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Novel approaches for monitoring and controlling gyspy moth, *Lymantria dispar* (L.) (Lepidoptera Erebidae), in Mediterranean cork oak forests

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Abstract

The gypsy moth, *Lymantria dispar* (L.), is the major threat to cork oak forest in Mediterranean area. In Sardinia (Italy), the management of gypsy moth population outbreaks have usually been conducted through the aerial applications of *Bacillus thuringiensis kurstaki*-based formulations, whereby more than 100.000 hectares of forests have been protected from pest defoliation. Nonetheless, continuous research aimed to develop new sampling approaches to identify infested areas and to improve the effectiveness of control strategies is needed. In line with this, the present three-year PhD work was conceived to advance available methods for monitoring and controlling the gypsy moth in Mediterranean area.

Firstly, the spatial distribution of *L. dispar* egg masses among cork oak trees was studied during the different pest population development phases and sequential sampling plans based on the count of egg masses on trees were developed.

Secondly, the effectiveness of different *Btk* strains sprayed at large scale was evaluated at different application doses. Our results showed the possibility of alternating different *Btk* strains for resistance management purposes and of applying lower doses than labeled.

Finally, the efficacy of a *Lymantria dispar multicapsid nucleopolyhedrovirus* (LdMNPV) formulation against larval infestations was tested in field conditions. Our results highlighted the potential use of LdMNPV as an alternative to *Btk*-based formulations for protecting Mediterranean cork oak forests.

Keywords: gypsy moth, pest management, sampling, IPM, *Bacillus thuringiensis*, baculovirus,

General introduction

The gypsy moth, *Lymantria dispar* (L.) (Lepidoptera Erebiidae), is one of the most damaging pests worldwide.[1-3] This species can feed on more than 300 plant species,[4] among which oak (*Quercus* L.) species are the main hosts.[3,5-7] In the Mediterranean region, the gypsy moth is mainly associated with downy oak (*Q. pubescens*), holm oak (*Q. ilex*)[8] and mostly with cork oak (*Q. suber* L.), which grows in pure and mixed stands covering approximately 2.5 million hectares across Europe and North Africa.[9] In this environments, *L. dispar* can defoliate large forest areas during its periodic outbreaks, leading to a general decrease in plant growth and to a significant reduction in cork production .[10-11] Additionally, affected trees become more susceptible to other pests,[11] and when defoliation occurs in conjunction with a prolonged drought period, tree health status can be seriously compromised causing tree death and potentially increasing the spread of the oak decline phenomenon.[11-14]

Given the negative impact of *L. dispar* on forest environments, different monitoring programs aimed at detecting gypsy moth distribution range and assessing the infestation level have been developed.[15-16] In North America, where *L. dispar* is a non-native invasive species, a monitoring network based on pheromone traps was established to detect low-density 'founder' populations for slowing or stopping gypsy moth spread from infested to uninfested areas.[15-17] Instead, the main goal of monitoring *L. dispar* in native areas is to identify the areas at risk of defoliation to plan microbiological control programs.[17-18] For this purpose, in Sardinia (Italy), a network of georeferenced monitoring sites distributed throughout all the oak forests covering the region was established in 1980.[7,19] At each of the sites constituting the monitoring network, the gypsy moth population density has been annually estimated by counting all the egg masses on 40 trees according to a sampling protocol developed in Maroccan cork oak forests.[20] This network was fundamental to estimate the population size and distribution, and to identify with higher chances

of severe infestations. In the most infested areas, *Bacillus thuringiensis kurstaki* (*Btk*) formulations have been applied at large scale from 2001 to control *L. dispar* infestations.[7,21] From the beginning of the control program in forest areas where applications were repeated more than once, the monitoring network allowed to protect approximately 200,000 hectares of cork oak forests in the last two decades.[7,22]

Although several experimental trials were conducted so far in Sardinia to develop technical and operative guidelines,[7,23-24] continuous research is needed to improve the effectiveness of both monitoring and control measures against gypsy moth in Mediterranean forests. Particular attention should be given to the estimation of the gypsy moth population density through a well-defined sampling protocol, which is fundamental to support the decision-making process on insecticidal applications, and to improve the control program from a technical and application point of view. In fact, a control program might involve the use of different microbiological formulations both to enhance the insecticidal effect against gypsy moth larvae and to prevent possible insect adaptation to *Btk* toxins.[25]

In order to comply with these objectives, the following three-year study was conceived to provide innovative approaches for monitoring and controlling gypsy moth in Mediterranean cork oak forests. Specifically, the aims were to (i) investigate the spatial distribution of *L. dispar* egg masses among trees for implementing sequential sampling plans; (ii) assess the effectiveness of different *Btk* strains sprayed at large scale at different application doses; (iii) evaluate the efficacy of a *Lymantria dispar multicapsid nucleopolyhedrovirus* (LdMNPV) formulation against gypsy moth larval infestations.

The count of egg masses is recognized as the most suitable method for estimating the gypsy moth population density,[24,26-27] mainly because they are easy to identify throughout autumn and winter. Moreover, egg mass density can be used to gather predictive information on the risk of defoliation

in the following year.[17-18] All different sampling protocols that have been used to estimate gypsy moth egg mass densities are particularly time-consuming, especially when high infestations occur,[28] so that they could be considered too much expensive for Integrated Pest Management (IPM) purposes. Since the decision whether or not to carry out a pest management program should be based on exceeding a specific threshold, sequential sampling plans should enable to noticeably save sampling time and cost.

Btk-based formulations are normally used to control gypsy moth infestation in Mediterranean forests,[7,21] as it significantly reduces larval population density,[21,29] having few biological and practical limitations.[7,30] However, the effectiveness of *Btk* application against *L. dispar* mainly depends on different aspects, including the application dose,[31-32] and the *Btk* strain.[33-34] In fact, although the insecticidal action of *Btk* is achieved exclusively whether enough bacterial spores and Cry proteins are ingested by larvae,[32] larval susceptibility to toxins can vary depending on strains.[33-34] The availability of formulations containing different *Btk* strains could improve long-term control strategy. Besides the use in rotation or combination of different strains, the application of lower doses than suggested in the label would allow treatment cost saving or the opportunity to use locally available budgets to increase the surfaces of the forest to be protected.

Lymantria dispar multicapsid nucleopolyhedrovirus (LdMNPV) is a baculovirus that has naturally co-evolved with gypsy moth and represents an effective biocontrol agent in North America and Canada.[1,35] Although its use appears promising in other environmental conditions,[36] commercial formulations are not easily available in Europe and the production of the viral material results particularly expensive. LdMNPV could represent a valid alternative to *Btk*-based formulations for protecting Mediterranean forests from *L. dispar* defoliations, mainly because species-specific affinity allows to exclude effects on no-target fauna.[35] Despite its slower insecticidal action, LdMNPV can affect the following pest generations as a result of vertical transmission.[37] With

regards to the variability of gypsy moth ecotypes and of the environmental conditions, specific field trials are needed to ascertain the efficacy of available gypsy moth baculoviruses in different areas.

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CHAPTER 1

Title:

"Development of enumerative and binomial sequential sampling plans for monitoring *Lymantria dispar* (L.) (Lepidoptera Erebidae) in Mediterranean cork oak forests"

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Ready to be submitted

Development of enumerative and binomial sequential sampling plans for monitoring *Lymantria dispar* (L.) (Lepidoptera Erebiidae) in Mediterranean cork oak forests

Running title: Sequential sampling plans for monitoring gypsy moth in cork oak forests

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Abstract

BACKGROUND: The gypsy moth, *Lymantria dispar*, is the main threat to cork oak forests as it causes complete defoliation over large forest areas. Sampling methods used for monitoring the pest population density are generally very time-consuming for practical purposes, such as delimitation of infested areas for control programs. For this reason, enumerative and binomial sequential sampling plans were developed using data collected from 1999 to 2011 in pure cork oak forests in Sardinia (Italy). The Taylor's power law (TPL) was used to evaluate the spatial distribution of gypsy moth egg masses among trees, whereas an enumerative sampling plan at a precision level of 0.10 and 0.25 was developed using the Green's method, and finally stop lines of binomial plans were computed by Wald's sequential probability ratio test. RESULTS: The gypsy moth population density was extremely variable, ranging from 0.05 to 59.4 egg masses/tree over the considered period. Egg masses on trees were aggregately distributed as regression slopes of TPL were significantly greater than 1 in all the gypsy moth population development phases. Following Green's method, only 31 cork oak trees are to be monitored at the damage threshold of 2.5 egg masses/tree with a precision level of 0.25. Binomial sequential sampling plans required lower sampling sizes (26.9-31.4 trees) than conventional sampling plans as well.

CONCLUSIONS: Enumerative and binomial sampling plans could represent suitable methods for sampling *L. dispar* egg masses in Mediterranean cork oak forests, having the practical advantage of lower cost and time consumption than standard sampling plans.

Keywords: gypsy moth, forest defoliator, pest monitoring, Taylor's power law, Green's method

Introduction

The gypsy moth, *Lymantria dispar* (L.) (Lepidoptera Erebidae), is one of the most harmful pests worldwide.[1,2] The pest completes one generation per year feeding on more than 300 tree species, including species belonging to *Betula*, *Eucalyptus*, *Larix*, *Populus*, and *Quercus* genera.[1,3-4] *Quercus* species are recognized as the most suitable hosts for gypsy moth, particularly the cork oak (*Q. suber* L.), downy oak (*Q. pubescens* Willd.), and holm oak (*Q. ilex* L.) in the Mediterranean area,[2,5-6] and the white oak (*Q. alba* L.) and northern red oak (*Q. rubra* L.) in North America.[7] *Lymantria dispar* population dynamics is described by a typical fluctuation pattern with a starting period of low-density population (latency), after which the pest density increases (progradation), reaches a peak (culmination), and then decreases until returning to low levels (retrogradation).[1,8] Periodic density fluctuations cause severe outbreaks at regular intervals, which occur from 6 to 13 years depending on the geographical area and anthropogenic pressure.[6,9-11] During outbreaks, *L. dispar* is able to defoliate thousand hectares of forests,[6] causing a general weakening of trees with a subsequent reduction in plant growth.[12] In the Mediterranean area, where gypsy moth mainly infests cork oak,[2,5] a decrease in cork production has been observed in the year following complete or partial defoliations.[13] In order to control gypsy moth infestations in native areas,[14] and slow its spread in newly introduced areas,[15-16] a number of control programs have been developed.[17-20] To support the decision-making process of insecticide application, estimation of the gypsy moth population density at different spatial scales through a well-defined sampling protocol is fundamental. Pheromone-baited traps have been used to investigate the spread of pest in newly established areas of North America rather than to estimate infestation levels,[17,21] as no correlation between trap counts and defoliation was observed.[22] Egg mass counts are the most suitable method for estimating the population density in outbreak suppression programs.[23-25] In fact, egg masses are easy to identify by color, present in the field throughout autumn and winter,

and their density is strongly related to defoliation in the following year.[22,26] Different sampling protocols have been used to estimate gypsy moth egg mass densities,[1] including the fixed-radius plot,[23] fixed- and variable-radius plots,[23] timed walks,[24] and direct counts on trees.[27-28] The fixed-radius plot is one of the most used methods to estimate gypsy moth egg masses due to its simplicity.[29] The method considers the count of all egg masses within repeated circular plots of a given radius to estimate the average density of gypsy moth egg masses in the stand.[23,29] The "fixed- and variable radius plots" method, which was mainly used until the mid-1980s,[29] is particularly time-consuming as it takes into consideration the count of all the egg masses on the forest floor and trees within the plots.[23] The “timed walk” protocol consists of counting all egg masses that are readily visible in a fixed length of time (e.g., five minutes) by an observer walking randomly in a stand.[24] Although this procedure could reduce the monitoring time, it is not recommended as density estimates are not adequately accurate and vary considerably according to observers.[30] The direct count of egg masses on trees is mainly used in Mediterranean forests[6,28] in which the population density of *L. dispar* is estimated by counting all the egg masses present on ten consecutive trees along the four cardinal directions starting from a common central reference point (i.e., 40 trees/site).[27] Some studies conducted in Mediterranean cork oak forests showed the strength of this method to estimate properly egg mass density and predict risk of defoliation.[26,28] In particular, significant defoliations were observed in the areas where average population density was higher than 100 egg masses on 40 trees (i.e., 2.5 egg masses/tree on average).[25,28] Nevertheless, this sampling method resulted to be particularly time-consuming, especially when both high and low infestations occurred,[31] and could be too expensive for IPM purposes. In fact, in this case, the exceeding of an action threshold (AT) would suffice to take the decision whether to control the pest or not. In order to reduce the sampling effort, the sequential sampling plans implemented up to now for gypsy moth management in North-East America considered

exclusively the sampling of egg masses through either area-based sampling procedures,[31-32] or timed walks.[33] Considering that sequential sampling should enable to noticeably save sampling time and cost, we developed sequential sampling plans based on the direct count of egg masses on trees in order to support decision-making for insecticide applications against *L. dispar* in Mediterranean forests. The specific aims of our work were to investigate the spatial distribution of gypsy moth egg masses to implement sequential egg mass sampling plans based on both enumerative and binomial samplings.

Materials and Methods

Study area and data collection

The study was carried out in Sardinia (Italy) which includes one of the most important cork oak forested areas in the Mediterranean basin.[34] In Sardinia, *Q. suber* occupies more than 128,000 hectares in pure or mixed forests, mainly associated with *Q. ilex* and *Q. pubescens*. The climate conditions where *Q. suber* grows are Mediterranean with a typical pattern of long dry season during summer and rainfall mostly distributed in winter. The average annual temperature and precipitation are 13.4 °C and 785 mm, respectively.

