Phoretic relationship between the myceliophagus mite *Microdispus lambi* (Acari: Microdispidae) and mushroom flies in Spanish crops

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Running title: Mushroom flies as vector ofmyceliophagus mites

Abstract

We studied the role played by the phorid *Megaselia halterata* (Wood) and the sciarid *Lycoriella auripila* (Winnertz) in the phoretic dispersion of the myceliophagus mite *Microdispus lambi* (Acari: Pygmephoridae). Twenty-four crops were monitorized during 18 months in commercial mushroom farms in Castilla-La Mancha (Spain). Adults of both species were collected weekly and the mites they carried were counted and identified. Both phorids (19.6%) and sciarids (4.4%) carried the mite *M. lambi*. The calculated load of each was 3.4 *M. lambi* mites per phorid and 1.9 per sciarid. The same percentage of male and female phorid was used as vector, but the load was lightly higher for females (1.86 mites per female compared with1.48 mites per male).

A mean of 7.2% of the phorids examined in winter were vectors of *M. lambi*, while in spring and autumn of the first year the average was higher than 22%. The mean load did not vary significantly between seasons. Inside the mushroom farms, less than 10% of a small initial population of phorids carried mites (less than 2 mites per phorid). As the cycle progressed, more than 35% of a larger population of emerging flies did so (average 3.5 mites per phorid vector). At the end of the growth cycle, the flies may fly off to colonize nearby farms, favouring the propagation of *M. lambi* from infected to uninfected crops.

Megaselia halterata is the principal vector of *M. lambi* in the mushroom farms of Castilla-La Mancha due to their high numbers, the high percentage carrying mites and the number of *M. lambi* they carry.

Key words: *Megaselia halterata*, *Lycoriella auripila*, *Agaricus bisporus*, mushroom mite, phoresis.

Introduction

The myceliophagus mite *Microdispus lambi* (Krczal) was detected for the first time in Spain in the summer of 1996 (Ferragut*et al.,* 1997). Since then this pest has become widely dispersed among Spanish mushroom growing farms. Previously, the mite had been described in New Zeland (Krczal, 1964), but had also been found in Australia and China (Clift & Toffolon, 1981; Gao *et al.,* 1986). It can develop and reproduce only on *Agaricus* species (Clift & Toffolon, 1981; Gao & Zou, 2001). Mite populations lead to the slow disappearance of the mycelium and substantial yield losses, sometimes leaving

farmers with no mushrooms to harvest at all. In Shanghai, contaminated mushroom spawn was a major source of mite infestation (Wu & Ma, 1988; Wu & Zhang, 1993), while in Australia, *M. lambi* was found to be phoretic on sciarid and phorid flies (Clift & Larsson, 1987).

A study of this pest in Spanish growing crops demonstrated that spawn, compost and casing materials cannot be considered as sources of contamination by *M. lambi* mite. Mite populations were detected on mushroom farms throughout the year, although the incidence declined markedly during the winter. In the *Agaricus bisporus* growing cycles, mites were first detected during the first flush but the initial infestation occurred soon after the application of the casing layer. Mite infestations were initially detected at the rear end of the room, near ventilation holes (Navarro *et al.*, 2004, 2010).

Dipteran species are some of the most serious arthropod pest problems affecting the cultivation of *A. bisporus* throughout the world (Sandhu & Bhattal, 1987; Tibbles *et al.*, 2005; Jess *et al.*, 2007; Erler *et al.*, 2009; Samshad, 2010). Mushroom yield losses are either directly due to the larvae of mushroom flies feeding on mycelia or mushrooms, or else due to other pests and diseases vectored by the flies (Erler & Polat, 2008). There is evidence of the transport of spores of different species of fungi by phorid and sciarid flies (White, 1981; Geels *et al.*, 1988; Shamshad *et al.*, 2009; Cloonan *et al.*, 2016). Similarly, both phorids as well as sciarids have been described as vectors of mites (Clift & Toffolon, 1981; Clift & Larsson, 1987; Keum*et al.*, 2015). In Spain, the species of mushroom flies commonly found in mushroom farms have been identified as *Megaselia halterata* (Wood) (Diptera: Phoridae) and *Lycoriella auripila* (Winnertz) (Diptera: Sciaridae), with a phorid to sciarid ratio of 4:1 (Navarro *et al.*, 2002). The predominance of phorid flies over sciarids in mushroom growing farms has been also described in Turkey (Erler & Polat, 2008) and in the Netherlands (Baars *et al.*, 2008). However, most authors that have studied mushroom flies describe sciarids as the major mushroom arthropod pest (Jess *et al.*, 2007; Fletcher & Gaze, 2008; Shamshad, 2010; Andreadis *et al.*, 2016; Eui & Seo, 2016). In Spanish mushroom farms the highest number of adult flies (phorids and sciarids) was collected in spring and autumn, while at sharp decrease in numbers was observed in winter (Navarro *et al.*, 2002), a situation also described in the literature by Jess *et al.* (2007) and Erler and Polat (2008). However, contrarly to those described by Jess *et al.* (2007), sciarid flies were not recorded throughout the year, but almost exclusively in spring. On the other hand, *M. halterata* was continuously detected in Spanish mushroom farms during the two years

