h, the number of germinated spores per 100 spores of each plate was assessed under the microscope (400× magnification). Germination was considered positive when the length of the germ tube was at least half the spore length. The viability of the *L. muscarium* spores used in the bioassays exceeded 92%.

For each experiment, 1 ml of spraying suspension at 1×10^8 spores ml⁻¹ was applied once on each leaf containing aphids using a 750 ml trigger water sprayer (Ampulla, Hyde, UK). This concentration was selected because it produced about 75% aphid mortality within 10 days and is corresponds to an application of 330 ± 40 viable spore/mm². The application was made at a distance of 15 cm between the sprayer nozzle and plant leaf to ensure that the fungal application covered the whole leaf and all aphids (Mohammed and Hatcher, 2016).

2.1.5 Obtaining a uniform age of aphids

In order to obtain a uniform age of third instar nymphs of *M. persicae* to be used for all experiments, adults of *M. persicae* were transferred from the stock culture onto 6-week-old Brussels sprout plants (20 adults per plant) using two 3-cm-diameter clip-cages and were allowed to produce nymphs for 1 day in a growth chamber at 20°C, 75 ± 2% RH and a photoperiod of 16: 8 (L: D) h. The adults were then removed and the offspring counted (50 nymphs were retained per plant) and allowed to develop on the plants for 4 additional days before the beginning of each experiment. If the number of nymphs was less than 50 per plant, the adults were kept on the leaves for another day until they produced a total of 50 nymphs (4 ± 1 days).

2.1.6 No-choice tests

2.1.6.1 Aphids first exposed to the parasitoid and then treated with the fungus

The objective of this experiment was to determine the development and sex ratio of *A. colemani* adults that had emerged from aphids exposed to the parasitoid first and then to *L. muscarium*. Aphid mummies were collected randomly from the parasitoid rearing cages and kept individually in 0.5 ml Eppendorf tubes and adult emergence checked daily. After emergence, adult parasitoids were examined under a binocular microscope to determine their sex. Male and female parasitoids were paired individually and kept in an Eppendorf tube with a cotton swab soaked in a honey: water mixture (20:80) as a food source for 24h to mate (Aqueel and Leather, 2013). The males were then removed from the tubes, and the female parasitoids were released onto the plants. Each plant was infested with 50 third instar aphid nymphs was covered with perforated plastic bags (perforations 0.8 x 0.8 mm) (Cater For You Ltd., High Wycombe, Buckinghamshire, UK) and then exposed to 3 females of *A. colemani* (Karatolos and Hatcher, 2009) for 3 days in order to confine individuals of *M. persicae* and its parasitoids and kept in a plant growth chamber at 20°C, 75 ± 2%RH and a photoperiod of 16:8 (L: D) h. These plastic bags allowed neither aphids nor parasitoids to escape, and plants always remained healthy. Afterwards, the parasitoid adults and plastic bag covers were removed and the plants were left with aphids in the same plant growth chamber. Plants were sprayed with *L. muscarium* at 3, 4, 5, 6 or 7 days after the parasitoids were added. Seven plants (with 50 third instar aphids per plant) for each treatment were used. In addition, seven Brussels sprout plants each with 50 third instar aphids were sprayed with sterile water and 0.02% Tween 80 only, as a control treatment, and seven Brussels sprout plants each with 50 third instar aphids were sprayed with fungus only. After fungus application, the plants were kept at room temperature for 1 h to dry and then they were transferred to the same plant growth chamber in the same conditions as described above.

During the ten days following the fungal application, dead aphids were counted and removed daily. Dead aphids were surface sterilized by rinsing twice with 70% ethanol for 30

seconds and then with sterilized distilled water, and thereafter placed on water agar (3g of agar l⁻¹ of water) in Petri dishes for 5 days to confirm infection by *L. muscarium*. A cadaver was regarded as having died from infection by this fungus when conidia of *L. muscarium* were recovered from it (Aiuchi et al., 2012). The number of aphids mummified was also recorded and then mummies were kept individually in Eppendorf tubes until adult parasitoid emergence, and then their sex was determined.

2.1.6.2 Aphids first treated with the fungus and then exposed to the parasitoid

To determine the effect of prior infection with an entomopathogenic fungus on the subsequent parasitization of the aphid by *A. colemani*, the parasitoid was released simultaneously, 1, 2, 3 or 4 days after applying the fungus (seven plants in each treatment, each plant had 50 third instar aphids as above). In addition, seven plants each with 50 third instar aphids were sprayed with sterile water and 0.02% Tween 80 only, as a control treatment, and seven plants each with 50 third instar aphids were sprayed with fungus only. The same method as described in 2.1.6.1 was used to release the parasitoids, confirm fungal infection and determine adult parasitoid emergence and sex ratio.