For this study, data collected from 1999 to 2011 in a permanent monitoring network covering the main Sardinian cork oak forests were used.[9] This network was established in 1980 and currently includes more than 680 sites, where gypsy moth egg masses are estimated using the procedure developed by Fraval and colleagues.[27] At each site and year, the sampling take into consideration the counts of all egg masses on 40 trees selected on the main cardinal directions from a common starting point (i.e., 10 trees for each cardinal direction). This sampling technique allows to properly estimate the egg mass density in the surrounding 5 ha.[27]

Estimating spatial distribution

The Taylor's power law (TPL)[35] was used to analyze the spatial pattern of *L. dispar* egg masses among trees. The TPL, which has been commonly considered for estimating spatial distribution pattern of pests in different environments, describes the specific relationship between mean (*m*) and variance (s^2) by a power function as:

$$s^2 = am^b \quad (1)$$

where *a* and *b* are parameters, both contributing to describe spatial aggregation.[36] In particular, the term *b*, which is also defined as Taylor's aggregation index, is conventionally used to indicate a uniform ($b<1$), random ($b = 1$), or aggregated ($b>1$) distribution.[37] Parameters *a* and *b* were estimated after the natural logarithm transformation (*ln*) of *m* and s^2 by the linear regression:

$$\ln(s^2) = \ln(a) + b\ln(m) \quad (2)$$

Mean and variance of egg masses abundance per tree were calculated for each sampling site from 1999 to 2010 (n = 547) and linear regressions were fitted separately for data collected during each phase of population dynamics and overall data. ANOVAs were performed separately for each regression line to test whether slopes statistically differed from 1, which indicates either a uniform ($b<1$) or clumped ($b>1$) spatial distribution pattern. In order to evaluate different spatial distribution patterns among different phases of population dynamics, a factorial analysis of covariance (ANCOVA) was applied considering the gradation phase as a covariate. Differences in slopes among linear regressions were tested by evaluating the significance of the interaction term in the ANCOVA model ($p<0.05$).

Enumerative sampling plan

The fixed-precision sequential sampling plan was developed with a total of 547 datasets collected from 1999 to 2010 in 161 monitoring sites. Optimum sample size (*N*) for Green's plan[38] was calculated at the levels of precision (P = SEM mean^{-1}) of 0.10 and 0.25 using the equation proposed by Karandinos:[39-40]

$$N = 1/P^2 am^{(b-2)} \quad (4)$$

where *N* is the number of samples (i.e., oak trees) that are necessary to estimate a density of egg masses equal to *m*, *P* is the desired precision level, and *a* and *b* are the TPL coefficients. Stop lines, indicating the number of trees required to estimate egg mass density at a defined precision level, were calculated as:

$$T_n \geq (an^{1-b}/P^2)^{1/(2-b)} \quad (5)$$

where T_n is the cumulative number of egg masses sampled and *n* is the total number of sampled trees. Stop lines were then generated by plotting the values of T_n against the correspondent values of *n*.

Validation of Green's sampling plan was carried out using the resampling for validation of sample plan (RSVP) software,[41] which allows to resample each validation dataset until the sequential stop lines are crossed. The minimum sample size for both precision levels (0.10 and 0.25) was set at ten trees. The mean precision and mean sample size were estimated using 500 iteration runs and were used to calculate the overall mean precision and overall mean sample size. A total of 55

independent datasets collected in 2011 were used to validation process. Validation was done on datasets with at least an average density of 1 egg mass/tree.

Relationship between egg masses density and occupied or infested trees

A positive relationship between the population density and occupancy *sensu stricto* is a consequence of the spatial distribution of individuals of each species.[42] In order to evaluate the congruence of this pattern for gypsy moth infesting cork oak trees, the relationship between the density of egg masses and percentage of both occupied and infested trees was assessed. A cork oak tree was classified as "occupied" when 1 or more egg masses were observed, whereas it was considered as "infested" when 3 or more egg masses were counted. The latter corresponds to the integer value closest to 2.5 egg masses/tree (i.e., action threshold of 100 egg masses/site). Before the analyses, data were separated depending on the phase of gypsy moth population development (i.e., progradation, culmination, retrogradation), and explored for data distribution. Given the nature of the relationship following an exponential model, linearization was carried out by log(x+1) transformation. Linear regression models followed by analysis of variance (ANOVA) ($p<0.05$) were performed separately for each type of gypsy moth development phase using the cumulative number of egg masses as dependent variable and the percentage of either occupied (i.e., percentage of trees with 1 or more egg masses) or infested trees (i.e., percentage of trees with 3 or more egg masses) as predictors.

Binomial sampling plan

A binomial sampling plan was generated using datasets used for developing Green's enumerative sampling plan. Two different binomial plans were developed relying on the relationship between

the percentage of occupied (i.e., percentage of trees with 1 or more egg masses) and infested trees (i.e., percentage of trees with 3 or more egg masses), and pest density. Two different binomial sampling plans were generated using the tally thresholds of 1 and 3. The tally threshold is the minimum number of egg masses required to classify a tree either as occupied (1) or infested (3) by *L. dispar*. Validation was made separately for each population development phase (i.e., progradation, culmination, retrogradation). Wald's sequential probability ratio test (SPRT)[43] was used, and stop lines were generated by using RSVP software.[41] Stop lines were generated considering the upper (θ_1) and lower (θ_2) boundaries for the decision action threshold and α (type I) and β (type II) errors, which indicate either the probability of treating when pest density is below the defined AT or the probability of not treating when pest density exceeds the AT, respectively. The θ_1 and θ_2 were set at 10% above and below the AT, respectively, whereas a value of 0.10 was used for both α and β errors. Validation process was made using 500 resampling iterations with replacement, and the minimum sample size was arranged considering the *x*-axis intercept of the lower stop line.

For both sampling plans, operating characteristic (OC) functions and average sample number (ASN) were calculated separately for each phase of gypsy moth population development and were used to validate the binomial sampling plans. The OC function estimates the probability of not taking action when the pest population reaches a particular density,[44] and allows to determine the accuracy of a binomial sampling plan, whereas the ASN is used to outline the efficiency of a binomial sampling plan as it indicates the sample size necessary to make a decision.[40,45] The OC functions were determined by regressing the values obtained from RSVP against the proportion of either occupied or infested trees and fitting a four-parameters log-logistic model[46] corresponding to the function:

$$f\big(x,(b,c,d,e)\big) = c + \frac{d-c}{1+\exp\big(b(\log(x)-\log(e))\big)} \quad (6)$$

where *x* is the proportion of infested trees, and *b*, *c*, *d*, and *e* the parameters of the function.[46] Model fitting were performed using the drc package[47] in R software.[48] Moreover, the actual α and β errors were calculated from OC curve. The 1 - OC value at θ_2 gives the actual α, whereas the OC value at θ_1 gives the actual β.[49]

Finally, a decision probability matrix was built to evaluate the precision of binomial sequential sampling plans.[50] The probabilities of taking (i.e., "to treat") or not taking (i.e., "not to treat") the correct decision were assessed by comparing the observed proportion of either occupied (trees with 1 or more egg masses) or infested trees (trees with 3 or more egg masses) with the estimated proportion obtained from the simulations. Probability matrices were calculated for all the different phases of gypsy moth population development. A probability matrix is composed by four cells indicating the correct decision to treat (A) or not to treat (B), and the incorrect decision to treat (C) or not to treat (D). The decision to treat or not to treat is correct when observed and estimated pest densities are above and below the AT, respectively.[50] Since the decision can be exclusively right or wrong, either A+B or C+D will be equal to 1 in the matrix.[50] For this reason, the probability A will equal to 1−OC and B will be OC when pest population density exceeds the AT. Contrarily, when population density is too low to justify a treatment, the probability C and D will be equal to 1-OC and OC, respectively.[50] The probability of making a correct decision with a given tally threshold is:

$$\sum p_i(A_i + D_i) \quad (7)$$

where p_i is the proportion of n datasets represented by dataset i, A_i is the probability of making the correct decision to treat, and D_i is the probability of making the correct decision to not treat.

Results

Spatial distribution

The mean population density of gypsy moth was extremely variable among monitoring sites and years, ranging from 0.05 to 59.4 egg masses/tree (Table 1). The highest and lowest mean values were observed in culmination (59.4 egg masses/tree) and retrogradation (0.01 egg masses/tree) phases, respectively. A strong correlation between mean and variance was observed for TPL in all population development phases (Table 1). *Lymantria dispar* populations had an aggregate distribution on trees as the slopes *b* of TPL were significantly greater than 1 when data were analyzed all together and separately for each phase (overall: $t = 29.66$; df = 1,545; $p<0.01$; progradation: $t = 18.01$; df = 1,179; $p<0.01$; culmination: $t = 9.05$; df = 1,172; $p<0.01$; retrogradation: $t = 22.84$; df = 1,190; $p<0.01$). ANCOVA showed that slopes of regression lines were not significantly different depending on the phases of gypsy moth population dynamics ($F = 1.13$, $p = 0.32$). Consequently, the parameters *a* and *b* estimated using overall data were used for implementing the enumerative sequential sampling plan.

Enumerative sampling plan

The optimum sample size (*N*) and sequential stop lines calculated with Green's method at different precision levels (0.10 and 0.25) are illustrated in Figure 1. The number of sampled trees decreased slightly as the population density increased at both precision levels (Figures 1A and 1B). The number of trees required to be sampled at a population density of 2.5 egg masses/tree (i.e., action threshold) was 31 at the precision level of 0.25 (Figure 1A), whereas 191 trees were needed at the

precision level of 0.10 (Figure 1B). At the same egg mass density, stop lines calculated at $P = 0.25$ and 0.10 indicated that sampling should be stopped at 76 (Figure 1C), and 479 cumulative egg masses (Figure 1D), respectively.

Results of validation of Green's sequential sampling plan are reported in Table 2. Mean precision of sequential sampling plan at $P = 0.25$ (0.183) was better than the desired precision, although it resulted slightly worse (0.190) than that at $P = 0.10$. Precision of sequential sampling plans was poorer than the desired levels in correspondence to the lowest values of population density both at precision level of 0.25 (Figure 2A) and 0.10 (Figure 2B). Mean sample size was 27.3 trees at $P = 0.25$ (Table 2) and it increased meaningfully to 164.7 trees at the precision level of 0.10 (Table 4). Mean sample size decreased with the increase of population density at both precision levels (Figures 2C and 2D).

Relationship between egg masses density and percentage of infested trees

Linear regressions indicate a significant relationship between the density of gypsy moth egg masses and percentage of both occupied and infested trees in all the different population development phases (Table 3), and the egg masses density grew up as the percentage of occupied and infested trees increased following an exponential curve (Figure 3). Threshold of 100 egg masses per site was reached when the percentage of trees occupied by at least 1 egg mass reached 58.5 ± 1.3% on average. This value was lower during progradation (57.3%) and higher during culmination (59.8%). Percentage of infested trees (with 3 or more egg masses) corresponding to a threshold of 100 egg masses per site was 40.1 ± 2.4% on average, with the lowest and highest values in culmination (37.6%) and in retrogradation (42.3%) phases, respectively.

Binomial sampling plan

Different action and tally thresholds were used to generate stop lines for the binomial sequential sampling plans according to monitoring of either occupied or infested trees. In particular, an AT = 58.5% (i.e., average percentage of trees occupied by at least 1 egg mass corresponding to a threshold of 100 egg masses per site) and a tally threshold of 1 were considered when occupied trees were sampled (Figure 4A). On the other hand, an AT = 40.1% (i.e., average percentage of trees infested by at least 3 egg masses corresponding to a threshold of 100 egg masses per site) and a tally threshold of 3 were used to develop stop lines for infested trees (Figure 4B). The intercept of the lowest stop line on the *x*-axis (i.e., the minimum sample size necessary to be examined to take a decision) was 7.3 and 16.5 for plans based on occupied and infested trees, respectively. Consequently, the minimum samples used for validating the binomial sampling plans were set at 8 using occupied trees and 17 for infested trees.

The OC and ASN values, which were determined separately for each binomial sequential plan and *L. dispar* population development phases, are reported in Table 4. For the binomial plan based on the sampling of occupied trees, the OCs were 0.496, 0.503 and 0.505 in progradation, culmination, and retrogradation phases, respectively. This indicates that the binomial sampling plans were marginally more conservative during progradation than the other population growth phases as the decision to treat occurred more often than the decision not to treat. The actual α and β values were lower than 0.10 in all different development phases (Table 4). Moreover, mean ASNs ranged from 26.9 trees in culmination to 31.4 trees in progradation. Validation of binomial plan based on the sampling of infested trees indicated that binomial plan resulted to be slightly conservative during both progradation (OC = 0.488) and retrogradation phases (OC = 0.486), whereas it was marginally tolerant when tested in culmination phase (OC = 0.509). The actual α and β values were lower than 0.10, as already observed for the binomial sampling plan of occupied trees,

whereas mean ASNs were 42.1, 35.3, and 42.4 in progradation, culmination and retrogradation phases, respectively.

The probability (A+D) indicating the correct decisions to treat or not to treat differed among plans and population development phases (Table 4). For the plan based on the percentage of occupied trees, (A+D) showed the lowest values (0.779) when the plan was applied during progradation, whereas it reached the highest values of 0.885 in culmination (Table 4). The probability of correct decision (A+D) was greater for the plan based on the percentage of infested trees and reached values higher than 90% during all different population development stages, with the highest value of 0.966 in culmination (Table 4).

Discussion

Sampling methods based on egg mass observation are largely used to estimate gypsy moth density in order to study its population dynamics, and planning control strategies aimed at reducing the damage caused by its infestations. Since counting all egg masses on trees can be as time consuming as the other sampling methods, time-saving sequential sampling plans useful for identifying promptly the infested forests are proposed in the present study. Sequential sampling plans were previously proposed for gypsy moth management-oriented programs in North America.[31-33] However, their implementation was conducted considering either area-based or timed walk samplings, whereas a sequential sampling plan based on egg mass counts on trees has not yet been proposed. The main technical limitation in using those sequential sampling plans was the high number of samples required to estimate gypsy moth density at low population levels (i.e., <250 egg masses/hectare).[31] Considering that sampling plans for decision-making in management programs based on insecticide applications indicate when to make the decision to spray or not to spray, the sampling method employed must be appropriate to the information required.[45]

Therefore, developing a sampling method that accurately estimates the occurrence of harmful pest densities rather than determining population densities is more appropriate to manage gypsy moth infestations. Although the sampling method we adopted does not provide information on population density per unit area, measuring egg masses per tree can be considered as one of the most suitable variable for prediction defoliation at a regional scale, especially for decision-making purposes in outbreak suppression programs.[22] As suggested by Liebhold and colleagues,[22] egg masses counts from a grid of permanent sampling sites distributed throughout the susceptible forests can be interpolated using different geostatistical techniques (i.e., kriging) to identify the areas where the action threshold is more likely to be exceeded.