of this previous study (Navarro *et al.*, 2002). A search for immature stages of phorids and sciarids in the substrates before filling of the farms and during the first few days of the crop demonstrated that, contrary to that described in the literature (Jess *et al*., 2007; Fletcher & Gaze, 2008; Erler *et al.*, 2009), neither the compost and nor casing materials can be considered as sources of contamination by phorids and sciarids in Spanish mushroom farms (Navarro *et al*., 2002, 2004).

Monitoring of the phorid and sciarid populations revealed that adult diptera mainly fly into the mushroom farms during application of the casing layer, although sometimes also during the incubation period. The usual route used by these species of flies to the farm was through ventilation holes (Navarro *et al.*, 2002, 2004).

Phoresy is one of the ways that wingless arthropod can disperse by attaching themselves to winged arthropods (Keum*et al.*, 2015). Thus, the dispersal of some mushroom mites might possibly depend on insects, although phoretic host specificity has not been clear in studies of most mushroom mites (Okabe, 2013). The aim of this paper is to increase our knowledge of the role of sciarid and phorid flies as vectors of the myceliophagus mite *Microdispus lambi.* It could also help to establish the way that other mushroom pests infest growing farms. Accurate determination of the sources and timing of infestations may provide an opportunity for an integrated pest management control strategy within mushroom production facilities.

Materials and methods

The study was carried out over a period of 18 months at 24 growing farms of Castilla-La Mancha (Spain) from March 1998 to August 1999, four crop cycles per season (Spring1: C1-C4; Summer1: C5-C8; Autumn: C9-C12; Winter: C13-C16; Spring2: C17-C20; Summer2: C21-C24). Each crop was located in a growing room (35x2.5x2 m) with a door for access at the front and a ventilation hole at the rear. Each crop was entirely grown in a single room and completed within 70 days.

Survey method

For each farm, a black light lamp (60 cm, Philips TLD 18w/08, Holland), equipped with a plastic sheet treated with a contact insecticide, was installed under the ventilation hole in order to collect flies. Each farm was visited weekly. On each sampling day a maximum of 48 flies was randomly collected in well-plates (IWAKI Glass, Japón) and

taken to the laboratory, where flies were identified (species and sex) by binocular microscope and mites that were phoretic on them were also identified and counted. The parameters defined for the study were the percentage of flies of each species carrying *M. lambi* mites, and the average load, defined as the number of *M. lambi* mites transported by each carrier fly (phorid or sciarid fly).

The factors studied were: species of fly (sciarid and phorid), sex (male and female), seasonal period in which the crop was grown (spring 98, summer 98, autumn 98, winter 99, spring 99, and summer 99), and the stage of the mushroom growing cycle. For this last category, the following growing stages were defined: before sowing (filling: day 0), after incubation (day 20 approx.), after the primordia had formed in the upper surface of the growing unit (induction: day 30 approx.), and after harvesting the first flush (F1: day 41 approx.), second flush (F2: day 48 approx.), third flush (F3: day 56 approx.), fourth flush (F4: day 63 approx.) and fifth flush (F5: day 70 approx.).

Data Analysis

An analysis of variance (ANOVA) was applied to study the effect of sex in the role of phorids as vectors of mites. Levene's test was used to check the homogeneity of variance, and a natural AsinR transformation was used to account for the heterogeneity of variance observed in the raw data related to the percentage of flies carrying mites.

