# Intensified agriculture favors evolved resistance to biological control

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**Increased regulation of chemical pesticides and rapid evolution of pesticide resistance have increased calls for sustainable pest management. Biological control offers sustainable pest suppression, partly because evolution of resistance to predators and parasitoids is prevented by several factors (e.g., spatial or temporal refuges from attacks, reciprocal evolution by control agents, and contrasting selection pressures from other enemy species). However, evolution of resistance may become more probable as agricultural intensification reduces the availability of refuges and diversity of enemy species, or if control agents have genetic barriers to evolution. Here, we use 21 years of field data from 196 sites across New Zealand to show that parasitism of a key pasture pest (*Listronotus bonariensis*, Argentine stem weevil) by an introduced parasitoid (*Microctonus hyperodae*) was initially nationally successful, but then declined by 44% (leading to pasture damage of c. NZD$160m p.a.). This decline was not attributable to parasitoid numbers released, elevation or local climatic variables at sample locations. Rather, in all locations the decline began 7 years (14 host generations) following parasitoid introduction, despite releases being staggered across locations in different years. Finally, we demonstrate experimentally that declining parasitism rates occurred in ryegrass *Lolium perenne*, which is grown nationwide in high-intensity pastures, but not in adjacent plots of a less-common pasture grass (*Lolium multiflorum*), indicating that resistance to parasitism is host-plant dependent. We conclude that low plant and enemy biodiversity in intensive large-scale agriculture may facilitate the evolution of host resistance by pests and threaten the long-term viability of biological control.**

Attack rates | GAMM | invasive species | meta-analysis | natural enemy

## Introduction

Global human population growth demands increased food production (1). This increasing need has led to increases in agricultural monocultures, which exacerbate yield losses to pest species (2, 3). Moreover, rapid evolution of pest resistance to chemical control (4), combined with the negative impacts of pesticides on human health and the environment, have increased calls for sustainable and acceptable pest management methods (5-7). Biological control of pests by native and introduced natural enemies is an ecosystem service worth billions of dollars annually (8), and it has been heralded as a powerful solution due to its low cost and long-term effectiveness, if initial control is achieved (9). Although pest evolution of resistance to microbial control agents has been documented (10), there are few if any examples of evolved resistance to introduced parasitoids or predators (11, 12), even though heritable variation in resistance to parasitoids exists and could be selected upon if the benefits outweigh any costs of resistance (13).

Several hypotheses can explain this absence of resistance (11). First, co-evolutionary arms races (natural enemies evolving counter-adaptations to the pest) may prevent host resistance from occurring (14, 15). Second, spatial and temporal refuges from attacks may reduce the overall selection pressure on the host, or allow source-sink evolutionary dynamics whereby vulnerable genotypes are maintained by immigration from refuges (16). In addition, combinations of different enemy species may exert separate selective pressure, and thereby prevent the pest from evolving resistance to any single enemy across its entire range (17).

However, these mechanisms that prevent resistance to biological control could in theory be undermined in large-scale homogeneous agricultural systems, which may have few refuges to sustain susceptible strains of the pest, low variability in attack rates, and low biodiversity of enemy species (9). Moreover, co-evolutionary arms races may favor one participant if mutation or recombination rates, or even available genetic diversity, differ significantly between enemy and pest. This could occur due to differences in population bottlenecks (e.g., if few enemy individuals are introduced) or in sexual versus asexual reproduction (18).

We therefore hypothesize that the conditions associated with agricultural intensification and expansion could favor the evolution of host resistance to biological control agents. Here, we use 21 years of data from a well-studied interaction between an exotic pest species [*Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae)], Argentine stem weevil] and its introduced parasitoid [*Microctonus hyperodae* Loan (Hymenoptera: Braconidae)] in exotic pasture in New Zealand to test whether parasitism shows changes congruent with this hypothesis. The pest was self–introduced, first discovered in 1927 and by the 1980s was causing NZD $74-251 million of damage per annum (19). A