2.1.7 Choice experiment

Five choice treatments were carried out on separate dates to investigate the parasitization of fungus-treated and untreated aphids by *A. colemani*. Five Brussels sprout plants (containing a total of 100-third instar aphids per plant) for each treatment were used. On each infested plant, one leaf with 50 aphids (applied to the plants as above, and to prevent nymphs moving between plant leaves the aphids were kept in 3-cm-diameter clip-cages until the parasitoids were released) was sprayed with 1 ml of *L. muscarium* at 1×10⁸ spores ml⁻¹ and another leaf (of equivalent age) also with 50 aphids was sprayed with sterile water and 0.02% Tween 80 as a control. To prevent the *L. muscarium* spray reaching the untreated

leaves, a piece of cardboard was used as a temporary shield during spraying. Plants were placed individually in a cage (45 x 45 x 45 cm) consisting of a wooden frame covered on all sides with muslin netting and clear Perspex top and a firm wooden base. The fungus application was made at 0, 1, 2, 3 or 4 days before releasing the parasitoid females. For parasitoid mating, the same method as described in 2.1.6.1 was used. Females of *A. colemani* were released onto the plant in each cage for 2 days (6 mated females per cage) and the plants were kept in the growth chamber at 20°C, 70 ± 2% RH and a photoperiod of 16: 8 (L: D) h. Afterwards, the plants were removed from the cages and placed in perforated plastic bags. The same method as described in 2.1.6.1 was used to determine the parasitoid emergence, its sex ratio and to confirm infection by *L. muscarium*. In this experiment, it was not possible to reliably measure parasitoid oviposition, which would be a true choice test, rather we measured the outcome of choice exhibited by mummification. We thus compared the results with expected values obtained from the appropriate no-choice experiment described in section 2.1.6.2 using the formula $E=100-R$, where R is the difference between the values from untreated and fungus-treated aphids in the no-choice experiment (2.1.6.2).

2.1.8 Susceptibility of parasitoid larvae to direct infection by *L. muscarium*

To determine the susceptibility of parasitoid larvae to direct infection by *L. muscarium*, 20 Brussels sprout plants containing 50 aphids per plant were used. The parasitoid females (2-3 day old), previously mated but with no previous oviposition were released onto the plants (3 females per plant). Brussels sprout plants were covered with perforated plastic bags for 3 days and kept in a plant growth chamber at 20°C, 75 ± 2%RH and a photoperiod of 16: 8 (L: D) h. Afterwards, 10 plants with parasitized aphids were sprayed with *L. muscarium* at 1×10^8 spores ml⁻¹. In addition, 10 plants with parasitized aphids were sprayed with sterile water and 0.02% Tween 80 only, as a control treatment. After fungus application, the plants were kept at room temperature for 1 h to dry and then the

plants were transferred to the same plant growth chamber in the same conditions described above.

Ten days following fungal application, the mummies produced were recorded and kept individually in Eppendorf tubes. Non-emerged mummies (recorded 15 days after parasitoids were added because the mean developmental time of *A. colemani* adults from oviposition until emergence at 20°C is 13 days) were surface sterilized by washing them in 70% ethanol for 1 min then dissected under a binocular microscope. The larval stages of *A. colemani* removed from these mummies were surface sterilized by rinsing them twice with 70% ethanol for 30 seconds and then with sterilized distilled water and then placed on water agar (3g of agar/L of water) for 5 days to confirm infection by *L. muscarium* (Aiuchi et al., 2012).

2.1.9 Effect of L. muscarium on the fecundity and longevity of the F1 generation of A. colemani

The fecundity and longevity of the F1 generation females of *A. colemani* were evaluated using ten parasitoid females that emerged from aphids treated with *L. muscarium* in the previous experiment (2.1.8). They were mated individually with the males that had emerged from aphids of the same experiment using the method described in 2.1.6.1. In addition, ten females that emerged from untreated aphids in the previous experiment were also mated as described in above (control). Mated females were raised individually on Brussels sprout plant infested with 50 third instar aphids and kept in a plant growth chamber at 20°C, 75 ± 2% RH and a photoperiod of 16: 8 (L: D) h. To determine the longevity of the F1 generation of *A. colemani*, the survivor parasitoid females were transferred onto new aphid-infested plants every 2 days until parasitoid death. To determine the fecundity of the F1

generation females of *A. colemani*, the number of mummies on each aphid-infested plant was counted 10 days after transfer of the parasitoid females.

2.1.10 Cage experiment to assess the effect of A. colemani on the transmission of L. muscarium

The objective of this experiment was to determine the effect of *A. colemani* on the transmission of *L. muscarium*. 6-week-old Brussels sprout plants each with 50 third-instar aphids were placed in pairs in a cage (45 x 45 x 45 cm). Potted plants were placed in water traps to prevent the movement of aphids between plants within each cage. The following treatments were tested: releasing 6 parasitoid females of *A. colemani* per cage for 3 days; exposure to *L. muscarium* containing 1×10^8 spores ml⁻¹ (one plant exposed to 2 ml and the other plant sprayed with 0.02% sterile aqueous Tween 80 as a control) or exposure to *L. muscarium* (one plant sprayed with fungus and the other plant sprayed with 0.02% sterile aqueous Tween 80 as a control) and then releasing *A. colemani* immediately after applying fungus. Eight replicate cages were prepared in total for each treatment. The cages were maintained for 10 days within a plant growth chamber at $20 \pm 1^\circ\text{C}$, $75 \pm 3\%$ RH and a photoperiod of 16:8 (L: D) h. Then the number of *L. muscarium*-sporulating cadavers and *A. colemani* mummies on each plant was assessed. The same method as described in 2.1.6.1 was used for non-sporulating cadavers to confirm infection by *L. muscarium*.