Generalized linear models (GLM) were used to evaluate the effects of season and stage of growing crop factors, and of the season * stage interaction on the percentage of phorid flies carrying mites and on load variables (Gbur *et al.*, 2012). A total of 216 observations were evaluated for each variable - a factorial treatment consisting of 6 seasonal periods and 9 growth stages, with 4 replicates. To test whether continuous variables fitted a normal distribution, data was examined using normal probability plot, standardized skewness and kurtosis, and the Kolmogorov–Smirnov test. A natural AsinR transformation was used to account some observed heterogeneity of variance in the raw data of percentage of flies carrying mites. An SQRT transformation was used to account some observed heterogeneity of variance in the raw data of load. The effect of each particular season and growing stage on variables such as percentage of phorids as vectors or load was tested using indicator variables (or dummy variables) in multiple regression analysis (González-Ochoa *et al*., 2004). These indicator variables (predictor variables) were the different seasons (k-1 indicator or dummy variables, k =6 levels of seasons), and the growing stages (k-1 indicator or

dummy variables, k =9 levels of growing stages), and the interaction of both. The general linear statistic test (F-test, Neter *et al*., 1996) was used to test some hypotheses about regression coefficients. All the statistical analyses were performed using the Statgraphics Centurion XV program (Statistical Graphics Corp., Princeton, NJ)

Results and discussion

Phoretic role of sciarid and phorid flies

8,927 flies were recovered from twenty-four farms with black light lamps (60 cm, Philips TLD 18w/08, Holland): 7,196 phorids and 1,731 sciarids. In half of the farms (C5, C9, C13, C14, C16, C17, C18, C20, C21, C22, C23 and C24) the presence of *M. lambi* carrier sciarids was not detected (Figure 1). In the remaining farms (12 growing cycles), the average percentage of phorids transporting *M. lambi* mites was always higher than the percentage of sciarid carriers. This occurred even in the crop C4, in which the number of examined sciarids was higher than that of phorids (566 sciarids and 152 phorids, data not shown).

[Figure 1]

With regard to the average load of *M. lambi*, the number of *M. lambi* mites per vector fly was higher for phorids than for sciarids in all of the twelve mushroom crops where vector sciarid flies were detected (Figure 2). On one phorid vector, 41 *M. lambi* mites were detected, whereas, in the case sciarid vectors, the maximum load detected was 9 *M. lambi* mites.

[Figure 2]

In general terms, 19.6% of the phorids and 4.4% of the sciarid captured carried *M. lambi* mites. The average load calculated was 3.4 *M. lambi* mites on each phorid and 1.9 mites on each sciarid vector.

The phoretic dispersion of mites on flies has been widely documented (Witch & Snetsinger, 1971; Binns, 1972, 1973; Clift & Toffolon, 1981; Binns, 1982; Keum *et al.*, 2015). In the case of this myceliophage mite, Clift and Larsson (1987) demonstrated that the phorid *M. halterata* was clearly a preferred host of *M. lambi* in Australian mushroom crops, although they also found that *L. mali* (Fitch) could act as vectors of this mite. In this current study the low percentage of *L. auripila* acting as vectors

suggests that there may be a lower level of importance of this species of sciarid fly in the phoretic dispersion of *M. lambi.* The lower number of total sciarid detected on farms could explain this fact, since the distribution of phoretic mites could be influenced by the availability of carriers (Glida *et al.*, 2003). However, the adaptive significance of the phoretic association between *M. lambi* and *M. halterata* could be, rather, that mushroom mycelium is the only source of food for both mushroom pests (Clift &Toffolon, 1981). Sciarids have less stringent nutritional requirement, consequently the mite-sciarid relationship may be weaker. However, the difference in the average load carried by phorids and sciarids also coincides with that described by Clift & Larsson (1987), although in their work, the values differmuch more (9.1 and 2.9 mites per phorid and sciarid vector, respectively).

The greater presence in mushroom farms of *M. halterata* flies rather than *L. auripila* (ratio 4:1, Navarro *et al.*, 2002), together with a greater percentage of phorids carrying *M. lambi* and with a higher carried average load, lend weight to the importance of studying phorids as vectors in the phoretic dispersion of *M. lambi* in Spanish mushroom farms.