The selection of a well-defined population threshold (i.e., the density level to be monitored) is fundamental for employing a sequential plan given that control measures should be applied only if pest density reaches this targeted level.[37] The action threshold of 2.5 egg masses/tree applied for cork oak forests in Sardinia was similar to that of 1.79 egg masses/tree under burlap bands in oak forests in North America,[22] mainly considering that *L. dispar* populations in North America did not co-evolve with their hosts and are not completely regulated by native predators and parasitoids.[51-52] Moreover, *L. dispar* can have a different development performance depending on host species,[53-54] so that the action threshold can change from one to another host. For all these reasons, effectiveness of a sequential sampling plan should be tested before its general application, and calibration of model parameters is highly recommended when significant differences in environmental conditions occur compared to those in which it was developed.

The spatial distribution of *L. dispar* egg masses in cork oak forests resulted to be aggregated and the pattern was not influenced by the different gradation phases, thus indicating that parameters are generally applicable even when gypsy moth development phase is unknown. However, parameters should be reassessed when different environment conditions (e.g., main host species)

occur because spatial distribution could be consequently affected.[32-33] Although egg mass spatial distribution was not influenced by gradation phases, sampling should be performed paying attention to further potential biological indicators, such as the average height of oviposition on trees above soil surface, which varies depending on the gradation phase in Mediterranean forests,[28] and length of egg masses. The latter represents an effective estimator of fecundity of gypsy moth,[55] and resulted to be more precise than egg mass density in predicting oak defoliation in North America.[56] These indicators should be taken into account especially when the estimated population density is close to the action threshold. Similar egg mass density can have different consequences in term of defoliation depending on the gradation phase. In Sardinia, an average density of approximately 2.5 egg masses/tree cause severe defoliation in progradation, whereas the same population density could lead to partial defoliation in retrogradation phase (authors' personal observation). This discrepancy is mainly due to both the effect on gypsy moth mortality by the natural enemy complex, which acts mainly after the culmination phase,[52,57] and fecundity (i.e., average number of eggs per egg mass), being generally lower in retrogradation than in other population development phases.[28-55]

Following the enumerative sequential sampling plan, a sample size of 31 trees is required for estimating an average density of 2.5 egg masses/tree (i.e., action threshold) with a precision level of 0.25. This value is lower than that of 40 cork oaks sampled with the conventional sampling protocol proposed by Fraval and collaborators.[27] Contrarily, when the level of precision of 0.10 was set, the number of trees needed to be sampled was considerably higher (191) than the standard sampling method. Since a level of precision of 0.25 is adequate for extensive monitoring protocols,[37] enumerative sampling plan at D = 0.25 can be suitably applied to minimize the effort in sampling gypsy moth egg masses in Mediterranean cork oak forests. Although a higher number of samples should be necessary to estimate the moth density at low population level, the proposed

method can be quite adequate to support control programs against *L. dispar* because it provides information about whether or not the pest reaches an action threshold.

For decision-making purposes, binomial sequential sampling plans is more suitable and less labor intensive than enumerative plans.[44] Validation procedures showed that both binomial sampling plans required an ASN higher than that of enumerative plans. In fact, ASNs in binomial plans ranged from 8 to 98 and from 17 to 212 when occupied (tally threshold = 1 egg mass) and infested (tally threshold = 3 egg masses) trees were considered, respectively (Table 4). Moreover, the binomial sequential plan based on infested trees in culmination phase showed an accuracy higher than 95% in making the correct decision to treat or not to treat (Table 4), whereas the probability of making an incorrect decision of not treating was lower than 5%. This result is of particular interest and shed light on the suitability of binomial sampling plans for decision-making purposes, mainly when gypsy moth population density exceeds the action threshold in culmination phase. In fact, *Bacillus thuringiensis* serovar *kurstaki* applications in culmination phase decreased significantly the damage on trees enhancing the long-term effectiveness of treatment as well.[8] In line with this, the binomial sequential plan developed on occupied trees was not as accurate as that developed on infested trees, as the probability of making an incorrect decision in culmination phase reached values of about 10%. Since the sample size is fundamental for selecting sampling procedures,[58] our results suggest that binomial sampling plans at both ATs can be considered as effective as the enumerative plan for decision-making purposes in Mediterranean environmental conditions. However, the availability of each sequential plan should be evaluated in field conditions by calculating the sampling time, as the amount of time required for either counting all the egg masses on trees or evaluating only the occurrence of occupied and infested trees varies largely.

Conclusions

The application of a well-defined sequential sampling plan for monitoring gypsy moth could notably implement IPM programs in Mediterranean area. With some differences in accuracy, all the sampling plans proved to be similarly effective regardless of gypsy moth population development phase so that they could be applied effectively even when the gradation phase is unknown. Both enumerative and binomial sampling plans could represent a better option for monitoring *L. dispar* egg masses in Mediterranean cork oak forests than the conventional sampling method currently adopted, mainly because of lower costs and time consumption. Future research will evaluate accurately the sampling effort associated with each sequential plan in order to determine the most suitable method and time saving.

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Author Contribution Statement

AL and RM conceived and designed the research. RM analyzed data. RM, MO, AC and AL wrote the manuscript. All authors read and approved the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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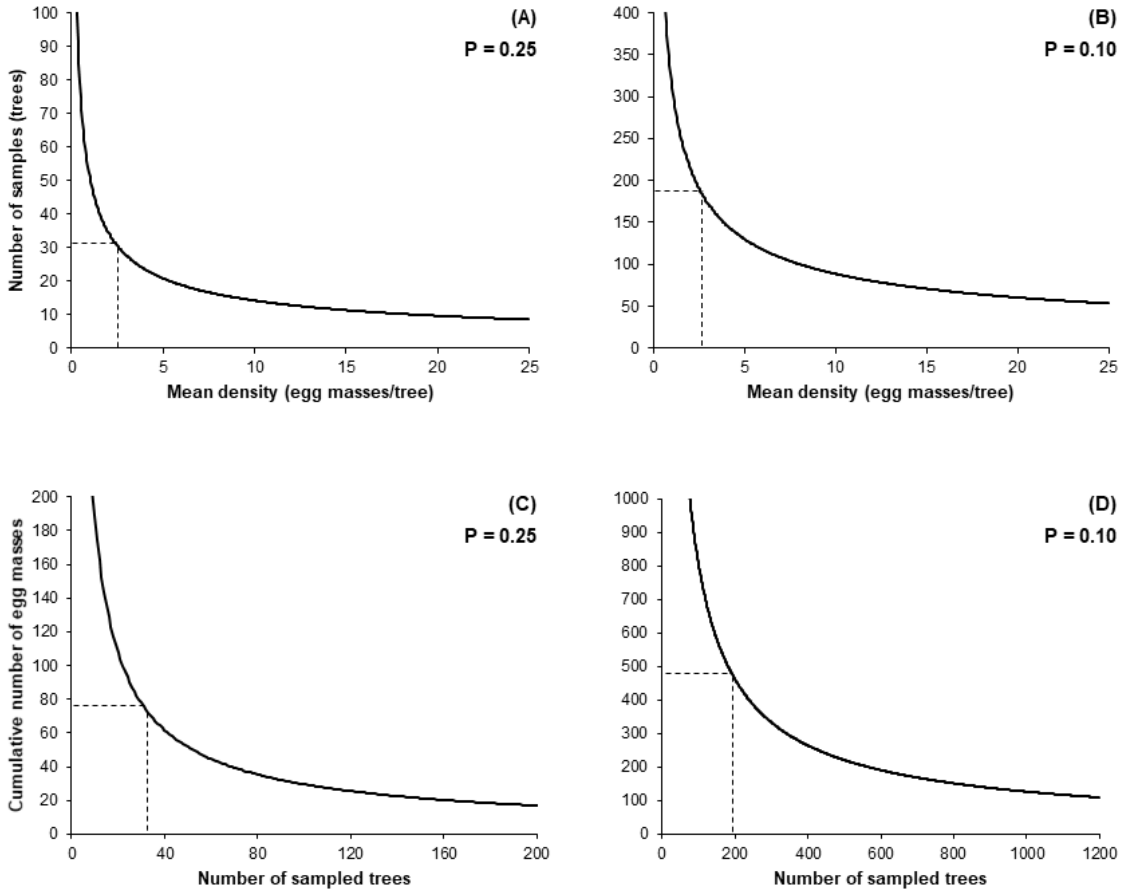

Figure 1. Sample sizes (A and B) and sequential stop lines (C and D) for the assessment of *Lymantria dispar* egg mass density on cork oak trees using Green's method at D = 0.25 (A and C) and D = 0.10 (B and D).

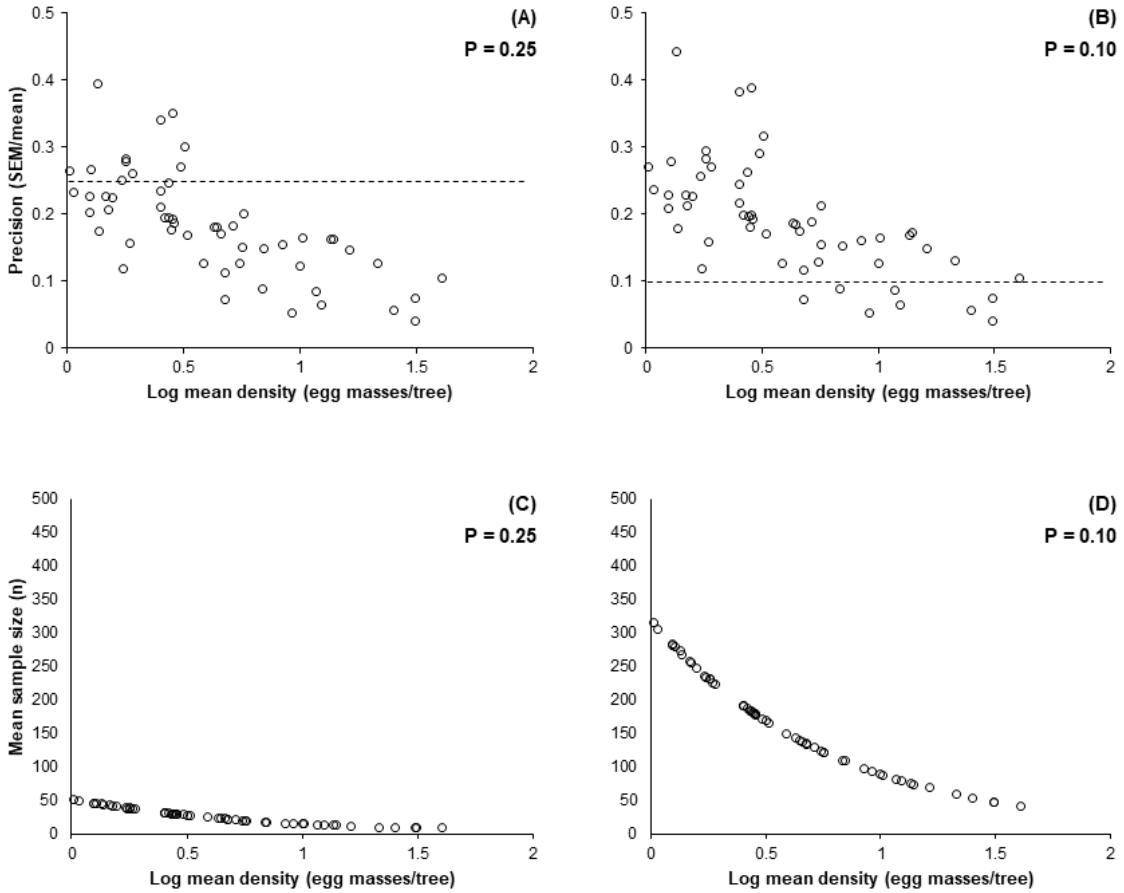

Figure 2. Validation of enumerative sampling plans to assess the infestation of *Lymantria dispar* on cork oak trees, based on Green's plan showing actual precision levels (A and B) and sample sizes (C and D) calculated at a fixed level of 0.25 (A and C) and 0.10 (B and D). Dotted lines indicate the desired precision levels of 0.10 (A) and 0.20 (B).