2.2 . Semi-field experiment

2.2.1 Preparation and experiment design

The objective of this experiment was to evaluate the efficacy of *L. muscarium* and either naturally occurring or introduced parasitoids separately and in combination against the green peach aphid *M. persicae*. The experiment was carried out in 1m square cages (Bridgend Garden Centre, Cupar, UK) from 6th May until 17th September 2015 in the experimental

garden at the Whiteknights Campus, University of Reading, UK. These cages had ground pegs for extra stability and security. Cages were covered with white plain voile net with a smaller mesh size (0.08 cm square) to prevent all natural enemies such as hoverflies, ladybirds and parasitoids from entering the cages. Access to cages was provided by zippers sewn on one side of the cage. Each treatment was replicated four times in a randomized complete block design. Each block included six plots with 2 m between blocks and 1 m between plots and each plot was represented by one cage. Brussels sprout plants were maintained in a glasshouse (at minimum 16°C night; 23-34°C day and 16 h light) and uniform sized plants were transferred to the field in cages when they were five weeks old. A total of 9 plants with a spacing of 30 cm between rows and 30 cm between plants were transferred to each cage. The soil in the field was slightly acid, slightly stony, and sandy loam overlaying gravel (Jarvis, 1968).

2.2.2 Infestation with *M. persicae* and treatments

One hundred and fifty laboratory-reared *M. persicae* (adults) were added to each plant in all cages on 21st and 22nd May using 3-cm-diameter clip-cages. The clip-cages were removed after 3 days. Following infestation, the aphids were allowed to freely reproduce on the plant for one month to build up a population. The population density of the aphids was assessed 1 day before treatments commenced by counting them on a 10 cm² portion of each of 20 randomly chosen leaves per cage. Just before treatments were added, the cages measuring the efficacy of naturally occurring parasitoids alone or in combination with *L. muscarium*, were re-covered with white plain voile net with a larger mesh size (0.2 cm square) to allow naturally occurring parasitoids to pass into the cages. Cages of each block were subjected to one of the following treatments: (1) exposure to *L. muscarium* containing 1×10^{10} spores ml⁻¹ (25 ml per plant) using a Diaphragm CP 15 sprayer (Agratech NW Ltd., Waterfoot, Lancashire, UK); (2) releasing 25 pairs of *A. colemani* per cage only; (3) exposure

to naturally occurring parasitoids only; (4) releasing 25 pairs of *A. colemani* and then spraying with *L. muscarium* at 1×10^{10} spores ml^{-1} after 7 days (25 ml per plant); (5) exposure to naturally occurring parasitoids and then spraying with *L. muscarium* at 1×10^{10} spores ml^{-1} per plant after 7 days (25 ml per plant) and (6) spraying with 25ml of sterile water and 0.02% Tween 80 per plant (control). The fungus was applied again 4 weeks after first application (26th July 2015) to the appropriate cages.

2.2.3 Evaluation

The number of living aphids and mummies were counted weekly on a 10 cm^2 area from each of 20 randomly chosen leaves per cage. No aphids or mummies were removed from the plots during this recording. To identify the natural parasitoid species, 5 mummies per cage were removed randomly every two weeks with a fine brush and placed individually in Eppendorf tubes to await emergence of the adult parasitoid or hyperparasitoid. Emerged parasitoids were then preserved in 70% ethanol, for taxonomic identification by the Natural History Museum in London. At the end of the experiment, the shoot dry weight of all plants was measured by placing the fresh shoots individually into tared, brown paper bags and then samples were dried in a forced-draft oven at 70°C for 2 days. Dry shoots were weighted using a Sartorius GMBH balance. The average high and low weekly temperature and relative humidity were recorded using a data logger (Tinytag Explorer 4.8, Gemini Data Loggers (UK) Ltd., Chichester, UK) which were $32.3 \pm 1.6^\circ\text{C}$ and $10.7 \pm 0.8^\circ\text{C}$, and 99.7 % and 50.3 % RH respectively

2.3 Data analysis

Statistical analyses were conducted using GenStat (version 16; VSN International, Hemel Hempstead, UK). For no-choice experiments, normality of data distribution was estimated using a Shapiro-Wilk test (W-test). The data were transformed using logit

transformation when it was necessary to meet the assumption of normality. The percentage of aphids mummified, emerged parasitoids and the female sex ratio was analyzed separately using one-way ANOVA. The means were compared using LSD test at 5% level of significance ($P < 0.05$). The percentage of emerged parasitoids was determined according to emerged adults from mummies.