Influence of sex in the role of phorid as vector

Approximately one-third of the examined 7,196 phorids were males. The statistical analysis of the data showed that there was no significant difference between the sexes in the percentage of vector flies (19.4% for males and 19.7% for females; $F_{1,426}$= 0.37; p = 0.5437; LSD = 3.64; SED: 1.31), meanwhile the average size of the carried load (1.48 and 1.86 mites per males and females, respectively) was statistically higher for females ($F_{1,426}$ = 4.34; p = 0.0378; LSD = 0.36; SED: 0,13).

The number of *M. lambi* attached to *M. halterata* males and females (6.4 and 8.0, respectively) led Clift and Larsson (1987) to distinguish between non-dispersing males and dispersing females for this phorid species. However, our work, with a much higher number of examined fly, stablishs much lower differences between sexs. It could be due to the smaller size of the males rather than active discrimination.

Percentage of phorid flies carrying mites

The GLM developed to assess the effect of seasonal period interacting with that of stage of the growing cycle on the percentage of flies carrying mites stablishes that there was a higher signification ($p < 0.0001$, F-test) in the influence of both factors, meanwhile

there was no signification for the interaction between the terms of “season” and “stage” ($p > 0.05$, F-test) (Table 1).

[Table 1]

Multiple regression analysis (Table 2) showed that “winter99” and “spring99” seasons were significant factors for “phorids as vector” variable, decreasing the percentage mainly due to the decreasing populations of phorids and mites inside the farms for winter, that which also influenced the levels of infestations in the next spring. “Induction” stage also reduced the value due to the beginning of the emergence of the first generation of phorids without a hard infestation of mites into the growing substrates. Meanwhile “F3” and “F4” stages was also significant factors, but increasing the value because, at this periods of time, mite population reached a very high level.

[Table 2]

Load (number of M. lambi mites transported by each carrier phorid)

Regarding to the load of mite on each vector phorid, the GLM developed to assees the effect of seasonal period interacting with that of stage of the growing cycle on this parameter demonstratedthat there was a higher signification ($p < 0.0001$, F-test) in the influence of only “stage” factor. The p-values for “season” and for the interaction of both factors were not significant ($p > 0.05$, F-test) (Table 1).

Multiple regression analysis (Table 2) showed that “filling” stage produces a significant drop in the number of mites carried by each phorid vector, showing a very low level of infestation at the beginning of the cycle. Meanwhile “casing”, “F4” and “F5” stages produced a significant increment, associated clearly to the increasing level of the mite infestation inside the growing substrates.

Phoretic relationship between phorid fly and myceliophagus mite

A study of mushroom pests in Spanish mushroom farmshas pointed to a direct relationship between the myceliophagus mite *M. lambi* and the phorid fly *M. halterata* (Navarro *et al.*, 2002, 2004, 2010). The progression of both pests in the growing crop is represented in Figure 3a, while the progression of phoretic parameters during the growth cycle studied in this paper is reflected in Figure 3b. Both figures show the average values obtained for 24 crop cycles that were studied.

[Figure 3]

During the initial stages of spawn running, mites were not detected inside the growing substrates, and a low number of phorid adults were observed in the farms (Fig 5a), since the compost shows low concentrations of mycelium and is not attractive to oviposition (Smith *et al.,* 2006). During this time, less than 10% of phorid flies carried *M. lambi* mites, and with a small number of mites per phorid (Fig 5b). During the casing period a greater number of phorids entered the room due to the high concentration of volatile substances, which would act as attractant (Grove & Blight, 1983; Pfeil & Mumma, 1993; Tibbles *et al.*, 2005) (Fig 5a), and 20% of them carried mites, with an average load of 2 mites per phorid (Fig 5b). Concurrently, oviposition by *M. halterata* occurs, being stimulated by mycelium development (Jess *et al.*, 2017). Mites take advantage of this to leave the vector and migrate to the compost, a substrate rich in food sources.