### Significance

**The need for agricultural production to meet the food demands of a growing human population will require sustainable and acceptable pest management, such as biological control, across 11% (1.5 billion ha) of the globe's land surface. However, the long-term viability of this ecosystem service can be threatened by the expansion and simplification of agricultural systems, which may facilitate the evolution of resistance by pests to their control agents. This study uses a national dataset to present evidence for the acquisition of resistance by a ryegrass weevil pest to its parasitoid wasp over the last 21 years. This resistance was not associated with differences in environmental conditions, but rather is specific to the most commonly-grown pasture grass species.**

**Reserved for Publication Footnotes**

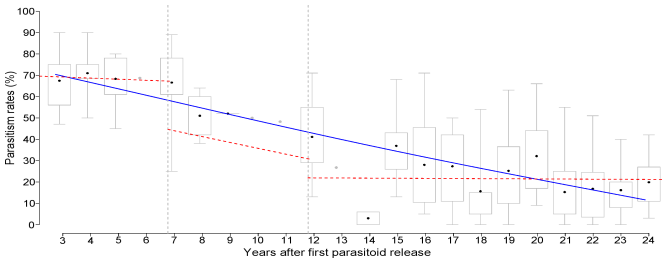


**Fig. 1.** Decline in overwintering *Listronotus bonariensis* parasitism by *Microctonus hyperodae* between 1994 and 2015. Best-fit using GAM (i.e. cubic smoothing spline with equivalent degrees of freedom = 2; solid blue line). Three OLS-CUSUM models fitted to the data (dashed red line) that account for estimated breakpoints (seven years and 12 years since first the release of the parasitoid; dashed grey lines). Grey points are individual plots in years where data were missing, which were estimated via cubic smoothing spline with a polynomial fit; individual mean values of parasitism rates (%) are shown in black points, the 25th to 75th percentile is shown by the grey box, and the range of values is shown by the lines outside the grey box.

**Table 1. Table and Table Captions Results of the best-fitting overwinter parasitism rates with explanatory variables recorded in each site (d.f. 309) via quasi-binomial Generalized Additive Mixed Model (GAMM).**

| | $GAMM_1$ |
|---|---|
| Intercept | **-1.1*** |
| **Years after first parasitoid release** | **-0.1***** |
| Year of sampling | -0.1 |
| **First year of parasitoid released** | **0.1***** |
| Number of parasitoids released | 0.1 |
| Elevation (m) | 0.1 |
| Mean annual precipitation (mm) | -0.1 |
| Growing Degree Days | -0.1 |
| Spatial autocorrelation term | -0.1 |
| **AIC** | **863.6** |

The full results of a comparison (using AIC) with other regression model families is presented in Table S1. Significant variables in any given model are shown in bold. The lowest AIC is shown in bold. Non-significant variables are shown in grey. The superscript refers to the statistical significance of the explanatory variable (*** P < 0.001, ** P < 0.01, * P < 0.05).

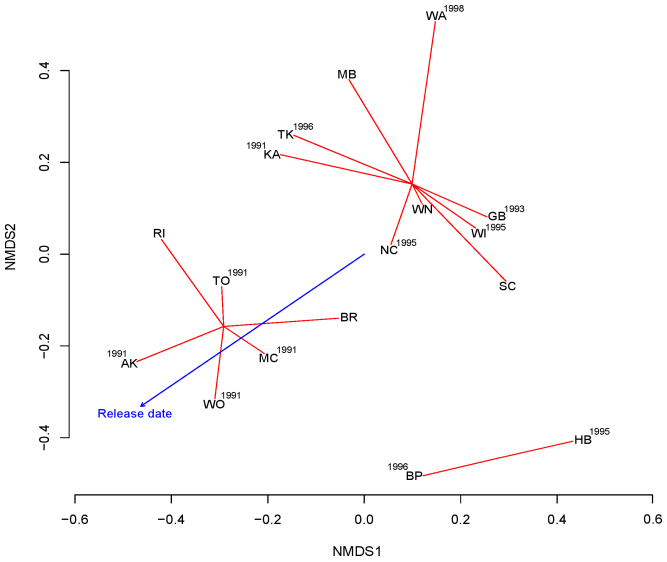


**Fig. 2.** NMDS ordination of New Zealand sub regions [*sensu* Crosby (52)] according to the temporal trend of overwintering *Listronotus bonariensis* parasitism by *Microctonus hyperodae* between 1994 and 2015. For graphical reasons, the resulting clusters were added to the ordination of the plots and connected to the group centroid using the function '*ordispider*'. Where the dates are shown, these represent the years of the first releases of *M. hyperodae*. No dates are shown where no release of *M. hyperodae* was made.