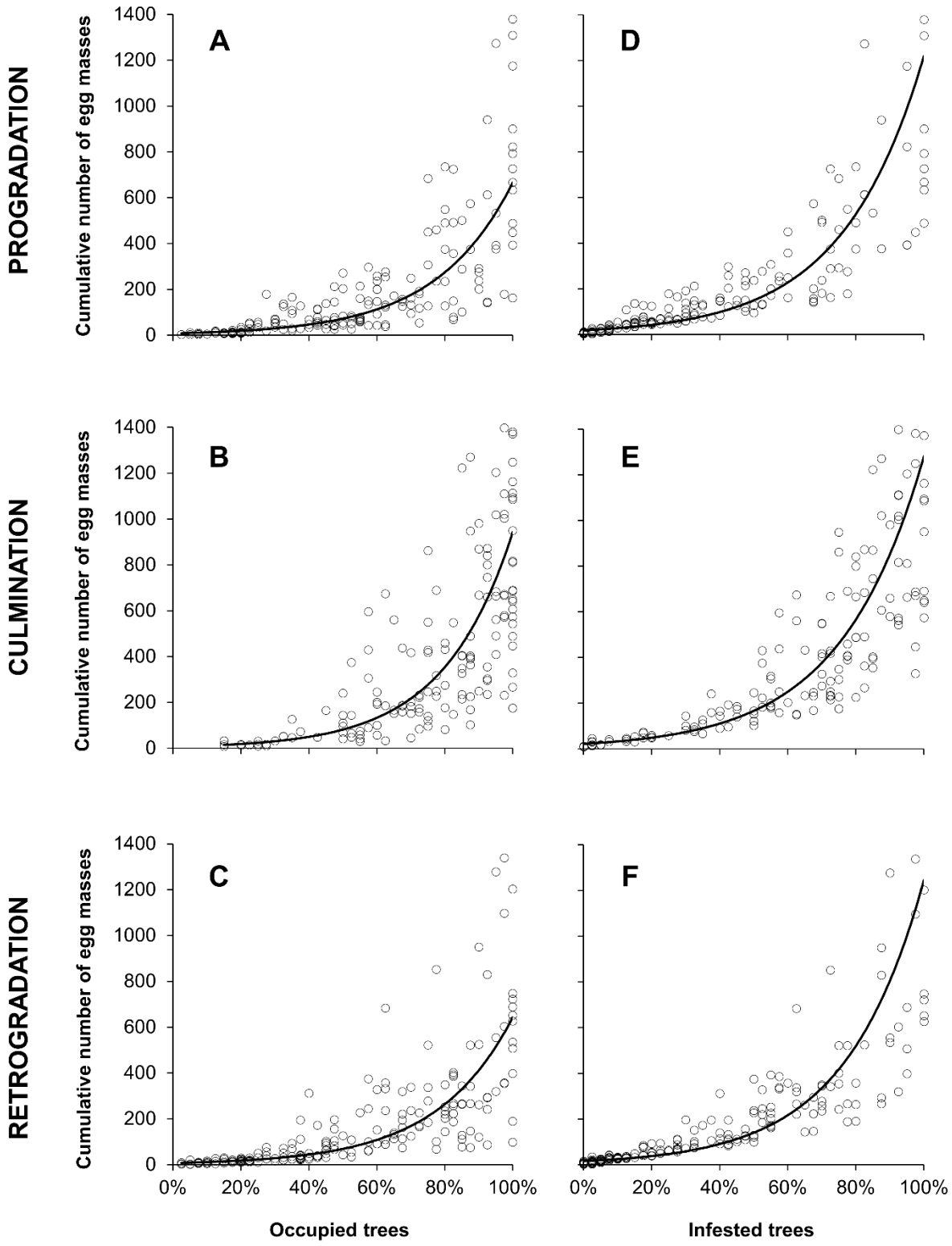

Figure 3. Relationship between the cumulative number of *Lymantria dispar* egg masses and the percentage of occupied (with one or more egg masses) and infested (with three or more egg masses) trees. Linear regressions were fitted separately for each phase of gypsy moth population development: progradation (A and C), culmination (B and D), retrogradation (C and F).

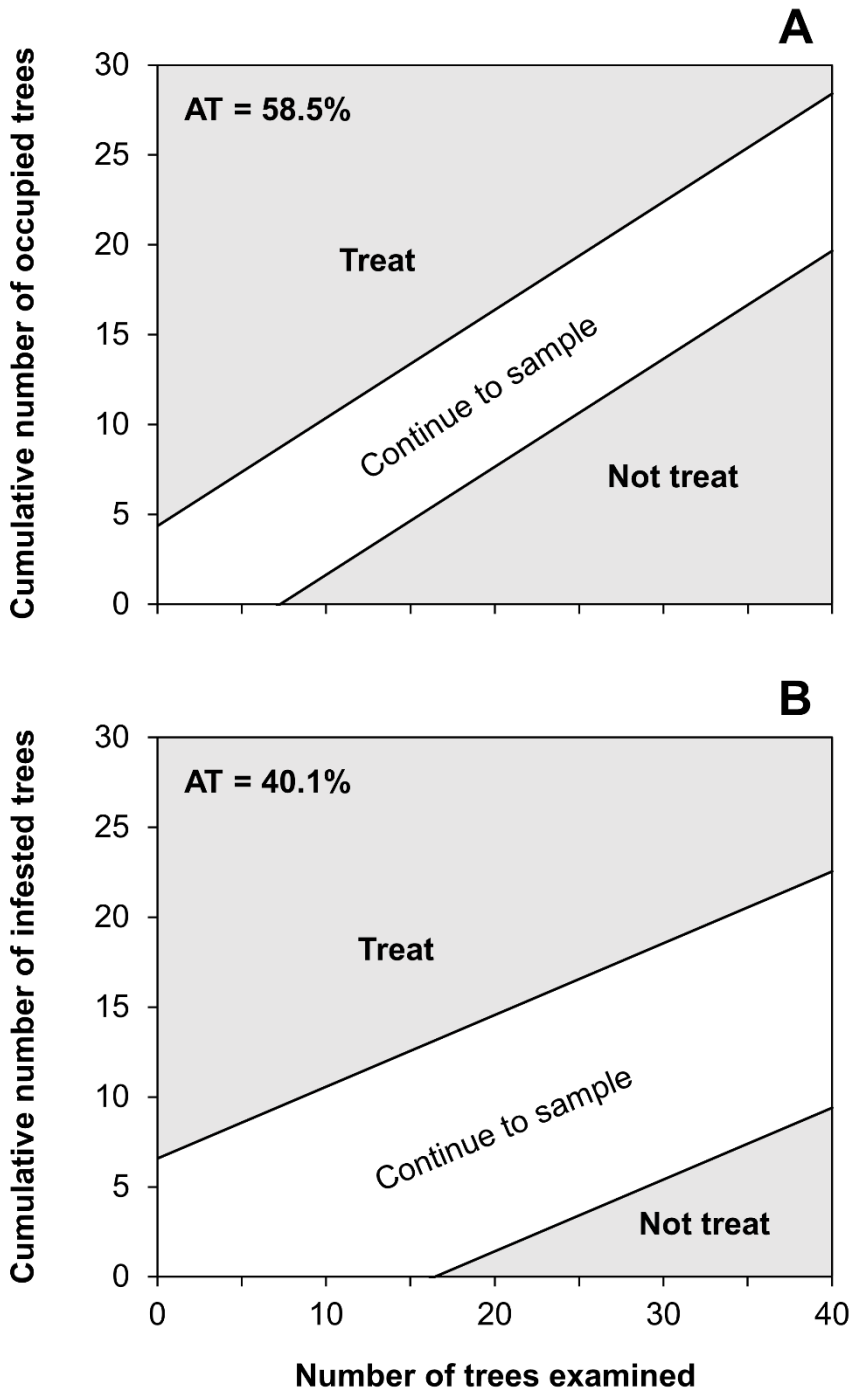

Figure 4. Decision stop lines for binomial sequential sampling plans of trees occupied (A) or infested (B) by *Lymantria dispar* in Mediterranean cork oak forests. Binomial plans were obtained from resampling validation analysis based on action thresholds of 58.5% (A) and 40.1% (B) of infested sample units, α and β = 0.1 and a tally threshold of 1 egg mass per tree (A) or 3 egg masses per tree (B).

Table 1. Dispersion indices for *Lymantria dispar* egg masses on cork oak trees in Sardinia (Italy) in 1999-2010.

Population development stage	Dataset (*n*)	Pest density range (egg masses/tree)	Taylor's power law			
			ln(*a*) ± SEM	*a*	*b* ± SEM	R^2
Overall	547	0.05 - 59.4	1.16 ± 0.04	3.18	1.45 ± 0.02	0.90
Progradation	181	0.07 - 38.2	1.15 ± 0.06	3.15	1.45 ± 0.04	0.88
Culmination	174	0.20 - 59.4	0.95 ± 0.11	2.61	1.51 ± 0.04	0.88
Retrogradation	192	0.01 - 45.1	1.22 ± 0.05	3.42	1.42 ± 0.03	0.91

Table 2. Validation of Green's sequential sampling plan based on resampling approach at fixed-precision levels of 0.25 and 0.10.

Pest density range (egg masses/tree)	Dataset (*n*)	Fixed-precision level = 0.25		Fixed-precision level = 0.10	
		Mean precision (range)	Mean sample size (range)	Mean precision (range)	Mean sample size (range)
1.02-40.55	55	0.183 (0.040-0.395)	27.3 (10-52)	0.190 (0.040-0.433)	164.7 (41-315)

Table 3. Results of linear regression analyses aimed at exploring the relationship between the number of *Lymantria dispar* egg masses (*y*) and the percentage of occupied (i.e., trees with 1 or more egg masses) or infested (i.e., trees with 3 or more egg masses) trees (*x*). Linear regressions were fitted separately for each phase of gypsy moth population development.

Model	**Population development stage**	***n***	**Equation**	**R^2**	**F**	***p***
Egg mass density ~ % of occupied trees	*Progradation*	181	$\ln(y) = 1.9284x - 0.8952$	0.77	602.5	<0.001
	Culmination	174	$\ln(y) = 2.1276x - 0.8466$	0.74	490.8	<0.001
	Retrogradation	192	$\ln(y) = 1.9350x - 0.8733$	0.81	789.9	<0.001
Egg mass density ~ % of infested trees	*Progradation*	181	$\ln(y) = 4.1957x - 2.9113$	0.82	842.7	<0.001
	Culmination	174	$\ln(y) = 4.0853x - 3.0704$	0.88	1310.6	<0.001
	Retrogradation	192	$\ln(y) = 4.3744x - 2.7541$	0.86	1134.3	<0.001

Table 4. Comparison of operation characteristics (OC value) and probabilities of correct (A and D) and incorrect (B and C) control decisions for sequential binomial sampling plans for cork oak trees occupied (AT = 58.5%) or infested (AT = 40.1%) by *Lymantria dispar* in progradation, culmination, and retrogradation phases.

AT (%)[1]	Tally threshold[2]	Population development stage	Dataset (n)	OC value[3]	Actual α[4]	Actual β[5]	ASN (range)[6]	A[7]	D[8]	A+D[9]	B[10]	C[11]	B+C[12]
58.9	1	*Progradation*	181	0.496	0.093	0.091	31.4 (8-93)	0.425	0.354	0.779	0.188	0.033	0.221
		Culmination	174	0.503	0.089	0.089	26.9 (11-98)	0.787	0.098	0.885	0.006	0.109	0.115
		Retrogradation	192	0.505	0.092	0.096	27.4 (8-95)	0.415	0.409	0.824	0.016	0.160	0.176
40.1	3	*Progradation*	181	0.488	0.098	0.090	42.1 (17-199)	0.387	0.519	0.906	0.072	0.022	0.094
		Culmination	174	0.509	0.091	0.099	35.3 (17-212)	0.770	0.196	0.966	0.023	0.011	0.034
		Retrogradation	192	0.486	0.101	0.092	42.4 (17-193)	0.409	0.508	0.917	0.021	0.062	0.083

[1] Action thresholds.
[2] Minimum number of egg masses necessary to classify a tree either as occupied (1) or infested (3) by *L. dispar*.
[3] Probability of not treating when the pest population density reaches the AT.
[4] Probability of treating when the pest density is below the AT (type I error).
[5] Probability of not treating when the pest density is above the AT (type II error).
[6] Number of samples required to make a pest control decision (i.e., to treat or not to treat).
[7] Correct decision to treat.
[8] Correct decision not to treat.
[9] Overall probability of making a correct pest control decision (i.e., to treat or not to treat).
[10] Incorrect decision to treat.
[11] Incorrect decision not to treat.
[12] Overall probability of making an incorrect pest control decision (i.e., to treat or not to treat).

CHAPTER 2

Title:

"Comparative efficacy trials with two different *Bacillus thuringiensis* serovar *kurstaki* strains against gypsy moth in Mediterranean cork oak forests"

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Comparative Efficacy Trials with Two Different *Bacillus thuringiensis* Serovar *kurstaki* Strains against Gypsy Moth in Mediterranean Cork Oak Forests

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Abstract: The efficacy of two formulations (Foray® 76B AVIO and Rapax® AS AIR) containing different *Bacillus thuringiensis kurstaki* (*Btk*) strains (ABTS-351 and EG-2348, respectively) was evaluated against *Lymantria dispar* larval populations in cork oak forests in Sardinia (Italy), in 2018 and 2019. The experimental design involved the following treatments: (I) untreated control; (II) Foray® 76B at the dose of 2.0 L/ha; (III) Foray® 76B at the dose of 2.5 L/ha; (IV) Rapax® AS AIR at the dose of 2.0 L/ha. Aerial applications were carried out using a helicopter equipped with four electronic rotary atomizers adjusted to sprinkle 160 micron-sized drops. *Btk* efficacy was evaluated by assessing the larval density reduction 7, 14, and 21 days after the application in each experimental plot in comparison with an untreated check. In addition to field surveys, the mortality of second and third instar larval samples, randomly collected from each plot after treatment and fed with foliage from the same plot, was determined in the laboratory. All *Btk* treatments were similarly effective, and no differences in larval density reduction among *Btk* strains and doses were found in either year. Twenty-one days after application, the average larval density reduction in the field was approximately 70% in all treated plots in 2018, whereas in 2019 it reached 80% only in areas treated with Foray 76B at 2.5 L/ha. Laboratory observations showed that all *Btk*-based products were effective against gypsy moth larvae, with significant differences in mortality between untreated control and the different *Btk* treatments. Our results shed light on the possibility of alternating different *Btk* strains for resistance management purposes and of applying lower doses than labeled, in order to achieve cost savings for product shipment and distribution and to reduce the environmental impact.

Keywords: *Lymantria dispar*; biopesticides; aerial application; entomopathogens; *Bacillus thuringiensis*

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1. Introduction

Cork oak (*Quercus suber* L.) is an evergreen species typical of Mediterranean pure and mixed forests or included in extensive agroforestry systems [1]. In addition to being biodiversity hotspots [2,3], cork oak forests are important economic resources in regard to cork production and their usefulness as pastureland.

A major threat to Mediterranean cork oak is the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Erebidae), one of the main forest pest defoliators worldwide [4–6]. Gypsy moth is a univoltine species whose larvae can feed and develop on more than 300 different plant species [7–9], even though *Quercus* species are the main hosts in natural environments [10,11]. Spring larval feeding behavior, mostly targeting young oak shoots, causes a significant loss of the photosynthesizing surface, which leads to a tree growth decrease and consequent cork production reduction of up to 60% in totally defoliated trees and 40% in partially defoliated trees [12]. Moreover, the ordinary development of infested trees might be affected in the following year too, resulting in delayed bud burst and additional cork growth reduction [13].