For choice experiments, the influence of fungal infection for each time interval on the percentages of aphids mummified, emerged adults and female sex ratio were analyzed using one-tailed t-tests. Paired t-tests were used for the analysis of choice tests. The effect of the fungus on the F1 generation females' longevity and fecundity was analyzed using one-way ANOVA. Chi-square tests were used to assess whether *L. muscarium* or an interaction between *L. muscarium* and *A. colemani* affected the number of fungus-killed aphids. A three-factor repeated measurement analysis ANOVA was used to test the individual effects of *L. muscarium*, *A. colemani* and naturally occurring parasitoids in addition to combined effects of *L. muscarium* with either naturally occurring parasitoids or *A. colemani* on the number of aphids over time under field conditions. Another two-factor repeated measurement ANOVA was used to compare the number of aphid mummies between the *A. colemani* and naturally occurring parasitoids cages individually or in association with *L. muscarium*. One-way ANOVA was used to determine the effect of different treatments on the plant dry weight. Treatment means were compared using LSD.

3. Results

3.1. Laboratory experiments

3.1.1. No-choice experiments

3.1.1.1. Aphids first exposed to the parasitoid and then treated with the fungus

The percentage of aphids mummified by 3 female *A. colemani* ranged between $69 \pm 2.4\%$ and $73 \pm 1.8\%$ but was not significantly different between treatments ($F = 0.32$; $df = 5$; $P = 0.895$) (Fig. 1A). The percentage emergence of adult *A. colemani* parasitoids was, however, significantly lower from those aphids treated with the fungus 3 and 4 days after the parasitoids were added than from untreated aphids or those fungus-treated later ($F = 7.83$; $df = 5$; $P \leq 0.001$) (Fig. 1B). The lowest percentage of females of the F1 generation of *A. colemani* was found in those which emerged from fungus-treated aphids 3 and 4 days after the parasitoid were added ($F = 26.15$; $df = 5$; $P \leq 0.001$) (Fig. 1C).

3.1.1.2. Aphids first treated with the fungus and then exposed to the parasitoid

There was a progressive decrease in percentage of mummified aphids with the increase in time after fungal application that the parasitoid was applied ($F = 238.41$; $df = 5$; $P \leq 0.001$), with the lowest mummification occurring when aphids were exposed to the parasitoids 3 or 4 days after fungal treatment (Fig. 2A). There was a progressive decrease in the proportion of parasitoids emerging from mummified aphids with the increase in time after fungal application that the parasitoid was applied ($F = 308.41$; $df = 5$; $P \leq 0.001$), with the lowest emergence of *A. colemani* from aphids exposed to *A. colemani* 4 days after exposure to *L. muscarium* (Fig. 2B). The percentage of female parasitoids that emerged was, however, significantly lower from those aphids exposed to *A. colemani* 3 or 4 days after exposure to *L. muscarium* than from untreated aphids or those exposed early ($F = 193.92$; $df = 5$, $P \leq 0.001$) (Fig. 2C).

3.1.2. Choice experiment

In all choice tests, there was no significant differences in the percentage of untreated aphids mummified ($F = 1.21$; $df = 5$; $P = 0.421$). When offered a choice between fungus-treated and untreated aphids, higher percentage of aphids mummified were recorded on

untreated aphids compared with fungus-treated aphids (Fig. 3A). The percentages of emerged parasitoids were significantly higher on untreated aphids compared with fungus-treated aphids, but the lower percentage was found when *A. colemani* released 4 days after fungal application (Fig. 3B). *L. muscarium* had a negative effect on the ratio of parasitoid females only when applied 2, 3 or 4 days before exposing to *A. colemani* compared with those emerging from untreated aphids (Fig. 3C).

3.1.3. Susceptibility of parasitoid larvae to direct infection by *L. muscarium*

About 82% of the parasitoid larvae which developed in aphids treated with *L. muscarium* 3 days after the parasitoid exposure were free of the fungal infection. No fungal infection was detected on the parasitoid larvae or pupae which developed in aphids that were not exposed to the fungus.

3.1.4. Effect of *L. muscarium* on the fecundity and longevity of the F1 generation of *A. colemani*

The mean fecundity of *A. colemani* F1 generation adult females which emerged from fungus-treated aphids was 83.6 ± 3.9 mummies, with no significant difference compared with those which emerged from untreated aphids (83.1 ± 4.3) ($F = 0.01$; $df = 1,19$; $P = 0.933$). The mean longevity of F1 generation adult females of *A. colemani* which emerged from aphids treated with the fungus 3 days after exposure to the parasitoids was 8.2 ± 0.4 days, with no significant difference compared with those which emerged from untreated aphids (8 ± 0.51) days ($F = 0.08$; $df = 1,19$; $P = 0.777$).

3.1.5. Cage experiment to assess the effect of *A. colemani* on the transmission of *L. muscarium*

There were significant effects of *A. colemani* on the number of fungus-killed aphids after 10 days of fungal treatment ($X^2 = 42.4$, $df = 5$, 41 ; $P \leq 0.01$), with a mean of 5.1 *L.*

muscarium-sporulating cadavers on non-fungus treated plants from the treatment containing both *L. muscarium* and *A. colemani*, and a mean of 0.25 *L. muscarium*-sporulating cadavers from the treatment containing *L. muscarium* alone (Fig. 4).