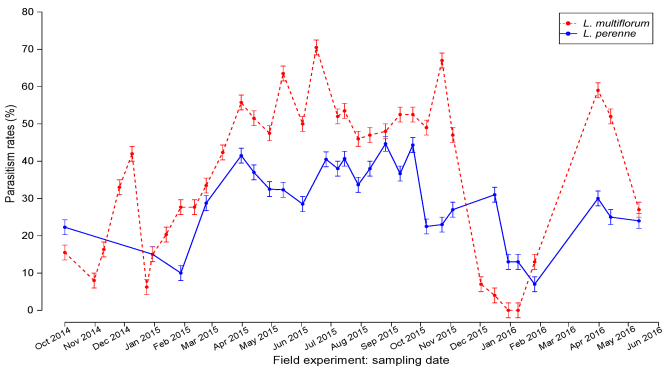


**Fig. 3.** *Microctonus hyperodae* parasitism (%) of *Listronotus bonariensis* collected between October 8, 2014 and May 19, 2016 from plots containing *Lolium multiflorum* (cv. Lush AR37 fungal endophyte; dashed red line) and the *L. perenne* (cv. Samson endophyte free; solid blue line) at the AgResearch Lincoln Research Farm (New Zealand). Values are the mean ± SEM of >14 individual weevils.

parasitoid species from its native range was introduced in 1991 and provided successful control with peak parasitism rates of 80-90% in the early years (20-22). However, there has been emerging evidence of recent declines in attack rates (23, 24). An obvious hypothesis is that these declines could be driven by abiotic (e.g. climate) or biotic (e.g. parasitoid-related) variables altering the host-parasitoid interaction (25). Alternatively, several conditions present in large-scale intensified pasture ecosystems suggest that evolved resistance to the parasitoid may have been possible during the c. 50 generations *L. bonariensis* has undergone since the first releases of its parasitoid (26).

Firstly, the parasitoid is parthenogenetic and suffered a severe population bottleneck during introduction [only 132 individuals comprising clonal lines from 7 geographically separate population were released into the country (21)]. Thus, we hypothesize that these factors would place it at an evolutionary disadvantage against the host, which undergoes sexual recombination during each of two generations a year (27), and theoretical work has shown that constraints to parasitoid evolution could rapidly lead to the evolution of resistance by hosts (28). Secondly, the spatial and temporal variability in attack rates that typically prevents the evolution of resistance (11, 28) may be reduced in crops with low species diversity, structural simplicity and a large, connected spatial continuum such as New Zealand's improved pastures. These occupy c. 10.6m ha of New Zealand [c. 40% of the total land area (29)]. In particular, c. 29% of improved pasture is intensively managed with low species diversity compromised of predominantly perennial ryegrass *Lolium perenne*, often with a single species of white clover, *Trifolium repens*. Such large-scale production systems of low cultivated diversity are common (e.g., monoculture cash crops, plantation forestry), and when the crop is structurally simple, as is often the case, spatial refuges from attack may be scarce.

Finally, control agent resistance to natural enemies is rare because diverse enemy assemblages in pests' indigenous ranges typically inflict varied selection pressure, such that no enemy species singularly exerts enough pressure (i.e., mortality) to drive the evolution of resistance (11). Again, this barrier to resistance may be reduced if enemy diversity is lower in high-intensity agricultural systems. For example, in New Zealand pastures,

grazing intensity is associated with a decline in the diversity and abundance of spider predators, which have approximately half the diversity of the fauna from similar sites in England (30), and invertebrate predators generally have low impact on *L. bonariensis* populations in New Zealand (31, 32). Insectivorous birds also have low abundance in the absence of native vegetation on New Zealand farms. Further, this vegetation (along with the proportion of 'unimproved' low-intensity pasture) has declined with intensified farm management over the past century (33). Hence, we hypothesize that this lack of alternative predation pressure, coupled with initially high parasitism rates, would also have imposed a strong selection pressure on the weevil population, further accelerating the evolution of resistance.