In order to reduce gypsy moth infestations and consequent damage to trees, different control strategies have been developed [14–18]. Of these, the application of *Bacillus thuringiensis* serovar *kurstaki* (*Btk*) is considered one of the most effective methods to reduce larval population density [16,17] with few biological and practical limitations [19,20]. *Btk* application success against *L. dispar* strictly depends on several technical aspects, including timing, which should target second instar larvae [19,21], appropriate dose [14,22], and droplet size [23]. In Sardinia (Italy), according to a multi-year experiment in which various strains and formulations were applied by employing diverse aerial distribution methods against different gypsy moth generations [24,25], *Btk* was able to protect cork oak forests from this pest [25]. Based on these studies, Foray® 48B and Foray® 76B (Valent Bioscience Corporation, Libertyville, Illinois, USA) suspension concentrates (SC) of strain ABTS-351 authorized against gypsy moth were the *Btk*-based formulations providing the highest efficacy [26]. Among these two products with equal effectiveness, Foray® 76B is recommended to be used at a nearly half dose, which allows cost saving for product shipment and distribution [19].

Btk-based formulations normally used to control Lepidopteran defoliators are suspension concentrates (SC) of spores and crystals, containing insecticidal *Cry* proteins, acting by ingestion. Activated *Cry* proteins bind to specific receptors covering the plasma membrane of the insect's midgut epithelium, determining the formation of amphiphilic pores and a subsequent abnormal flux of ions and water into the epithelial cells [27]. Consequently, affected gut cells lyse [28] and infected larvae undergo paralysis and death, eventually exhibiting bacterial septicemia [29]. Although *Btk* acts specifically against Lepidoptera, susceptibility to toxins can vary depending on *Btk* strains [30,31]. For example, the tent caterpillar, *Malacosoma neustria* (L.) (Lepidoptera Lasiocampidae), which is often considered a secondary pest of cork oak forests [5,11], was more susceptible to ABTS-351 strain than *L. dispar* in application experiments conducted in cork oak forests in Sardinia [18]. Such differences were shown to be related to different degrees of *Cry* toxin affinity for midgut receptors, resulting in different amounts of amphiphilic pore formation on epithelial cells in susceptible insect species [32]. On the other hand, full insecticidal action of a *Btk*-based formulation is achieved when a sufficient number of spores and *Cry* proteins are ingested by young larvae [22], which is strictly related to the intrinsic features of the formulation and the way it is applied [33]. Therefore, the characteristics of the formulation and the insecticidal traits of the *Btk* strain it contains represent key factors to maximize gypsy moth containment efficacy in the field, enabling a longer residual effect.

Since the dose, formulation, and strain of *Btk* products are recognized as the main features determining a successful application against *L. dispar*, large-scale comparative trials with different *Btk*-based products and doses were performed in 2018 and 2019 in two cork oak forests in Sardinia. Experiments, including field and laboratory work, involved the two *Btk* strains ABTS-351 and EG-2348, containing a different assortment of *Cry* toxins.

2. Materials and Methods

2.1. Experimental Products

Two aqueous suspension concentrate (SC) *Btk* formulations were used in this study: (1) Foray® 76B AVIO (Sumitomo Chemical Agro Europe S.A.S., Machelen, Belgium), containing 20 billion international units (BIU)/L of *Btk* strain ABTS-351 expressing the insecticidal crystal toxins Cry1A(a), Cry1A(b), Cry1A(c), and Cry2A and (2) Rapax® AS AIR (Biogard, CBC Europe S.r.l., Grassobbio, Italy), containing 24 BIU/L of *Btk* strain EG-2348 and Cry1A(a), Cry1A(c), and Cry2A proteins. Foray and Rapax were authorized for aerial applications in Sardinian forests by Reg. n. 17,191 and 17,190 in 2018 and by Reg. n. 17,393 and 17,394 in 2019, issued by the Italian Ministry of Health according to art. 53, p. 1, Regulation (CE) n. 1107/2009.

2.2. Study Area and Experimental Design

Trials were carried out in 2018 and 2019 in pure cork oak forests in northern and eastern Sardinia, respectively, according to a randomized complete block design with three replicates (plots) of approximately 200 ha each, including the following treatments: (1) Foray® 76B AVIO applied at the dose of 2.0 L/ha (hereafter called Foray 2.0); (2) Foray® 76B AVIO applied at the recommended dose of 2.5 L/ha (hereafter called Foray 2.5); (3) Rapax® AS AIR applied at the dose of 2.0 L/ha (hereafter called Rapax); (4) untreated check (control). *Btk*-based formulations were sprayed at an ultralow volume using a helicopter equipped with a 12-m long bar with four rotary atomizers (model AU) regulated to sprinkle 160 micron-sized drops. Spraying equipment included a flow control system allowing for the management of insecticide distribution rates at variable helicopter speeds. *Btk* applications were made on 12 May and 11 May in 2018 and 2019, respectively, under optimal weather conditions, corresponding to an average temperature ranging from 19 to 28 °C, 30–60% relative humidity, and wind speeds below 1–2 m/s. Treatments targeted a gypsy moth larval population mainly in the second instar, being highly susceptible to the *Btk* action [34].

In both years, the level of gypsy moth infestation in the study areas, estimated by assessing the number of egg masses along a standardized 40-plant transect (economic damage threshold = 100 eggs), clearly indicated this species fell in a retrogradation phase within its multi-year cycle, as determined by analysis of data from network stations established in Sardinia since 1980 [35] and including more than 680 permanent monitoring sites [36]. Based on the close correlation between egg mass density and defoliation level in the following spring, in Sardinia, total defoliation is expected when the 100-egg threshold is reached [19]. Despite being in a retrogradation phase, the average density of *L. dispar* recorded in both 2018 and 2019 was higher than the action threshold, thus justifying the need for a phytosanitary application.

2.3. Evaluation of Btk Treatments

The effect of *Btk* applications against gypsy moth larvae was evaluated under field and laboratory conditions. In the field, gypsy moth larval population density was estimated in each plot before spraying and 7, 14, and 21 days after spraying. Larval density was assessed by counting the number of larvae on four randomly chosen shoots (approximately 30 cm in length) per tree from 10 consecutive cork oak trees selected along a linear transect (40 shoots/site/sampling date). In addition, larvae were identified according to different instars. Larval density reduction in treatment x after t days (ΔD_{xt}) was calculated as

$$\Delta D_{xt} = \frac{D_{x0} - D_{xt}}{D_{x0}} \times 100 \quad (1)$$

where D_{x0} is the initial larval density in treatment x at sampling time 0 (i.e., before spraying), and D_{xt} is the larval density t days after the *Btk* applications in treatment x.

In the laboratory, the larval mortality was evaluated on second and third instars randomly collected from each plot a few hours after each spraying. Groups of one hundred larvae from each plot were transferred into aerated plastic containers 18 cm in diameter and 25 cm in height (Kartell™ Inc., New York, NY, USA) with a perforated cup. To prevent larvae escaping from the upper holes, a portion of non-woven fabric was inserted between the cap and the top edge of the container. Throughout the entire breeding period, the larvae were fed ad libitum with fresh cork leaves from their original treated plot. Gypsy moth larvae were reared until pupation and the number of live and/or dead larvae, as well as the total number of pupae, was recorded every 2–3 days.

2.4. Statistical Analysis

All statistical analyses were performed using R statistical software version 3.10 [37]. Differences among treatments in instars composition were evaluated before *Btk* application for all years using a χ^2 test for independence ($p < 0.05$). Moreover, one-way Analysis

of Variance (ANOVA) followed by Tukey post hoc test at 0.05 level of significance were performed to test for differences in larval infestation (i.e., average number of larvae counted on 40 shoots) before treatment.

The efficacy of different *Btk* treatments was evaluated by considering larval density reduction compared to initial density. In order to take into account the natural population decrease, larval reduction due to *Btk* treatments were corrected for natural mortality (C_{xt}) using the Schneider–Orelli formula [38]:

$$C_{xt} = \frac{\Delta D_{xt} - \overline{D}_{Ct}}{100 - \overline{D}_{Ct}} \times 100 \quad (2)$$

where ΔD_{xt} is the larval density reduction in treatment x after t days, and $\overline{D}_{Ct}$ is the average larval density reduction t days after the *Btk* applications in untreated control. Differences in corrected larval reduction among *Btk* treatments were tested separately within sampling times (i.e., 7, 14, and 21 days after application) using ANOVA followed by Tukey's test at significance level of 0.05.

Data obtained from laboratory assessments were used to evaluate both larval survival and density reduction under controlled conditions. Survival analysis was performed separately for each year of observation using a mixed effects Cox proportional hazard model using survival (version 3.2-11) [39], and coxme (version 2.2-16) [40] packages in R. In each model, treatments were considered as fixed factors and cage (i.e., replicate) as a random effect factor. Further post-hoc analysis was performed using multcomp (version 1.4-16) package in R [41], applying a Bonferroni correction for multiple testing. Larval density reduction corrected using Schneider–Orelli was calculated at 7, 14, and 21 days after treatment in order to evaluate the individual effect of *Btk* against gypsy moth larvae under laboratory conditions. One-way ANOVA followed by Tukey's test at 0.05 level of significance was used to test for differences among treatments.

3. Results

In both experimental years, *Btk* formulations were applied when the gypsy moth larval population was mainly in the second instar (Table 1). Statistical differences in instar distribution among treatments were found in 2018 ($\chi^2 = 108.3$, df = 6, $p < 0.05$), and 2019 ($\chi^2 = 282.4$, df = 6, $p < 0.05$). In 2018, plots treated with Foray 2.5 were characterized by a higher proportion of third instars than other plots, whereas plots sprayed with Rapax showed a significantly higher proportion of first instars (Table 1). In 2019, although treatments targeted a larval population mainly in the second instar, the presence of fourth instars was observed in all treated areas except those treated with Rapax (Table 1). No statistical differences in larval density among treatments were found before microbiological applications in either 2018 or 2019 (Table 2), indicating a similar level of infestation in all experimental areas.

Table 1. Occurrences of *Lymantria dispar* larval instars observed in 2018 and 2019 in untreated (control) areas and areas treated with Foray® 76B AVIO and Rapax® AS AIR 2018 in Sardinia (Italy). Pearson standardized residuals measuring the deviation from expected values are reported in brackets (+ = positive deviation; − = negative deviation).

Year	Treatments	First Instar	Second Instar	Third Instar	Fourth Instar
2018	Foray 2.0 L/ha	242	521	121	-
	Foray 2.5 L/ha	278	527 (−)	218 (+)	-
	Rapax	106 (+)	120	8 (−)	-
	Control	192	467 (+)	65 (−)	-
2019	Foray 2.0 L/ha	11 (−)	190 (−)	163 (+)	24 (+)
	Foray 2.5 L/ha	15	118	61 (+)	2
	Rapax	42	308	46 (−)	0
	Control	48	394 (+)	26 (−)	1 (−)

Table 2. Average (mean ± standard error) *Lymantria dispar* larval density occurred in 2018 and 2019 in untreated and *Btk* treated plots before insecticide applications. Analysis of Variance (ANOVA) statistics and *p*-value are reported.

Year	Foray 2.0 L/ha	Foray 2.5 L/ha	Rapax	Control	F	*p*
2018	294.7 ± 101.3	247.0 ± 19.2	78.0 ± 35.7	241.3 ± 65.9	2.20	0.17
2019	129.7 ± 61.6	65.3 ± 13.7	132 ± 12.2	156.3 ± 51.9	0.89	0.49

The analysis of larval density reduction in the field exclusively due to *Btk* treatments applied in 2018 showed a comparable percentage of reduction among treatments 7 ($F_{2,6} = 0.27$, $p = 0.77$), 14 ($F_{2,6} = 1.13$, $p = 0.39$), and 21 ($F_{2,6} = 0.12$, $p = 0.89$) days after spraying (Figure 1a). Similarly, no differences in corrected larval reduction were found in 2019 throughout the sampling period (7 days after application: $F_{2,6} = 3.31$, $p = 0.12$; 14 days after application: $F_{2,6} = 0.08$, $p = 0.93$; 21 days after application: $F_{2,6} = 0.88$, $p = 0.50$) (Figure 1b).

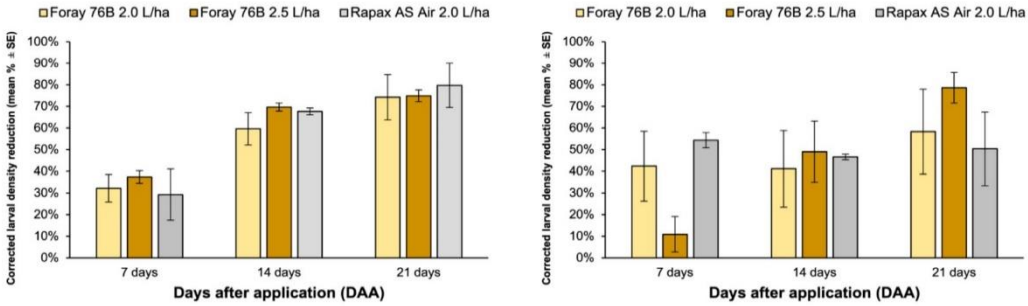

Figure 1. Percentage reduction of *Lymantria dispar* larval population caused by application of Foray 76B® (2.0 L/ha and 2.5 L/ha) and Rapax AVIO® 7, 14, and 21 days in (**a**) 2018 and (**b**) 2019 panel. Larval reductions were corrected with the Schneider–Orelli formula.

Survival of field-collected larvae maintained in the laboratory in 2018 was different among treatments ($\chi^2 = 210.03$, $p < 0.01$), with a significantly higher survival rate in untreated control than other treatments. Survival observed in larvae fed with foliage sampled from areas treated with Rapax was comparable to larvae from Foray 2.0-treated areas ($z = -2.48$, $p = 0.08$), whereas it was significantly lower in respect to larvae fed with Foray 2.5-treated foliage ($z = -3.96$, $p < 0.01$).