3.2. Semi-field experiment

Before exposure to the parasitoids, there was no significant difference in the number of aphids per leaf between treatments ($F=0.81$; $df = 5, 23$; $P = 0.544$). Although the average number of aphids per leaf in the control treatment was significantly increased between week 3 and 7 post-treatment, there was a significant decrease in all control cages afterwards (Fig. 5). Plants that were treated only with *L. muscarium* showed about 19% reduction in the number of aphids between week 2 and 7 post-treatment, compared to the control treatment ($P \leq 0.001$). The density of aphids exposed to either naturally occurring or introduced parasitoids (*A. colemani* only, naturally occurring parasitoids only) was significantly lower every week post-treatment, compared to the control or those aphids receiving *L. muscarium* only. Furthermore, the density of aphids exposed to *A. colemani* and later treated with *L. muscarium* was significantly lower every week post-treatment, compared to those aphids exposed to *A. colemani* only ($P \leq 0.05$). The density of aphids exposed to either naturally occurring parasitoids alone or in combination with *L. muscarium* was not significantly different every week post-treatment. (Table 1 and Fig. 5).

The parasitoid identification by the Natural History Museum (London) only found *Praon volucre* (Haliday) and *Aphidius matricariae* (Haliday) as naturally occurring aphid parasitoids. Aphid mummies were only observed on plants exposed to *A. colemani* or natural parasitoid populations and did not begin appearing until 10 days after exposure to the parasitoids. There was no significant difference in the number of aphid mummies between those aphids exposed to either naturally occurring or introduced parasitoids and later treated

with *L. muscarium* and those exposed only to the parasitoids (*A. colemani* only, naturally occurring parasitoids only, *A. colemani* + *L. muscarium* and naturally occurring parasitoids + *L. muscarium*) (Fig. 6). However, the number of weeks post-treatment had a significant effect on the number of aphid mummies, with a significant decrease for all treatments beginning 3 weeks post-treatment. The interaction between treatments and number of weeks post-treatment also differed significantly (Table 2).

The shoot dry weight of plants receiving either naturally occurring or introduced parasitoids alone or in combination with *L. muscarium* was significantly greater than plants in other treatments ($F = 6.88$; $df = 5$; $P = 0.002$) (Fig. 7).

4. Discussion

This study showed that *L. muscarium* had no effect on *A. colemani* development, emergence and sex ratio when the its aphid host exposed to fungus 6 or 7 days after parasitoids were added. In the caged plants experiment under laboratory conditions, our results showed that the persence of *A. colemani* adults enhanced the spread of *L. muscarium* from infected aphids on treated plants to healthy aphids on untreated plants. There can be a positive interaction between added *A. colemani* or exposure to naturally occurring parasitoids and added *L. muscarium* in semi-field conditions resulting in a significantly greater reduction in the aphid population than produced by the use of one agent alone, but the overall effect on the aphid was less than additive.

The percentage of aphids mummified was not affected by *L. muscarium* treatment 3 days or more after the parasitoids were added (Fig. 1A). This confirms the findings of Aqueel and Leather (2013) who demonstrated *L. muscarium* treatment 3 days after exposure to *A. colemani* did not significantly affect the percentage parasitism on cereal aphids (*Rhopalosiphum padi* and *Sitobion avenae*), compared with untreated aphids. In the present

study, two possible reasons can be given for the non-significance difference in the percentage of aphid mummified between treatments and control. First, the presence of a fungistatic substance secreted by the parasitoid larvae into the hemolymph of the host could impede the development of fungus (El-Sufty and Führer, 1985). Or second, the change in aphid cuticle composition during parasitoid development, with greater cuticle thickness possibly preventing penetration by *L. muscarium* (Aiuchi et al., 2012). In contrast to these findings, Rashki et al. (2009) reported a 20 to 70% reduction in percentage of aphid mummies arising from female *A. matricariae* when aphids were treated with *B. bassiana* 1, 2, 3 or 4 days after parasitism, compared to untreated aphids. Emami et al. (2013) also reported a 75% reduction in the number of aphid mummies arising from female *A. colemani* when the aphid host *M. persicae* was treated with *B. bassiana* 1 day after parasitism compared with untreated aphids. The differences may be due to the allowance of 3 days for oviposition on the aphid hosts by female *A. colemani* before fungal application and/or different entomopathogenic fungus species applied where the above studies used *B. bassiana*, while *L. muscarium* had been tested in our study.

L. muscarium, however, had a negative effect on *A. colemani* emergence and female sex ratio when the time interval between aphid parasitization and fungal application was less than 5 days (Fig. 1). Because eggs deposited in unsuitable hosts are destined to die or produce inferior adults, endoparasitoid females are expected to distribute their fertile (female) eggs optimally in suitable hosts (Bernal et al., 1999) which may have led *A. colemani* females in our experiment to lay more infertile (male) eggs in fungus-infected aphids rather than fertile eggs. *L. muscarium* can reduce the nutritional quality of aphid hosts and this consequently could have a greater effect on emerging *A. colemani* females, due to their higher growth rate and larger body size compared with males (Rashki et al., 2009; Martins et al., 2014). Similarly, Aqueel and Leather (2013) reported that the percentage of *A. colemani* emergence

and female sex ratio from fungus-treated aphids were lower compared to untreated aphids. In another study, Kim et al. (2005) demonstrated that the percentages of *A. colemani* emergence were significantly reduced from aphids exposed to *V. lecanii* up to 3 days after parasitization.