We begin by reporting on a significant decline in *L. bonariensis* parasitism by *M. hyperodae* in the last 21 years, and we examine whether this pattern is more consistent with variation in abiotic or biotic conditions or with the hypothesis of acquisition of resistance by the weevil to the parasitoid. We then explore whether parasitism rates are more similar in sites that co-occur spatially, or whether the release date of the parasitoid (i.e. time available for the acquisition of resistance) better explains present-day similarities in attack rates across sites. Subsequently, we use a field experiment to test the hypothesis that declining attack rates are specific to the predominant grass species used in intensified pastures, as indicated earlier in greenhouse studies (34).



## Results

### Long-term declines in field parasitism rate

We found that overwintering parasitism rates declined significantly (*rho* = -0.68, $P < 0.001$) with time elapsed since the first parasitoid release. The best-fitting model was a cubic smoothing spline ($\lambda = 1.34$, Df = 2), with breakpoint analysis highlighting the significant ($P < 0.001$) presence of two breakpoints (seven years and 12 years since first the release of the parasitoid; Fig. 1). The best-fitting model with lower AIC belonged to the GAMM family (Table 1) and a comparison with this model is presented in Table S1, indicating that both a non-linear relationship with time and the inclusion of region-level random effects improved model fit. Within the GAMM family, the best-fitting model ($GAMM_1$; AIC = 863.6) indicated that the years elapsed since first release had a significantly negative, but non-linear effect ($P < 0.001$) on overwintering parasitism rates. The $GAMM_1$ results also indicated that the first year of parasitoid release had a significantly positive, but non-linear effect on parasitism rates ($P < 0.001$). There was no significant interaction effect between release date and time since release (Table S1), indicating that parasitism rates at a given location began to decline seven years post release, irrespective of the release date at that particular site. None of the remaining predictors included in the model (year of sampling, total number of parasitoid individuals released, several measures of local climate, elevation, or a spatial autocorrelation parameter) showed any significant relationship with parasitism rates (Table 1).

### Determination of parasitism rates: ordination and pattern

The NMDS ordination indicated that trends in the parasitism rates clustered together according to the major regional release dates (Fig. 2). A gradient was observed whereby there was a cluster of points associated with the 1991 parasitoid releases versus another cluster that comprised the five regions (see Fig. S1 for a map with the region codes given in Fig. 2) in which the parasitoid was released later (i.e. 1993, 1995, 1996 or 1998). This cluster of later-release-date sites also contained one region where there was a low number of parasitoids released (< 1000 individuals; KA) and three where the parasitoid was not released and had instead arrived later through natural dispersal (MB, SC and WN; Fig. 2).

Overall, even sites that were spatially highly separated and climatically very different (with different amounts of pasture in the region) grouped together (e.g. sub regions such as MC and BR, respectively on the dry east and wet west coasts of the South Island grouped with RI, TO, WO and AK of the central and northern North Island, because all received parasitoid releases in 1991 or later via natural dispersal). This demonstrates that attack rates declined through time at different sites based on the local date of parasitoid release rather than their spatial proximity. Finally there was another cluster generated by regions where the parasitoid was released in 1995 or 1996 (i.e. BP and HB) but where few or no *L. bonariensis* were found in the recent sampling campaigns (Fig. 2).

### Field experiment: influence of pasture type on parasitism rate

Fig. 3 shows rates of parasitism in our experimental plots, measured at fortnightly intervals during the period 2014-2016. Notably, this frequency of sampling accommodated the large fluctuations in parasitism rates that occur with interacting population dynamic processes of both the weevils and the parasitoid (35). Regardless, there were significant differences in the rates of parasitism across the two grass types (RSS = 1222.3, $P < 0.001$) and significant similarity in the pattern of parasitism rates (Cross-correlation = 1, $P < 0.001$).

It could be possible that differences in host abundance across grass species were the cause of parasitism differences, rather than the consequence of differences in attack rates. However, previous work showed that the searching efficiency of the parasitoid was very high even at very low weevil ground densities (36), which suggests that density-dependence is unlikely to explain the patterns observed here. Nevertheless, to test specifically for dependence on host density we used historical data from Goldson, Proffitt and Baird (26), and tested for a correlation between host abundance and parasitism rate. This revealed no significant relationship ($\rho = -0.1$, $P = 0.5$), which suggests that attack rates were unlikely to have been host density dependent.