No difference in survival was found between larvae fed with Foray 2.0- and Foray 2.5-treated foliage ($z = 1.49$, $p = 0.81$). The survival of larvae fed with untreated foliage was approximately 80% at the end of the laboratory observations (30 days), in contrast to 24.7%, 10.0%, and 6.3% in larvae fed with foliage treated with Rapax, Foray 2.0, and Foray 2.5, respectively (Figure 2a). A similar pattern was observed in laboratory observations conducted in 2019 (Figure 2b), in which the survival of larvae fed with *Btk*-treated foliage after 30 days was slightly lower than that observed in larvae from untreated areas (Foray 2.0 = 1.3%; Foray 2.5 = 4.3%; Rapax 2.0 = 10.0%; untreated control = 68.3%). In these experiments, significant differences in survival among treatments were found ($\chi^2 = 339.92$, $p < 0.01$), with a higher survival rate of larvae fed on untreated foliage than those fed with Foray 2.0 ($z = -17.25$, $p < 0.01$), Foray 2.5 ($z = -15.66$, $p < 0.01$), and Rapax ($z = -15.76$, $p < 0.01$).

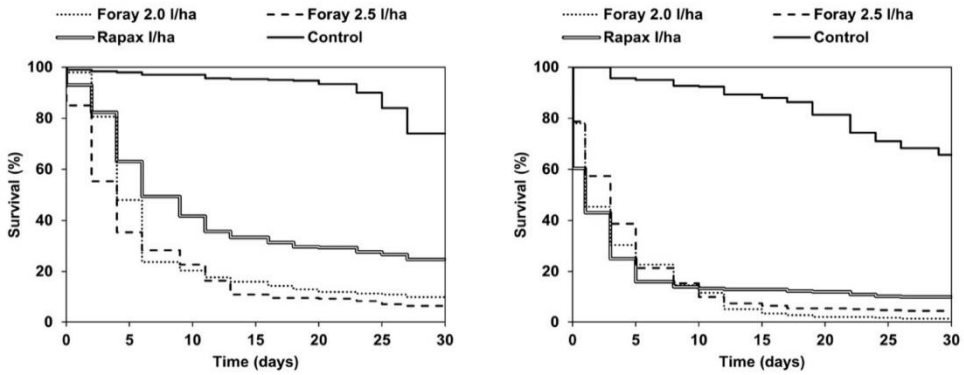

Figure 2. Survival of *Lymantria dispar* larvae fed in the laboratory with untreated and *Btk*-treated foliage in (**a**) 2018 and (**b**) 2019.

The corrected larval mortality observed in the laboratory in 2018 and 2019 is illustrated in Figure 3a,b, respectively. In 2018, larval mortality attributable exclusively to *Btk* formulations was similar 7 ($F_{2,6} = 3.79$, $p = 0.09$) and 14 ($F_{2,6} = 5.24$, $p = 0.05$) days after their application in all treatments (Figure 3a). However, significant differences among *Btk*-based formulations were found 21 days after sprayings, with a lower mortality in larvae fed with Rapax than those fed with Foray at both assayed doses ($F_{2,6} = 9.69$, $p = 0.01$). In 2019, the average corrected larval mortality observed in the laboratory seven days after application was, respectively, 75.4%, 73.9%, and 59.2% for larvae fed with Foray 2.0, Rapax, and Foray 2.5. Larval density decreased over time and the reduction of reared larvae reached more than 80% in all treatments 21 days after the application. No differences in larval mortality among treatments attributable to *Btk* were found at different times of observation (7 days after application: $F_{2,6} = 0.51$, $p = 0.62$; 14 days after application: $F_{2,6} = 1.03$, $p = 0.41$; 21 days after application: $F_{2,6} = 2.39$, $p = 0.17$) (Figure 3b).

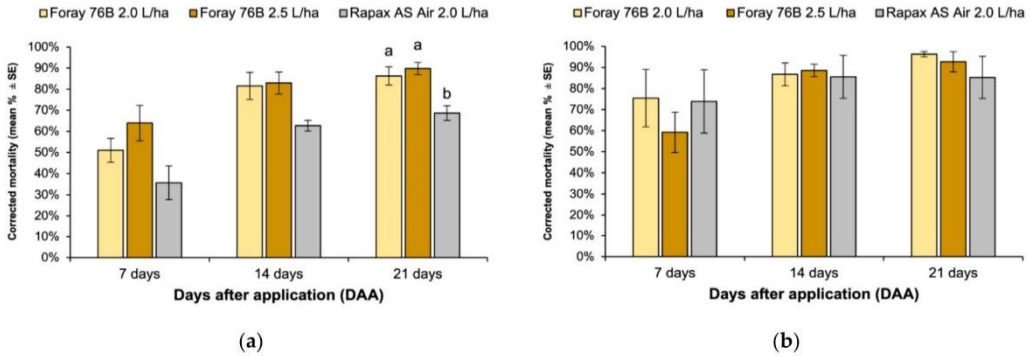

Figure 3. *Lymantria dispar* mortality (%) under laboratory conditions caused by Foray 76B® (2.0 L/ha and 2.5 L/ha) and Rapax AVIO® 7, 14, and 21 days in (**a**) 2018 and (**b**) 2019 panel. Mortality was corrected with the Schneider–Orelli formula.

4. Discussion

All *Btk* applications in this study successfully contributed to the reduction in *L. dispar* larval populations in the forest. Foray® 76B AVIO, commonly used in large-scale gypsy moth management programs in Sardinia [19], caused a significant decrease in larval density

when applied either at a standard (2.5 L/ha) or lower (2.0 L/ha) dose. The efficacy of Rapax® AS Air, which is characterized by a different *Btk* strain (i.e., EG-2348), was generally comparable to Foray® 76B AVIO (i.e., ABTS-351). However, the latter, as a whole, appeared more effective in controlling the *L. dispar* larval population. On the other hand, the efficacy of these *Btk* products observed in 2018 and 2019, compared to the untreated control areas, was less evident than expected due to the target population being under retrogradation. Another aspect affecting efficacy comparisons between the two experimental years is the larval age at the time of application, which is a factor correlated to *Btk* effectiveness [22]. In 2019, the larval population at the time of application was characterized by a higher larval age than that in 2018. Therefore, to achieve the same level of mortality when targeting older larvae, a higher dose would have been needed [14,22]. Nevertheless, differences in larval age are also related to other natural control factors affecting population. Older larvae which survive *Btk* treatments are exposed for a longer time to several biotic and abiotic mortality factors, which act more consistently during a gypsy moth retrogradation phase [42]. Among these, the biological control agent community, whose population is host-density dependent, expresses its highest potential during retrogradation [42–45]. The effects of the complexity of natural phenomena affecting *Btk* efficacy evaluations based on population density measurements before and after treatments and during retrogradation were mitigated in this study by including laboratory observations of larval samples collected from treated plots. This approach allowed the demonstration of clear treatment efficacy, with larvae feeding on non-treated foliage showing a significantly higher survival rate than that of larvae from treated areas in both years (Figure 2a,b).

Based on this study, both *Btk* formulations were equally effective in attaining larval density reduction in the field. The EG-2348 strain characterizing Rapax® AS Air formulation was previously assayed against *L. dispar* and *M. neustria* populations, proving to be effective when applied on individual cork oak trees [17]. However, in those experiments, *Btk* suspension was diluted with water and applied from the ground with an atomizer at a volume of 10 L/100 m^2. No recommendations for aerial application of this product were available, but our results demonstrated that the same procedures in use in Sardinia since the 1990s for aerial application of Foray® [19,24,25] can also be used effectively for Rapax®. This aspect is not secondary to achieving an appropriate size and density of suspension droplets, which is strictly dependent on the type of distribution and the physico-chemical features of the formulation [33]. These results provide important practical information for the aerial application of Foray® 76B AVIO and Rapax® AS AIR, as both are aqueous suspensions. Our results align with previous trials reporting the effectiveness of *Btk*-based SC formulations on different Lepidopteran species affecting forest and agricultural crops, under different field conditions [17,46].

Another relevant finding emerging from this work is the availability of two formulations containing *Btk* strains bearing different *Cry* genes, both resulting in a satisfactory pest control capability. This relates to the need to develop a longer-term control strategy involving the alternate or combined use of different strains, according to a resistance management approaches based on modes of action diversification. Insect resistance to *Bt* toxins was in fact reported in both laboratory and field conditions [47–49], being developed by different mechanisms, most of which are still under investigation [32]. Possible adaptation to *Bt* toxins by natural populations should therefore also be considered in the forest environment, where the selection pressure caused by the application of the same active substance for several years may facilitate the onset of resistance [50]. In addition to strains rotation, resistance management should include the maintenance of treatment-free areas as a reserve of *Bt*-sensitive populations.

The present study indicated similar efficacy of using the standard dose of 2.5 L/ha or a reduced dose of 2.0 L/ha for Foray® 76B AVIO. This dose was also adequate for Rapax® AS AIR. The consistent employment of reduced doses is recommended in order to minimize the possible environmental impact and the costs related to product shipment and distribution [19].

In conclusion, our results confirmed the high efficiency of *Btk* applications to manage *L. dispar* infestations in the Mediterranean area, which represents an effective method to protect forests from defoliation. The two formulations containing different *Btk* strains (i.e., ABTS-351, EG-2348) did not show significant differences in efficacy against gypsy moth larval population, providing a choice of alternative products to be considered in insect resistance management strategies. In addition, Foray® 76B AVIO proved to be equally effective at a lower dose than that labeled, which allows treatment cost saving or the opportunity to use locally available budgets to increase the surfaces of the forest to be protected. On the other hand, the use of different doses might be calibrated according to the typical spatial heterogeneity of gypsy moth infestations, applying lower doses in less infested areas and standard doses in areas with higher population density.

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CHAPTER 3

Title:

"Gypsy moth management with LdMNPV baculovirus in cork oak forests"

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Gypsy Moth Management with LdMNPV Baculovirus in Cork Oak Forest

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Abstract: (1) Research Highlights: Applications of a species-specific baculovirus is a promising method to control the gypsy moth and regulate its population dynamics in forest ecosystems. (2) Background and Objectives: Cork oak protection against the Lepidopteran defoliator *Lymantria dispar* requires an appropriate forest ecosystem management program, involving the application of eco-sustainable microbial products during population outbreaks. The species-specific multicapsid nucleopolyhedrovirus (LdMNPV), agent of natural epizootics in gypsy moth populations, represents an option that was investigated in a multi-year field study, involving viral applications either from the ground or by aerial treatment. (3) Materials and Methods: Efficacy trials against *L. dispar* populations were conducted in 2018 and 2019 in Sardinia, according to a randomized block design. Each year, two trials were conducted, applying a baculovirus commercial formulation with an atomizer from the ground and assessing the effects of different doses and application timing, respectively. An aerial application trial distributing LdMNPV at ultra-low volumes (2 L/ha) was also conducted in 2019 to assess the virus efficacy at a larger field scale. (4) Results: In both years, a significant increase in larval mortality was detected in plots treated with higher viral occlusion body (OB) doses and with an earlier application targeting younger larvae, in comparison with untreated controls. Due to an observed retrogradation phase of the target pest in 2019, no significant differences in larval density between areas treated from a helicopter and control were detected, but in the few weeks following application, a meaningful vitality decrease in larval samples from treated plots was observed. (5) Conclusions: Based on the results of this study, the use of LdMNPV in forest protection programs against gypsy moth can be worth consideration in multi-year integrated program strategies to modulate population dynamics.

Keywords: biocontrol; bioinsecticide; entomopathogen; microbial; ecosystem

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1. Introduction

Lymantria dispar (L.) (Lepidoptera: Erebidae), also known as gypsy moth, is a univoltine species whose larvae, hatching from overwintering eggs, cause significant damages to cork oak leaves. The combination of their feeding behavior and a high biotic potential are the cause of periodic outbreaks, determining wide forest defoliations [1]. In order to reduce such deleterious effects, the implementation of appropriate biocontainment measures is necessary. Accordingly, the application of bioinsecticides was proven to be a successful approach to counteract this pest action, ensuring limited environmental impact [2]. For this purpose, available formulations based on the entomopathogenic bacterium *Bacillus thuringiensis* exploit the highly specific mode of action of bacterial toxins selectively targeting moth larvae [3]. On the other hand, the risks of possible side-effects on non-target lepidoptera inhabiting the forest ecosystem have sometimes been reported [4]. Another group of entomopathogens is represented by baculoviruses, very specific microorganisms co-evolved with their host [5] and able to cause fatal infections to larvae after the ingestion of viral particles. The bioinsecticidal activity is associated with crystalline occlusion bodies

that, after being ingested by susceptible insects, release occlusion-derived viruses (ODVs) acting on the host midgut epithelial cells. The infection spread in the host body relies on the production of a second type of virions, namely, budded viruses (BVs) [6].

Lymantria dispar multicapsid nucleopolyhedrovirus (LdMNPV) is specifically associated with gypsy moth, being co-evolved with this species [7]. This biocontrol agent represents a natural regulator of the defoliator population, as a result of periodic viral epizootics, especially under high-density conditions [8]. Hence, the baculovirus, reproduced in the laboratory on live larval material and applied in the field, becomes a tool able to significantly affect the population dynamics and therefore be used artificially to counteract gypsy moth outbreaks [9]. While this strategy appears promising, the use of LdMNPV is relegated to specific contexts where commercial products are available (i.e., Canada, USA). Due to the high costs associated with the production of viral material and the lack of available products in the market of several global regions (i.e., Europe) where local *L. dispar* ecotypes represent a constant threat to the different tree forest species, experimental work is needed to implement the use of baculovirus in different environmental conditions.

The present study had the purpose of evaluating the efficacy of a LdMNPV formulation under use in other world areas, against European *L. dispar* in cork oak forests affected by this pest. The study was conducted through two steps in different years, involving either small-scale viral applications from the ground, and larger-scale aerial treatments.