Aphids treated with *L. muscarium* were less likely to be mummified when they treated with fungus first than untreated aphids (Fig. 2A). Although it is possible that aphids treated with *L. muscarium* might be nutritionally unsuitable as hosts for various parasitoids (Martins et al., 2014), the failure of parasitoids to utilize hosts treated with entomopathogenic fungi has usually been attributed to premature death of their aphid hosts or their direct infection by the fungus. The lower percentage of aphids mummified from fungus-treated aphids especially 3 and 4 days before exposure to *A. colemani* may also be related to the parasitoid rejection of infected hosts for oviposition, because the endoparasitoids may have the ability to detect the hyphal bodies or fungal metabolites in their aphid hosts using their ovipositors (Mesquita and Lacey, 2001). When offered the choice between healthy and different stage of the fungus-infected aphids in our study, the percentage of aphids mummified was significantly lower on fungus-treated aphids compared with untreated aphids in, suggesting that female *A. colemani* had ability to discriminate between healthy and fungus-treated aphids. Or, the lower mummification rate on fungus-infected aphids may be resulted from premature death of aphid hosts and/or nutritionally unsuitable as hosts for various parasitoids. Parasitoid rejection of fungus-infected hosts has been demonstrated by Fransen and Van Lenteren (1993) who reported that the parasitoid *Encarsia formosa* laid fewer eggs in the greenhouse whitefly, *Trialeurodes vaporariorum*, infected by a fungus 4, 7, 10 or 14 days before parasitism compared to uninfected hosts. Rashki et al. (2009) demonstrated that the parasitoid *A. matricariae* laid fewer eggs in *M. persicae* infected with *B. bassiana* than uninfected aphids.

Few studies have addressed the possible direct susceptibility of parasitoid larvae to entomopathogenic fungi. The ability of entomopathogenic fungi to invade the parasitoid

larval tissue was first observed by Keller (1975), but he was not able to determine if the death of parasitoid larvae was due to the fungus or if the fungus had colonized the parasitoid larvae after death. Our results indicate that about 82% of *A. colemani* larvae which developed in aphids treated with *L. muscarium* 3 days after the parasitoid exposure were free of fungal infection. There are three possible mechanisms to explain the inability of *L. muscarium* to penetrate the tissues of *A. colemani* larvae. The parasitoid cuticle may contain physicochemical properties which may prevent direct penetration by entomopathogenic fungi (Askary and Brodeur, 1999), or fungistatic factors produced in the insect midgut may decrease the viability of fungal bodies and their capacity to penetrate the insect host via epithelial cells (Dillon and Charnley, 1991) and inhibit enzymatic degradation and allow normal parasitoid development and emergence (Li et al., 2012). Thirdly, endoparasitoid wasps introduce maternal factors into the body of their host upon parasitisation to manipulate host physiology. These include calyx fluid containing virus or virus-like particles, such as polydnaviruses (PDVs), and protein secretions from venom glands (Edson et al., 1981; Moreau and Asgari, 2015). These maternal factors are used by the parasitoid to overcome the host immune defenses and have been shown to be essential for successful parasitism and development of the parasitoid inside the host (Digilio et al., 2000), may also play a direct or indirect role in inhibition fungal germination and development in parasitized hosts. However, we found that 18% of *A. colemani* larvae were infected with *L. muscarium*, suggesting that dense aggregation of *L. muscarium* hyphal bodies may have induced strong mechanical pressure, noticeable by an inward depression the cuticle of some parasitoid larvae, and favored localized penetration of the fungus. Furthermore, larvae of *A. colemani* feeding on host hemolymph and other tissues also consume blastospores and hyphae of *L. muscarium*. This confirms other work which demonstrated the ability of *V. lecanii* (= *Lecanicillium* spp.) to infect 25% of *A. nigripes* larvae, another endoparasitic species, developed in potato aphids,

Macrosiphum euphorbiae when it was applied at spore concentration 1×10^7 spores ml^{-1} (Askary and Brodeur, 1999). In addition, Furlong (2004) found that the parasitoid pupae of *D. semiclausum* were infected by the pathogen only at high spore concentration of *B. bassiana* but did not hinder their development.

There is little evidence in the literature indicating that parasitoids act as direct vectors of entomopathogenic fungi by transferring conidia to their insect hosts. In our experiments we found an increased number of fungus-killed aphids on non-fungus-treated plants in the presence of *A. colemani* (Fig. 4), suggesting that the parasitoid is vectoring the spores from infected aphids or sprayed leaves. This is in agreement with Fuentes-Contrera et al. (1998) who demonstrated that the presence of *Aphidius rhopalosiphi* increased the fungal infection of *S. avenae* when parasitoids foraged in an experimental arena with sporulating aphid cadavers compared with foraging in the absence of sporulating cadavers.