The emergence of the first generation of flies developed inside of the growing medium and those coming from eggs laid during the days of casing (Lewandoski *et al.*, 2012), starts with the first flushes (F1-F2) (Fig 5a). At that time, the population of mites in the casing layer, a substrate from which most flies emerge (O'Connor &Keil, 2005), is still very low (Navarro *et al.*, 2010). Therefore, only a small percentage (<10%) of the high number of emergent flies (200-500 adults captured per plate and day) carries mites and the average load is small (approximately 1.5 mites per phorid) (Fig 5b).

The third flush coincided with a peak in the population of phorids (almost 800 adults captured per plate per day). The incidence of *M. lambi* in the substrates is also clearly greater (150-200 mites/sample, approx.), so a greater percentage of emerging flies transporting mites was detected (15-20%), and with a high average load size (2-3 mites per phorid). Finally, in the final stages of the cycle (F4-F5), the presence of mites in the cultivation substrates increases considerably (300-600 mites/sample) (Figure 5a), and they can be observed in large numbers on the casing layer. Thus, not only the flies that come from the casing but also those which continue to enter the farms are more likely to carry mites. For this reason, the percentage of phorid vectors increases considerably (up to 40% approx.), at the same time that an increase in the value of the average load transported is detected, with almost 4 mites on each carrier phorid. After the cycle, these flies, attracted by the volatiles from the growing mycelium of new

productive cycles, may colonise nearby crops, favouring the spread of *M. lambi* from infected crops to uninfected farms.

Other studies describe this same behaviour in other phoretic species on diptera, in which mites present in a substrate adhere to the diptero-vector at the moment in which the adult emerges from the pupa (Binns, 1973). Bortolon *et al*. (2016) considered that mites apparently attach themselves preferentially to females because after becoming adults, the flies return to the substrate to lay their eggs. This differential phoretic role of the sexes was not evident in this case, since the recorded differences could be due to the smaller size of the male rather than active discrimination.

The monitoring of pest populations and the determination of potential infestation sources are important prerequisites for establishing viable control strategies for crop pests (Jess *et al.*, 2017). Sanitation and exclusion practices are vital to integrated pest and disease management (Martín *et al*., 2016), because such control will not only minimises the risk of introductions from outside the farm but also reduces the chances of pest spreading on the farm from affected to uninfected crops (Shamshad, 2010). Insect pest control must be applied during the early stages of mushroom production to avoid significant damage and consequent yield losses (Jess & Bingham, 2004). Preventing flies from accessing the farm to lay their eggs, at least at the time of fruiting induction, would delay the onset of the first generation of diptera from the growing medium, while *M. lambi* infestation could be delayed and prevented from reaching high levels. Likewise, interruption of the crop after harvesting the third flush would prevent the spread of both pests.

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Table 1. R^2, residual deviance, degrees of freedom and p-value for each variable in the GLM. GLM was used for each parameter.

Parameter	R^2	Variable	Res. Des.	d.f.	p-value
Phorids as vector	56.92	Model	33471.6	53	0.0000
		Season	4727.0	5	0.0001
		Stage	19254.0	8	0.0000
		Season*Stage	9490.6	40	0.0504
Load	42.66	Model	29.39	53	0.0001
		Season	2.58	5	0.0712
		Stage	14.49	8	0.0000
		Season*Stage	12.33	40	0.1771

Table 2. Regression coefficients for predictor variables of the percentage of phorids as vectors and load