2. Materials and Methods

2.1. Tested Formulations

A suspension concentrate formulation of LdMNPV, commercially authorized in Canada, was provided by Andermatt Biocontrol AG (Switzerland) for experimental applications from the ground or by helicopter. The concentration of the active substance was 2.8×10^{10} OB/L. Foray 76B (Sumitomo Chemical Agro Europe S.A.S), containing 20 billion international units (BIU)/L of *Bacillus thuringiensis kurstaki* (*Btk*) strain ABTS-351, was used as a reference product.

2.2. LdMNPV Applications from the Ground

Two different trials were conducted to evaluate the effects of the baculovirus: (1) time–response and (2) dose–response. Treatments and application details are summarized in Tables 1 and 2, respectively. Time–response and dose–response trials were conducted in different experimental fields in the same year.

Table 1. Treatments in the time–response trial.

Treatment	Description	Application Date		Application Rate
		2018	2019	
Untreated Check	Not treated	-	-	-
LdMNPV Early	Earlier application	9 May	4 May	2 L/ha
LdMNPV Later	Later application	16 May	11 May	2 L/ha
Foray 76B	Reference product	16 May	11 May	2 L/ha

Table 2. Treatments in the dose–response trial.

Treatment [a]	Description	Application Rate
Untreated Check	Not treated	-
LdMNPV Low	1/3 standard rate	0.66 L/ha
LdMNPV Standard	Standard rate	2 L/ha
LdMNPV High	3× standard rate	6 L/ha
Foray 76B	Reference product	2 L/ha

[a] All applications were made on one date (9 May 2018, and 11 May 2019).

The trials were conducted in 2018 and 2019 in forests in north-western Sardinia (Italy) in compliance with Good Experimental Practice (GEP) guidelines established by the European and Mediterranean Plant Protection Organization (EPPO PP 1/210(1), Efficacy evaluation of insecticides—Defoliators of forest trees). The completely randomized experimental design involved four plots (100 m^2) per each treatment. Gypsy moth larval density was recorded before treatments and during the following three weeks (7, 14, and 21 days after LdMNPV application). Assessments were based on counting the number of larvae in eight 30 cm long branches randomly sampled from each plant. Defoliation levels in plots were also evaluated after treatments.

In the time–response trial, early application was conducted one week earlier (9 May 2018, and 4 May 2019), targeting eggs and just-hatched first instar larvae, while standard applications (16 May 2018, and 11 May 2019) targeted first and second instar. In the dose–response trial, applications were made on one date (9 May 2018, and 11 May 2019). Baculovirus applications were carried out with a motorized atomizer (M3 series, Cifarelli SpA, Italy), with a volume of 10 L per plot.

2.3. Aerial Applications

Aerial applications were carried out on 11 May 2019 on a forest area located in the Centre of Sardinia (Abbasanta, Italy). Treatments were performed in ultra-low volumes (ULVs), employing a helicopter (LAMA SA 315/B) equipped with 4 electronic Micronair rotary atomizers (model AU) treating a 20 m wide lane. Treatments were performed early in the morning so that environmental conditions ranged within sub-optimal limits. During product application, a global positioning system (GPS) was employed to trace and record the helicopter route, ensuring accurate and homogeneous distribution. Untreated check plots were compared with plots (around 100 ha each) treated with LdMNPV or *Btk* (Foray 76B). Direct assessments were based on counting the number of larvae on four 30 cm long branches per each of ten plants randomly sampled in each experimental plot. In addition, samples of larvae (n = 100) were collected from each plot and maintained in the laboratory on foliage collected from the same plots after treatment, in order to compare insect survival. The experiment involved three replicates.

2.4. Data Elaboration and Statistical Analysis

Overtime differences in average larval density among treatments in application experiments from the ground were tested using repeated measures ANOVA (PROC MIXED), and means were separated by LSMEANS comparison (adjust = Tukey), using SAS software (version 9.1) [10] with the significance level set at $\alpha = 0.05$. Analysis of variance (ANOVA) followed by least significant difference (LSD) test ($p < 0.05$) was used to compare efficacy data on a specific date and defoliation levels among treatments.

For different datasets in this study, in order to verify assumptions of normality and heteroscedasticity, the Shapiro–Wilk [11] and Levene's tests [12] were performed, respectively. If necessary, data were transformed as the arcsine of the square root of the percentage.

Field treatment efficacy was evaluated in terms of larval density reduction, where percent reduction in treatment x after t days (ΔD_{xt}) was calculated as:

$$\Delta D_{xt} = \frac{D_{x0} - D_{xt}}{D_{x0}} \times 100 \quad (1)$$

where D_{x0} is the initial larval density in treatment x at sampling time 0 (i.e., before application), and D_{xt} is the larval density t days after applications in treatment x. Efficacy differences between treatments were tested separately for each sampling date (i.e., 7, 14 and 21 days after applications) using one-way ANOVA. Tukey's test at a significance level of 0.05 was used for means separation if necessary.

Aerial application trial data obtained from laboratory observations on field-collected larvae were analyzed by a mixed effects Cox proportional hazard model using survival [13], and coxme [14] packages in R software [15]. In each model, treatments were considered

as fixed factors and the larval cage (i.e., replicate) as a random effect factor. Further post hoc analysis was performed using the multcomp package in R [16], applying a Bonferroni correction for multiple testing. Moreover, larval density reduction (%) was corrected for natural mortality to take into account the effect of natural population decreases (C_{xt}) using the Schneider-Orelli formula [17]:

$$C_{xt} = \frac{\Delta D_{xt} - \overline{D}_{Ct}}{100 - \overline{D}_{Ct}} \times 100 \quad (2)$$

where ΔD_{xt} is the larval density reduction (%) in treatment x after t days, and $\overline{D}_{Ct}$ is the average larval density reduction (%) in untreated control t days after applications. After this correction, transformed data were used to evaluate the merely effect of *Btk* or LdMNPV against gypsy moth larvae as assessed in the laboratory. Student's t-test at the 0.05 level of significance was used to test for differences between different treatments 7, 14, and 21 days after application.

3. Results

3.1. LdMNPV Applications from the Ground

Comparing the different plots involved in trials, a homogeneous larval density was observed before insecticidal applications in both years for time–response (2018: $F_{3,15} = 0.31$; $p = 0.82$; 2019: $F_{3,15} = 1.33$; $p = 0.31$) and dose–response (2018: $F_{4,15} = 0.26$; $p = 0.90$; 2019: $F_{4,15} = 0.16$; $p = 0.95$) trials.

In the time–response trials conducted in 2018, no significant changes in larval density were observed one week after the application of LdMNPV in the "LdMNPV early" experimental thesis. On the other hand, a significant larval density reduction was found during the following two weeks in the same plots, in comparison with the untreated check ($F_{9,63} = 15.07$; $p < 0.01$). No significant changes in larval density were instead associated with the "LdMNPV later" thesis (Figure 1). A significant dose-dependent effect was observed in the trial conducted in 2018 ($F_{12,79} = 19.13$; $p < 0.01$). A higher larval density reduction was associated with a higher LdMNPV dose, and this decrease became more significant as time advanced (Figure 2).

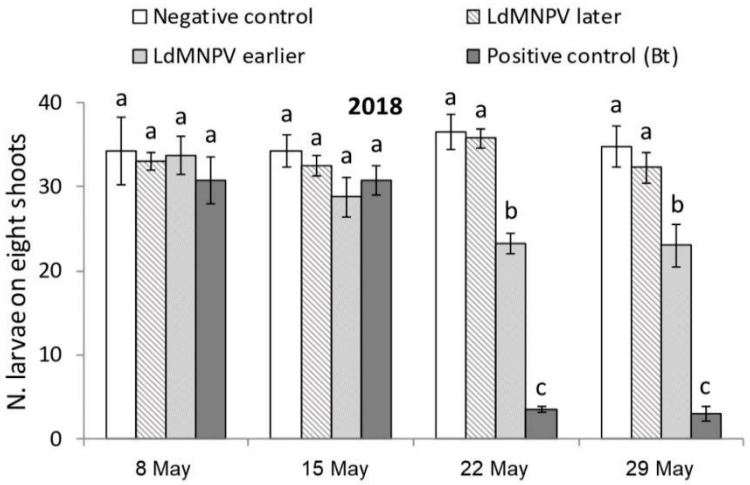

Figure 1. Larval density (mean ± SE) assessed by sampling 8 shoots/plot in the time–response trial with LdMNPV applications from the ground in 2018. Different letters (a, b, c) above bars indicate significant differences among means within each sampling date (ANOVA Mixed Proc., LSMEANS, $p < 0.05$).

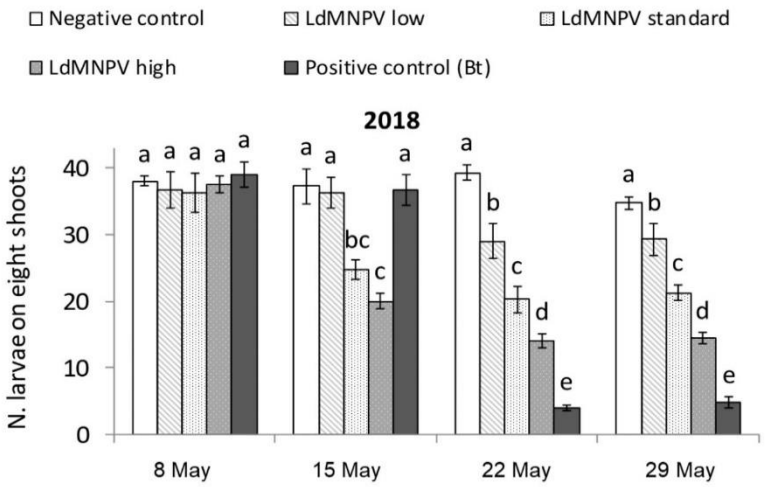

Figure 2. Larval density (mean ± SE) assessed by sampling 8 shoots/plot in the dose–response trial with LdMNPV applications from the ground in 2018. Different letters (a, b, c, d, e) above bars indicate significant differences among means within each sampling date (ANOVA Mixed Proc., LSMEANS, $p < 0.05$).

These results were comparable to the output of trials conducted in 2019. In the case of the time–response trial, a significant larval density decrease was achieved by both early and later applications of the baculovirus ($F_{9,63} = 16.62$; $p < 0.01$), with a higher and faster effect of the earlier treatment in comparison to the untreated check (Figure 3). A good efficacy was also observed in the dose–response trial, in which the LdMNPV doses assayed showed a significant biocontrol action on gypsy moth larvae with a dose-dependent effect, in comparison with the untreated control ($F_{12,79} = 9.43$; $p < 0.01$). A greater protection of trees was associated with the highest doses applied (Figure 4).

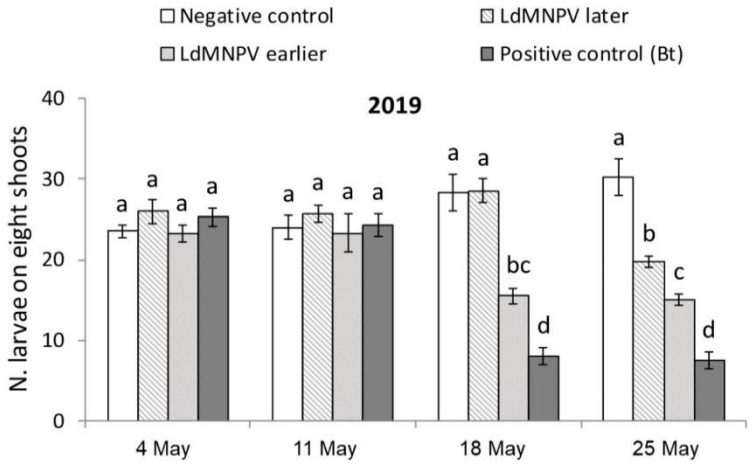

Figure 3. Larval density (mean ± SE) assessed by sampling 8 shoots/plot in the time–response trial with LdMNPV applications from the ground in 2019. Different letters (a, b, c, d) above bars indicate significant differences among means within each sampling date (ANOVA Mixed Proc., LSMEANS, $p < 0.05$).

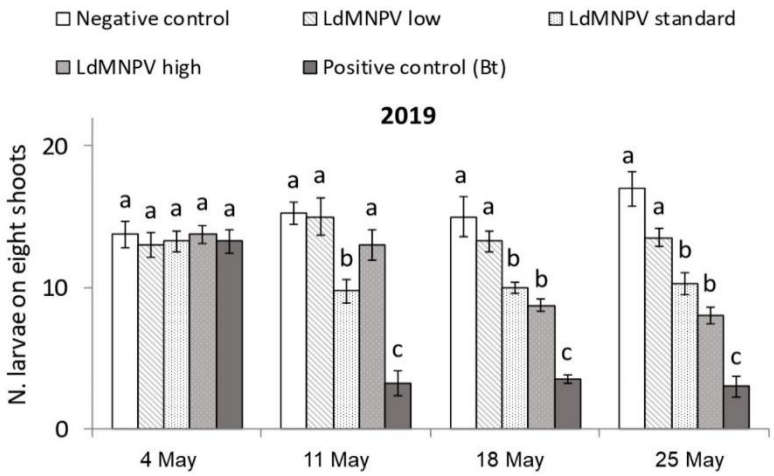

Figure 4. Larval density (mean ± SE) assessed by sampling 8 shoots/plot in the dose–response trial with LdMNPV applications from the ground in 2019. Different letters (a, b, c) above bars indicate significant differences among means within each sampling date (ANOVA Mixed Proc., LSMEANS, $p < 0.05$).

In general, a higher percentage of defoliation was found in the untreated check, while a significant protection was associated with higher LdMNPV doses (2018: $F_{4,19} = 24.50$; $p < 0.001$; 2019: $F_{4,19} = 15.43$; $p < 0.01$) and earlier treatments (2018: $F_{3,15} = 33.08$; $p < 0.01$; 2019: $F_{3,15} = 47.61$; $p < 0.01$) (Figure 5).