The results of the semi- field experiment showed that 1×10^{10} spores ml^{-1} of Mycotal[®] alone was an unsatisfactory strategy to control *M. persicae*. However, this fungal concentration caused 100% mortality in 3rd instar of *M. persicae* within 9 days of treatment under the laboratory conditions (Mohammed and Hatcher, 2016). A comparison between laboratory and field results reveals retardation in the development of disease caused by the fungus and a low mortality response in the field experiment, probably because of poor environmental conditions such as temperature, rainfall and relative humidity during the treatment period, factors known to affect spore germination and/or penetration of the aphid host by *L. muscarium* (Fargues et al., 2003). For example, the average daily temperature was $9.4 \pm 1.6^\circ\text{C}$ during the experimental period which could significantly reduce the germination and growth of fungal conidia. Or second, fungal treatment did not directly impact aphid reproduction in the short-term and has no physiological effect on the developing progeny of *M. persicae* (Mohammed and Hatcher, 2016). Moreover, it is possible that spore density on

entire plants was considerably reduced over that observed the in leaf bioassay (Mesquita et al., 1996).

We hypothesized that entomopathogenic fungi and the parasitoid populations will cause an additive effect which could increase the level of aphid control (Mesquita et al., 1997). The semi-field experiment shows that the co-applied *A. colemani* and *L. muscarium*, do not greatly inhibit each other, resulting in a reduction of aphid population significantly higher than produced by one agent alone (Fig. 5). However, we found that the combined effects of naturally occurring parasitoids and *L. muscarium* did not significantly increase aphid mortality, compared to the outcome of exposure to naturally occurring parasitoids alone. Contrary to our findings, Mesquita et al. (1997) reported that the combination of *A. asychis* and *P. fumosoroseus*, resulted in a significantly greater reduction of the Russian wheat aphid *D. noxia* in the field, compared to the use of one agent alone.

In the semi-field experiment, *L. muscarium* had no detrimental effect on *A. colemani* or natural parasitoid wasp populations when the time interval between aphid parasitization and fungal application was 7 days. These data were compatible with the laboratory results. The lack of detrimental fungal effects on the parasitoid efficacy could be related to the effective time interval between parasitization and fungus application (Milner, 1997). In addition, the climatic conditions such as temperature and relative humidity in the field may be suboptimal for fungal development which prevented fungal epizootics. Fungal epizootics are often harmful for the parasitoid population because of the premature death of the host due to fungal infection (Brooks, 1993). In terms of shoot dry weight of treated and untreated plants, the treatments involving the parasitoids (*A. colemani* only, naturally occurring parasitoids only, *A. colemani* + *L. muscarium* and naturally occurring parasitoids + *L. muscarium*) presented a significant difference in dry weight of plants, compared to fungal and control treatments (Fig. 7). This can be explained by the effects of the parasitoids on the

reduction of aphid populations. These results are consistent with Mesquita et al. (1997) who reported that the dry weight of barley plants treated with *P. fumosoroseus* and later exposed to *A. asychis* was significantly higher relative to the control.

In conclusion, our results showed that combining *A. colemani* and *L. muscarium* (Mycotal®) to control *M. persicae* may be feasible for control of *M. persicae* in greenhouse situation as a result of environmental conditions, especially temperature that favor to fungal growth which can be controlled and also parasitoids can be kept in. However, fungal applications should be timed carefully with the later developmental stages of the parasitoid to conserve the parasitoids within the system. Another aspect which could influence the integration of *A. colemani* and *L. muscarium* for controlling *M. persicae* is, the aphid host plant species. Studies have shown that this plays an important role in either aphid-fungus-interaction or aphid-parasitoid interaction. For example, Shishehbor and Brennan (1995) showed that the highest level of parasitism of *Trialeurodes ricini* by *Encarsia formosa*, occurred on aubergine, while the lowest was found on pumpkin and potato. Also, Tkaczuk et al. (2007) demonstrated that virulence of *Pandora neoaphidis* against *Acyrtosiphon pisum* was greater on dwarf bean than on field bean, pea or lucerne. Therefore, more studies on this aspect of the host plant species-*M. persicae*-*A. colemani*-*L. muscarium* system are necessary to improve overall control efficacy of *M. persicae*. In addition, large scale open field studies of combination effects of the fungus and either naturally occurring or introduced parasitoids on aphid population growth are required to improve methods for combining these two biological control agents.

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- Figure 1.** Percentages of (A) mummified aphids, (B) emergence of F1 *A. colemani* and (C) F1 *A. colemani* that emerged as females (mean $\pm$ SE, n= 7) when aphids were first exposed to

the parasitoid and then sprayed with *L. muscarium* at 3, 4, 5, 6 or 7 days after the parasitoids were added. Different letters above columns indicate significant differences in the values at $P < 0.05$, using LSD test. AC = *A. colemani*; LM = *L. muscarium*.