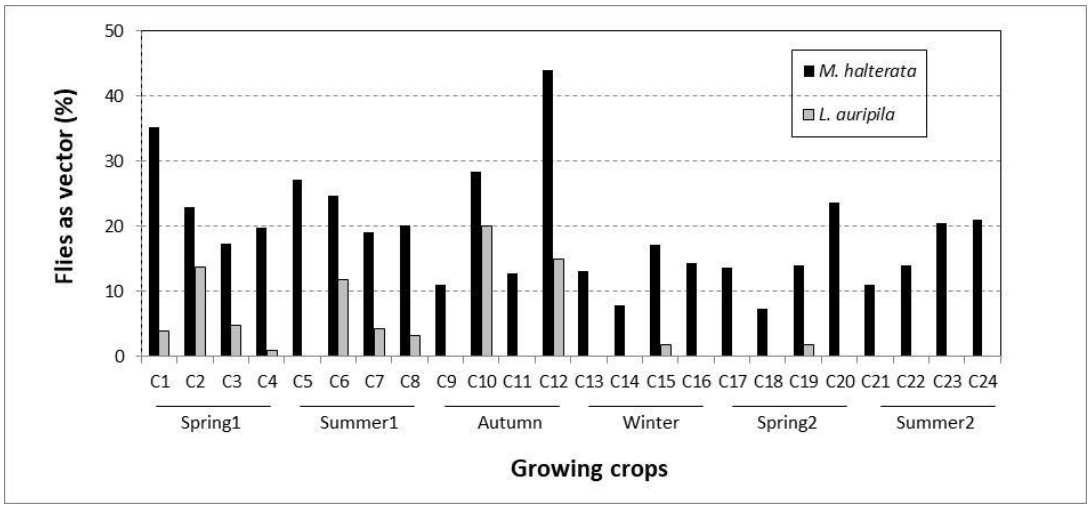
	Phorids as vectors (%)	Load
Constant	18.7	1.8
Winter99	-13.6	
Spring99	-10.4	
Filling		-1.1
Casing		0.7
Induction	-11.1	
F3	18.1	
F4	22.9	0.8
F5	-11.2	1.7
(Autumn98)*(F3)		2.3
(Winter99)*(Casing)		-2.5
(Spring99)*(Induction)	17.1	

Figure legends

Figure 1. Percentage of flies of both species as vector of *M. lambi*, for each of the 24 growing crops.

Figure 2. Load (number of *M. lambi* carried per phorid and sciarid) for each of the 24 growing crops.

Figure 3. a) Progression of the incidence of *M. lambi* (mites/20 g of substrate sample) and *M. halterata* (adults captured per trap and per day) in the different periods of the growth cycle. (b) Progression of the phorid as vector of *M. lambi* mites(%) and the load (mite per vector phorid) in the different periods of the growth cycle.



447

448 Figure 1

449

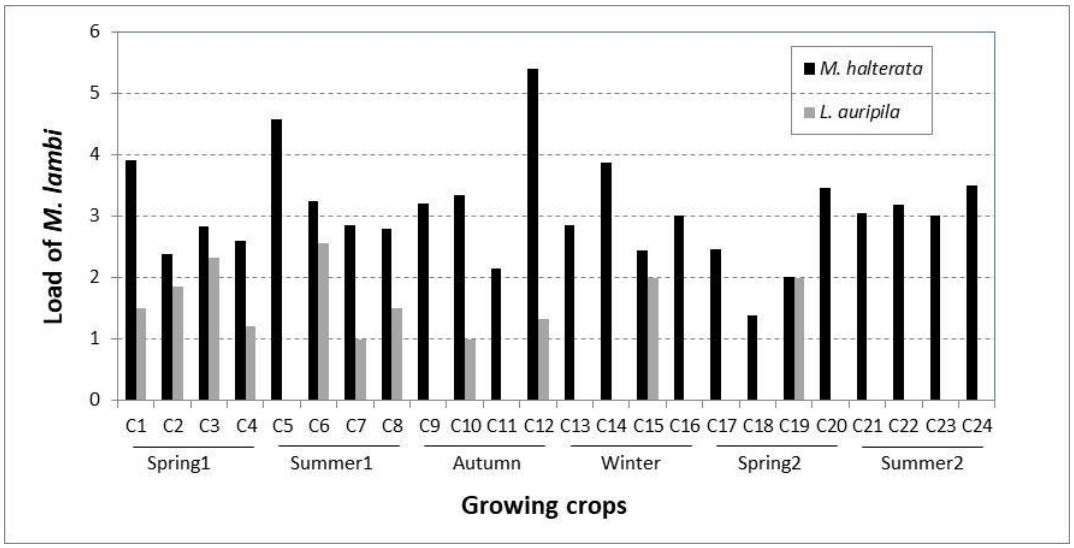

Load of M. lambi
0
1
2
3
4
5
6
M. halterata
L. auripila
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 C13 C14 C15 C16 C17 C18 C19 C20 C21 C22 C23 C24
Spring1
Summer1
Autumn
Winter
Spring2
Summer2
Growing crops