In all trials, the decrease in larval density and the protection against defoliation in plots treated with the *Btk* reference product was the best and associated with greater and faster action (Figures 1–5).

3.2. Aerial Applications

LdMNPV formulation applied at a dose of 2 L/ha appeared to be well and homogeneously distributed in the treated plots.

In 2019, a general drop in larval density during the season was observed in the experimental area involved in the aerial application study, outlining a retrogradation phase of gypsy moth population in this forest ecosystem in Sardinia. Accordingly, such a reduction was observed in all plots, with no differences among treatments 7 ($F_{2,8} = 3.52$, $p = 0.13$), 14 ($F_{2,8} = 0.95$, $p = 0.46$), and 21 ($F_{2,8} = 1.85$, $p = 0.27$) days after applications (Table 3).

Table 3. Percentage (mean ± SE) of larval density reduction in the field at different time intervals after bioinsecticidal application, in respect to pre-treatment. Percentage data are corrected using the Schneider-Orelli formula.

Days [a]	Treatment			F	*p*
	Foray 76B	LdMNPV	Untreated Check		
7	64.40 ± 8.82 [b]	31.71 ± 4.00	39.66 ± 17.02	3.52	0.13
14	70.28 ± 8.94	34.34 ± 34.33	46.87 ± 18.57	0.95	0.46
21	75.30 ± 9.11	45.59 ± 5.83	50.58 ± 20.39	1.85	0.27

[a] Days after application. [b] No significant differences among means were observed (ANOVA, $p > 0.05$).

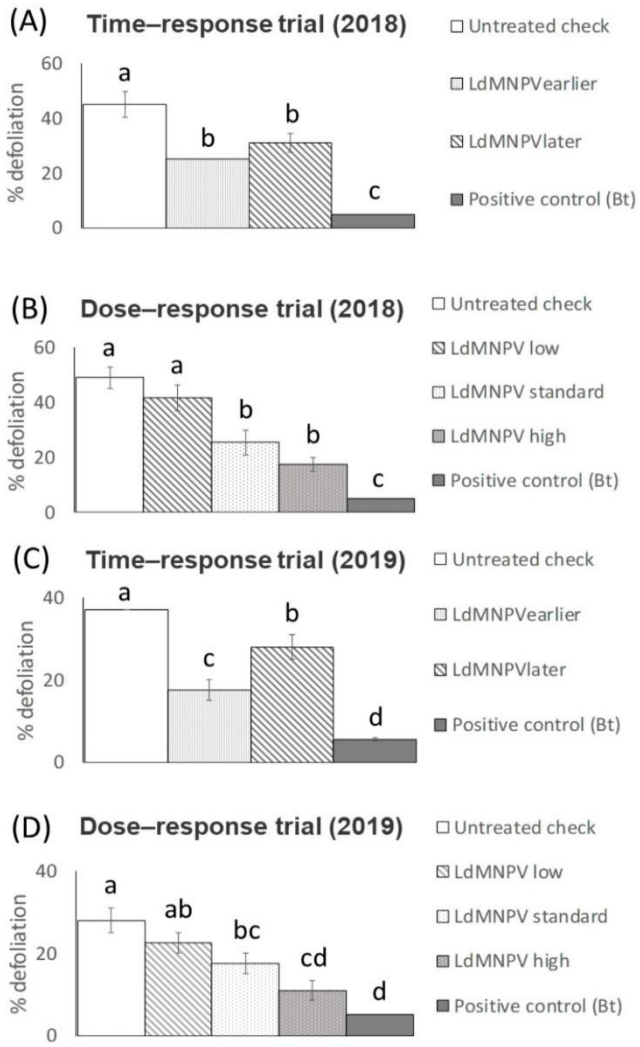

Figure 5. Defoliation percentage (mean ± SE) in different plots treated with LdMNPV from the ground in 2018 (**A**,**B**) and 2019 (**C**,**D**). Different letters (a, b, c, d) above bars indicate significant differences among means (ANOVA, Tukey test, $p < 0.05$).

On the other hand, significant differences in survival rate were observed in the laboratory on the field-collected larvae from different plots ($\chi^2 = 486.79$, $p < 0.01$), with a significant reduction associated with larvae from plots treated with either LdMNPV or *Btk* (Figure 6). In more detail, the survival rate achieved at the end of the observation period was higher for LdMNPV (12%) than *Btk*-treated larvae (0.7%) ($z = -15.73$, $p < 0.01$). The highest survival rate (52%) was instead associated with larvae from untreated plots ($z = -9.08$, $p < 0.01$). The reduction in surviving larvae attributable exclusively to *Btk* and LdMNPV was significantly different between these formulations, either 7 ($t = 7.16$, $p < 0.01$) and 14 ($t = 13.44$, $p < 0.01$) days after applications. Instead, no statistical differences in

corrected larval reduction were found between *Btk* and LdMNPV, 21 days after application (t = 2.75, $p = 0.10$) (Table 4).

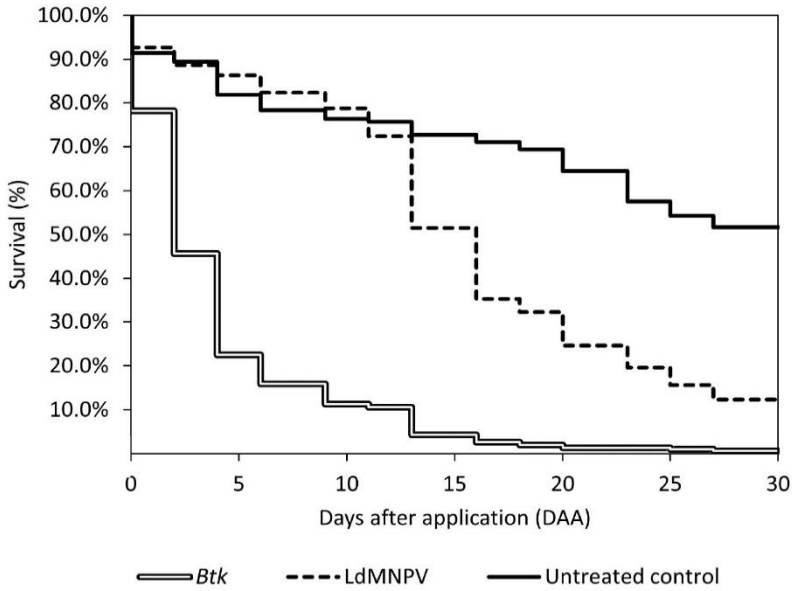

Figure 6. Survival rate of field-collected *Lymantria dispar* larvae from plots treated with Foray76B, LdMNPV formulation, or untreated (control).

Table 4. Reduction percentage (mean ± SE) of surviving larvae in the laboratory attributable exclusively to treatments at different time intervals from bioinsecticidal application.

Days [a]	Treatment [b]		t	*p*
	Foray 76B	LdMNPV		
7	75.44 ± 4.55 [a]	10.05 ± 2.67 [b]	7.16	0.004
14	86.74 ± 1.80 [a]	21.94 ± 2.12 [b]	13.44	<0.001
21	96.33 ± 0.41 [a]	70.19 ± 5.47 [a]	2.75	0.010

[a] Days after application. [b] Different letters in each line indicate significantly different means (Student's *t*-test, $p < 0.05$).

4. Discussion

Baculoviruses represent natural and selective bioinsecticides and have successfully been used against several Lepidopteran pests worldwide. However, their use is limited to niche contexts, due to their narrow host range, a delayed insecticidal action in respect to synthetic chemicals, and economical issues related to industrial production technologies still necessarily relying on the use of living insects as substrates for virus replication [18].

Lymantria dispar multicapsid nucleopolyhedrovirus (LdMNPV) formulation used in this study showed good efficacy against gypsy moth larval populations in Sardinian forest areas, where this pest is the cause of important defoliations during its periodic outbreaks [19]. In the experiments conducted with applications from the ground, the lethal effects were dose- and time-dependent, with a higher efficacy achieved with higher doses and earlier treatments. These results align with a pathogenic process that begins with the ingestion of occlusion bodies (OBs) releasing occlusion-derived viruses (ODVs) that act in the midgut, infecting epithelial cells [20]. Accordingly, a stronger and faster effect is

expected as a consequence of the earlier ingestion of a higher number of viral particles [21]. It follows that in order to ensure baculovirus' short-time effectiveness, an early application in the season, possibly against the first instar larvae, is of primary importance.

While a good baculovirus efficacy was achieved in these experiments, larval mortality was significantly lower in comparison with plots treated with *Btk*, which was confirmed to be a powerful bioinsecticide against gypsy moth [22,23].

Higher scale experiments involving larger areas and aerial applications of the bioinsecticidal products employing standard doses (2 L/ha) confirmed a reduced survival rate of baculovirus-treated larvae, in respect to the untreated control. Additionally, in this case, *Btk* treatments generated a higher lethal-effect. This greater knock-down power relates to the mechanism of action of solubilized and activated bacterial crystal toxins (Cry proteins) interacting with and disrupting midgut epithelial cells, which leads to insect paralysis and death [24]. This direct toxicity caused by *Btk* is in antithesis with a slower action of the baculovirus depending on an infectious process involving replication of the virus and its spread within the insect body through the tracheal system [20]. Everything considered, a milder action of the virus compared to *Btk* clearly emerged in field trials. Despite such differences, larval population density in 2019 was affected by a natural reduction associated with all treated and untreated plots and related to gypsy moth population retrogradation in Sardinian forest. Accordingly, a more evident efficacy of baculovirus applications in large areas is expected during population progradation, when the baculovirus can express its full potential as a natural regulator of moth population dynamics [25]. Thus, a higher host density triggers horizontal transmission processes, determining a greater number of infected individuals [26]. While these microparasites can naturally regulate periodic cycles of host abundance, their artificial introduction in the forest ecosystem by early applications in the season would produce a similar effect, under appropriate density dynamic conditions. Such density-dependent containment ability has also been demonstrated in laboratory experiments, in which different degrees of resistance to the baculovirus were associated with diverse larval densities [27].

Besides an action normally contained during the season of application, the virus introduced into the forest environment is expected to produce an additional impact on the following generations as a result of sub-lethal effects and vertical transmission [28]. This expectation supports the use of baculovirus against gypsy moth even if the efficacy in the application season is limited. Following an integrated approach to forest management, baculovirus with a slow action, but a detectable midterm impact on subsequent generations, could be combined with applications of *Btk* that generate a more immediate knockdown effect. However, such an emerging hypothesis needs specific multiyear studies to be appropriately documented.

On the other hand, however, it is important that the application of these microbiological control agents is calibrated on the basis of the actual conditions of population dynamics at a given time, in order to produce the desired pest containment effects and make these low-environmental impact interventions even more economically viable. Thus, gypsy moth baculoviruses are good candidates to be introduced in gypsy moth multi-year management programs aiming at interfering with their natural population dynamics.

5. Conclusions

Based on the obtained results in small-scale trials, *L. dispar* showed a significant susceptibility to the LdMNPV formulation, when applied at higher doses and against younger larvae. The highest dose achieved a good efficacy in protecting the crop, albeit at a lower degree than the *Btk* reference product. Such efficacy was not confirmed in larger-scale trials conducted by aerial applications, partly due to population dynamics affected by a natural retrogradation phase. However, a significant increased mortality of larvae collected in plots treated with the baculovirus was detected. Given a higher susceptibility of younger larvae, earlier applications are recommended.

Everything considered, the use of LdMNPV in forest protection programs against the gypsy moth is worth further consideration under different infestation conditions. Its efficacy in regulating population dynamics during outbreaks is expected to be maximized under progradation [29]. This ecological effect could be exploited in a multi-year integrated program involving the combined use of *Btk* to contain infestations and of the baculovirus to modulate population dynamics.

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General conclusions

Socio-environmental and economic concerns related to *Lymantria dispar* infestations in Mediterranean forests foster the development of effective and environmentally sustainable methods of monitoring and management. Accordingly, sampling methods and microbiological control approaches have been studied to implements IPM strategies against gypsy moth outbreaks. The proposed sampling plans proved to be of significant support to decision making on insecticide application when the action threshold is exceeded, and the population development phase is unknown. Compared to traditional sampling methods used so far in Mediterranean conditions, both the enumerative and binomial sampling plans could reduce the amount of time required for estimating gypsy moth infestation without affecting the reliability of the monitoring. However, the availability of the methods we proposed should be verified in field conditions by calculating the sampling time to determine the sustainability of sequential sampling plans to monitor *L. dispar* at large spatial scale.

Bacillus thuringiensis kurstaki (*Btk*) proved to be an effective and low impact technique to contain gypsy moth infestations and, at the same time, protect cork oak trees from defoliation. The *Btk* strains we tested were equally effective against *L. dispar* larvae, with significant differences in larval density decrease compared to untreated control. In addition, *Btk* applications carried out using Foray® 76B AVIO at a lower dose than the one suggested in the label, was successfully considered. These results highlight the reliability of using different products to either prevent or reduce insect resistance to a specific *Btk* strain, as well as the possibility of applying a lower dose to save costs of shipment and distribution.

The evaluation of the species-specific multicapsid nucleopolyhedrovirus (LdMNPV) against *L. dispar* in Mediterranean conditions showed that the use of viruses-based formulations is worth of further consideration. In fact, although LdMNPV control efficacy in containing *L. dispar* larval

population density was lower compared to *Btk*, a significant larval mortality was observed in treated compared to untreated plots. Since the results we obtained were in line with the time-dependent effect of the pathogenic process, LdMNPV should be used against early larval stage to enhance its efficacy. Further research is needed to evaluate LdMNPV effectiveness during diverse gypsy moth population development phases, in the prospect of supporting its use as an alternative or in combination with *Btk* in a multi-year integrated pest management program.

In conclusion, the results obtained with the present PhD thesis contribute to increase knowledge on *L. dispar* monitoring and control strategies. Overall, the usefulness of our findings can be extended outside the Mediterranean forest area, even if the robustness of the proposed sampling and control methods should be appropriately calibrated in areas with different environmental conditions and where this pest is a nonnative species.