Figure 2. Percentages of (A) aphid mummies, (B) emergence of F1 *A. colemani* and (C) female sex ratio of *A. colemani* (mean $\pm$ SE, $n = 7$) when aphids were first sprayed with *L. muscarium* and then exposed to the parasitoid for 3 days 0, 1, 2, 3 or 4 days later. Different letters above columns indicate significant differences in the values at $P < 0.05$, using LSD test. AC = *A. colemani*; LM = *L. muscarium*.

Figure 3. Mean percentages of (A) aphids mummified, (B) emergence of *A. colemani* and (C) *A. colemani* that emerged as females (mean $\pm$ SE, $n = 5$) on treated and untreated plant leaves when aphids were treated with *L. muscarium* 0, 1, 2, 3 or 4 days before releasing the parasitoids in choice tests, compared to those only exposed to *A. colemani*. (N.S.) indicates $p > 0.05$, single asterisk (*) indicates $p < 0.05$; double asterisks (**) indicate $p < 0.01$; and triple asterisks (***) indicate $p < 0.001$. AC = *A. colemani*; LM = *L. muscarium*.

Figure 4. Mean number ($\pm$ SE) of *L. muscarium*- sporulating cadavers recorded after 10 days from treatments containing either *L. muscarium* only or both *L. muscarium* and *A. colemani* $n = 8$. Different letters above columns in each treatment indicate significant differences in the values at $P < 0.05$, using LSD test.

Figure 5. Weekly density of *M. persicae* (mean $\pm$ SE) on 10 cm² of Brussels sprouts leaf exposed to *L. muscarium* (Mycotal®) at 1×10^{10} spores ml⁻¹, natural parasitoid populations and *A. colemani* individually or together. AC = *A. colemani*; LM = *L. muscarium*; NP = naturally occurring parasitoids

Figure 6. The effect of combining an entomopathogenic fungus and either naturally occurring or introduced parasitoids on the mean number of aphids mummified ($\pm$ SE) on 10

778 cm² of Brussels sprouts leaf on different weeks after spraying with *L. muscarium*, compared
779 to those aphids exposed only to the parasitoids. AC = *A. colemani*; LM = *L. muscarium*; NP =
780 naturally occurring parasitoids.

781 **Figure 7.** Mean of shoot dry weight ($\pm$ SE) of Brussels sprouts growing in field and exposed
782 to *A. colemani* or natural parasitoid populations individually or in association with *L.*
783 *muscarium*. Values followed by the same letter(s) are not significantly different at $P < 0.05$,
784 using LSD test.

785 AC = *A. colemani*; LM = *L. muscarium*; NP = Natural parasitoid population.

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Table 1. Three-factor repeated measurement analysis ANOVA of individual and combined effects of either of *A. colemani* or naturally occurring parasitoids with *L. muscarium* and weeks-posttreatment on weekly density of *M. persicae* on Brussels sprouts under field conditions during the time of experiment.

Source	df	SS	MS	F-value	P
Block	3	91.63	30.5	0.43	0.82
LM	1	7394.1	7394.1	127.8	≤ 0.001
Parasitoid	1	74188.2	74188.2	1282.3	≤ 0.001
Parasitoid source	1	2364.4	2364.4	45.5	≤ 0.001
LM*Parasitoid	1	520.6	520.6	58.4	≤ 0.001
LM*Parasitoid source	1	169596.4	169596.4	1.1	0.61
Residual	15	3293.7	219.5	17.8	
Weeks	12	51898.4	4324.8	334.4	≤ 0.001
LM*Weeks	12	3293.7	274.4	21.2	≤ 0.001
Parasitoid*Weeks	12	292678.5	12194.9	1194.7	≤ 0.001
LM*Parasitoid*Weeks	12	4667.6	194.49	19.05	≤ 0.001
Parasitoid*Parasitoid source*Weeks	12	2680.1	223.3	21.8	≤ 0.001
LM*Parasitoid*Parasitoid source*Weeks	12	58.0	4.8	0.4	0.92
Residual	216	1862.1	8.9		
Total	311	312970.2			

AC = *A. colemani*; LM = *L. muscarium*; NP = Natural parasitoid population

Table 2. Two-factor repeated measurement analysis ANOVA of the effect of different treatments (naturally occurring and introduced parasitoids individually or in combination with *L. muscarium*) and weeks post-treatment on the number of aphid mummies in Brussels sprouts under field conditions.

Source	df	SS	MS	F-value	P
Block	3	30.3	10.1	2.5	0.8
Fungal treatment (FT)	1	1.5	1.5	0.38	0.55
Parasitoid source (PS)	1	33.2	33.2	8.2	0.01
Residual	9	36.0	4.0	1.6	
Weeks	11	1580.2	1398.2	572.7	≤ 0.001
FT *Week	11	102.6	9.3	3.8	≤ 0.001
PS* Weeks	11	602.0	54.7	22.4	≤ 0.001
FT*PS *Week	11	8.8	0.8	0.3	0.97
Residual	132	322.2	2.4		
Total	191	16518.1			