## Discussion

It has been argued that biological control provides sustainable long-term pest suppression, because the diversity of selection pressures, refuges from attack, and the co-evolutionary arms races that are present in most ecosystems prevent the evolution of resistance to their natural enemies (11, 17). Indeed, the time and expense associated with pre-release testing of control agents is predicated on an assumption that rapid evolution of resistance is unlikely. Yet, here we have demonstrated a significant decline in control rates of an economically-important pest by its introduced parasitoid control agent. Importantly, the measured decline was not associated with local abiotic conditions, nor did it originate in one location and subsequently spread to other nearby locations. Rather, the declines began simultaneously across the country, seven years (approximately 14 host generations) after release of the parasitoid at each given location (22), and reached a plateau at present-day attack rates after 12 years. A seminal experimental study of host-parasitoid evolution (37) found similar marked and rapid declines in attack rates (by 40-68% in 8-20 generations, depending on the specific experimental treatment), and these declines were consistent across replicates, as they were across locations in our study. Given our findings of national-scale uniformity in time to resistance, and the lack of spatial clustering in resistance patterns, it is unlikely that resistance occurred via the spread of a novel mutation. Rather, it likely involved a selective sweep on resistant genotypes that existed at lower frequencies in the background population, however, this hypothesis requires testing with genomic techniques.

The mechanism of resistance is not yet clear, however, there is sufficient evidence to posit several hypotheses. First, there may

have been the evolution of some form of escape behavior, as behavioral change can rapidly generate new phenotypes (38). For example, a study of field crickets (*Teleogryllus oceanicus*) on the Hawaiian islands showed that genetically-based resistance in this species had occurred twice involving separate genetic changes on different islands in the same archipelago (39). The crickets stopped stridulating (after about 24 generations) because such activity attracted the parasitic fly (*Ormia ochracea*) and this species exerted negative selection pressure. The potential for escape behavior in Argentine stem weevil has been suggested by previous work which showed that, in the presence of the parasitoid, the weevils tended to move off the foliage towards the soil in upright potted plants (40). This leads to the hypothesis that plant physical structure may affect the ability of the weevil to employ its escape behavior. If correct, this would suggest that resistance could be related to the 3-dimensional structure of the dominant plant species (in this case, *L. perenne*). However, this hypothesis was not supported by a more recent study by Goldson and Tomasetto (34), which showed that parasitism rates did not differ significantly between vertically- versus horizontally-positioned grass tillers in laboratory cages. Thus, in spite of an apparent lack of impact of plant structure, plant species did indeed significantly affect parasitism rates. Goldson and Tomasetto (34) showed that in the presence of *L. perenne,* parasitism rates were significantly lower (c. 46%) than in the presence of the far less common short-rotation pasture species *L. multiflorum* (c. 75%), whereas empty control cages showed 35% parasitism. These laboratory results accord with the field experimental results presented here, wherein parasitism rates were lower in weevils on *L. perenne* than in *L. multiflorum* (Fig. 3). If these differences are not caused by plant structure, they may be caused by differences in plant chemistry such as volatiles, which can be important in attracting natural enemies of herbivores (41). Although *L. multiflorum* alone has been shown experimentally not to be attractive to our parasitoid species *M. hyperodae* (42), plant feeding by the weevil may nevertheless stimulate the release of herbivore-induced plant volatiles that elicit a parasitoid or weevil response. This requires further investigation.

An alternative hypothesis for the mechanism of resistance is encapsulation of the parasitoid egg by the host immune system (43). However, encapsulation is unlikely to have generated the observed differences in parasitism rates across host-plant treatments (in both the field and laboratory), and in fact no evidence of parasitoid encapsulation has been observed despite thousands of weevil dissections by numerous workers (35).

Whatever the mechanisms of resistance are, our finding that attack rates on weevils remained higher (though with the same seasonal dynamics) in experimental plots of an uncommon pasture species (*L. multiflorum*) may provide a possible opportunity to off-set the impacts of resistance of the weevil to its parasitoid. Specifically, given the evidence that the mode of resistance is host-plant dependent, there may be opportunities for the introduction of pasture-species diversity, which may allow attack rates to approach their previous levels. At the same time this study's finding also serves as a warning that low crop diversity (such as the single species of grass dominating New Zealand pastures) may facilitate adaptation by pests to their enemies.