Figure 2

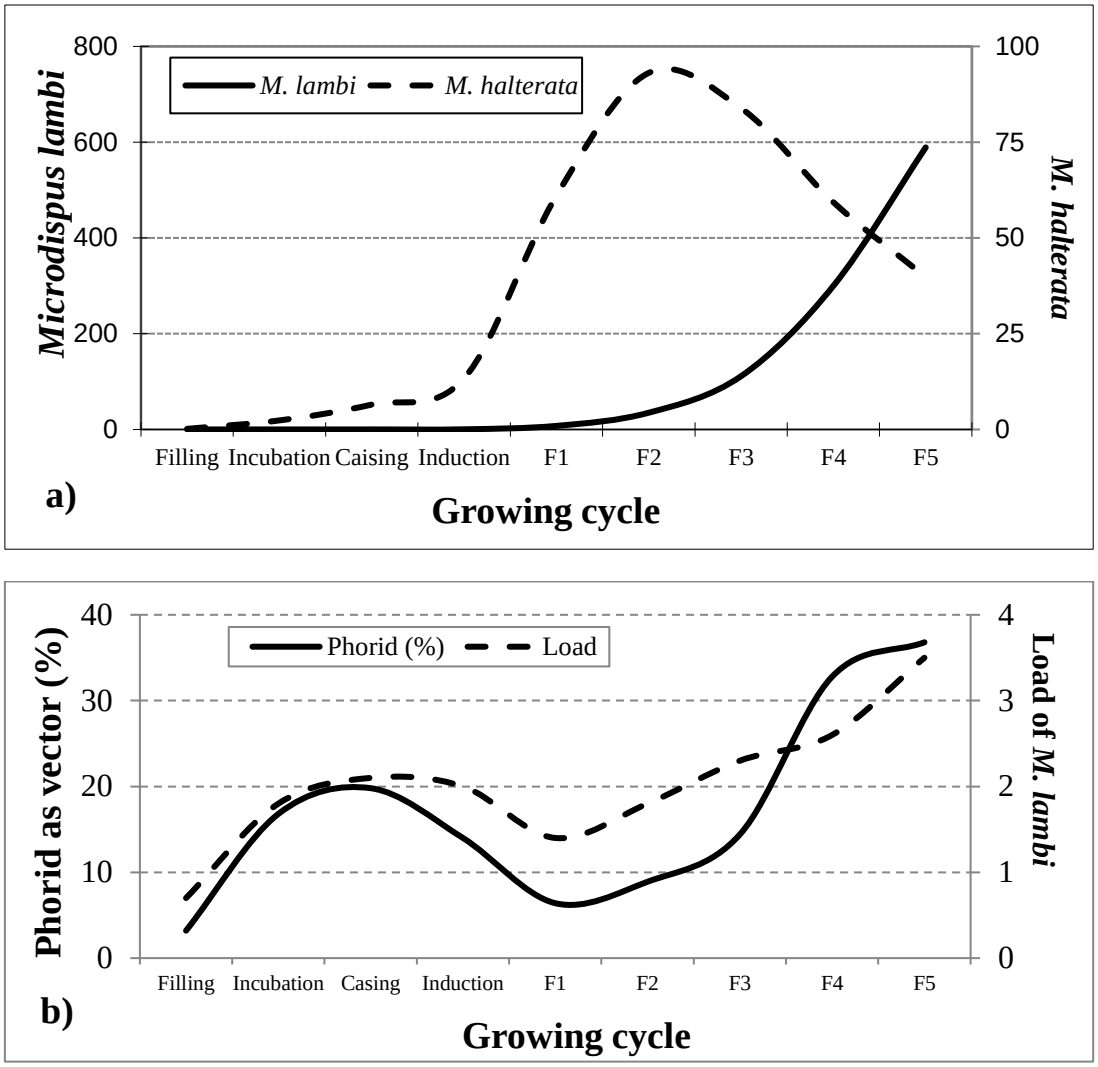

800
600
400
200
0
100
75
50
25
0
M. lambi
M. halterata
Microdispus lambi
M. halterata
Filling
Incubation
Caising
Induction
F1
F2
F3
F4
F5
a)
Growing cycle
40
30
20
10
0
4
3
2
1
0
Phorid (%)
Load
Phorid as vector (%)
Load of M. lambi
Filling
Incubation
Casing
Induction
F1
F2
F3
F4
F5
b)
Growing cycle

Figure 3