To our knowledge, there are no clear cases of evolved resistance to introduced biological control parasitoids (11, 17, 44), and the only other possible example may have been caused by the introduction of new host strains (45). Irrespective, the absence of evidence may not be evidence of absence. Insufficient post-release monitoring of biological control introductions means that long-term efficacy of control remains unclear (46). Although not necessarily deliberately selected for in biological control, parthenogenetic parasitoids are common within the Hymenoptera and are therefore sometimes used in biological control (47). Moreover, the typically low natural enemy diversity in intensified agriculture is likely to increase the selection pressure imposed by control agents (48). Both factors increase the likelihood of evolved resistance. Thus, we hypothesize that any sustained success of biological control introductions will be lowest in situations where the agent is parthenogenetic, crop biodiversity is low, the crop is grown over a large spatial extent, the pest and control agent are specific to a single crop type, and there has been considerable time since parasitoid release. Even in the absence of long-term monitoring, these hypotheses could be explored, for example using a meta-analysis of biological control parasitism rates worldwide. Moreover, our results suggest that pre-release assessments should consider the available variation in pest susceptibility (on which selection could operate), the genetic diversity of agents being released, and the diversity of existing enemies (i.e., sources of alternative selection pressure).

Biological control has the potential to be a sustainable method of long-term pest suppression. However, its efficacy depends on a suite of mechanisms that prevent the appearance of resistance to parasitoids and predators. These mechanisms may break down in intensive agro-ecosystems with low biodiversity (11, 17). Although resistance to insecticides is explicitly managed against, the same is not presently true for biological control using predators and parasitoids, and we hope that our findings will stimulate discussion on this topic. Agro-ecosystem biodiversity offers a variety of benefits for biological control (49), such as resources for natural enemies and greater pest suppression via enemy diversity (50, 51). In addition, crop and enemy biodiversity may be crucial for the maintenance of co-evolutionary regimes that prevent the resistance of pests to their natural enemies, and maintain the multi-billion dollar ecosystem service of biological control (8).

## Materials and Methods

### Data collection and extraction

We assembled published data on the percent parasitism of *L. bonariensis* by *M. hyperodae* collected from 18 New Zealand biological 'sub regions' (52) from 1994 to 2015 (data will be made available to readers upon request). The parasitism rates used were measured during overwintering diapause when levels were found to have stabilized [e.g., 20, 53]. Parasitism rates during the New Zealand summer months are known to fluctuate greatly due to the interplay between episodes of weevil emergence and varying parasitoid attack rates driven by its own patterns of emergence [e.g., 20, 23]. Conversely, diapausing overwintering parasitism rates are stable, as there is neither any weevil eclosion nor adult parasitoid activity (53). We were therefore able to use these diapause collected parasitism data as a conservative proxy-measure of overall parasitoid activity and impact. There are different times of onset of both weevil and parasitoid postdiapause activity in the North and cooler South Island areas, due to differences in the rates of heat accumulation above a temperature development threshold of c. 10°C (54). Therefore, parasitism data were collected between May and August inclusively in the North Island and May and September inclusively in the South Island. Data were available for all the 21 years of this study except for four years (1997, 2001, 2002 and 2004).

Based on the above time periods, a total of 336 published and unpublished records of *M. hyperodae* parasitism of *L. bonariensis* from all of the sampled regions of New Zealand were used (SI1). Firstly, we extracted the parasitism rates and the collection dates (i.e. years) from graphs (e.g. scatter plots or histograms) using DATATHIEF III software (http://datathief.org/). Where these data were not available in the publication, we obtained them directly from the corresponding authors of the studies (4 contacted, 4 replied). We supplemented these published data with unpublished data obtained by dissecting frozen archived weevil samples as part of ongoing national parasitism surveys over the last 21 years. In total, these data were obtained primarily from weevils collected in *Lolium* spp. pastures across 196 sites. This amounted to examining by dissection a total of c. 11,000 individual weevils. The dissections for parasitoid eggs and larvae in all data sources followed the protocol used in published studies elsewhere (55).

### Temporal and spatial analysis of field parasitism rate

We used this 21-year weevil parasitism dataset to examine whether attack rates changed significantly over time, whether any changes were linear, and whether they could be explained by parasitoid-related factors or local environmental conditions rather than adaptation by the host. To achieve this final model, we sequentially determined specific aspects of a model chosen through a process described below.

First, to characterize any temporal trend in the overwintering parasitism rates, we investigated different fitting lines to the data, and this process selected a cubic smoothing spline with a polynomial fit. Because there were duplicated points in the response variable, i.e., different studies measuring parasitism rates in the same location and at the same date, we also applied a generalized cross-validation method in order to reduce any potential bias.

We based the final model on a logistic model implemented through a quasi-binomial generalized linear model using R software (R 3.3.1; http://cran.r-project.org; Accessed April 10, 2016) and the function '*lme*' in the additional package "gamm4". This was extended by adding a random effect (γ) in the linear predictor to account for possible error due to the nested structure (arising from the different regions in New Zealand) in a generalized linear mixed-effects model (GLMM). Visual inspection of the data suggested non-linear changes in parasitism through time, so we allowed for the possibility of a non-linear influence of time by using a generalized additive model (GAM) using the function '*gam*'. This latter model family was extended to include random effects in the predictor terms via a generalized additive mixed model (GAMM) using the function '*gamm*'. We ran a second specification for all of these families of models (i.e., GLMMs, GAMs and GAMMs) by introducing an interaction term between the years elapsed since first release and the first year of release. To compare the suitability of the models, we used the Akaike Information Criterion (AIC).

Based on the parameterization discussed above, we were able to generate an appropriate model with which to test for the effects of abiotic and biotic variables on parasitism rates. We fitted binomial models with quasi-likelihood to account for the over-dispersion and to explain the proportion of overwintering parasitism ($y$, converted to the linear predictor η via a logit link function). We modelled this as a function of 1) biotic variables: the first year of parasitoid release; total number of parasitoid individuals released; the year of sampling and the years elapsed since first release in that sub region; and 2) abiotic variables: elevation extracted from the digital elevation model; the mean annual precipitation; the mean annual temperature and the growing degree days above 10°C at the location from which the data were collected. The climate data were obtained using the National Institute of Water and Atmospheric Research climate maps (NIWA).

During these analyses of parasitism rates over time, it was also possible to demonstrate the adequacy of an autoregressive model of order 1, AR(1), to account for temporal autocorrelation (56). Such adequacy was confirmed by an inspection of the standardized residuals, their autocorrelation function and the p-values of the Ljung-Box statistic (57). To capture this temporal autocorrelation, we therefore included the AR(1) component in all of the subsequent models.

Our sampling design (Fig. S1) meant that there was also the potential for spatial autocorrelation to affect the parameter estimates and error probabilities, such that they could distort the variance-covariance matrix. In order to test for this, we used a Moran's *I* test using the "spdep" package and found low but significant autocorrelation in the parasitism rates (Moran's $I$ = 6.95, $P < 0.001$). To account for this, we adjusted our models by including a spatial auto-covariate term (SAC). In our case, the SAC term accounted for spatial autocorrelation originating from the potential movement of *M. hyperodae* and its host *L. bonariensis* between sampling sites. We computed the SAC term for each sampling location using a neighborhood boundary of 300 km, weighted by inverse distances among neighboring observations and visually assessing the degree to which our models accounted for unexplained spatial variation by plotting a semivariogram of the normalized residuals.

Because our model indicated non-linear changes in parasitism, we tested whether parasitism rates changed abruptly over the duration of this study and attempted to identify the year(s) in which any abrupt changes occurred. To do this, we fitted linear models by ordinary least squares (OLS) regression to the data and an OLS-based CUSUM [Cumulative Sums of standard OLS residuals process; (58)] to investigate possible structural changes in the models (i.e., breakpoints). This analysis was conducted using the "strucchange" package. A formal test for the presence of breakpoints was conducted by adopting the methodology developed by Bai and Perron (59) through a minimization of the residual sum of squares with a Bayesian Information Criterion (BIC).

**Determination of parasitism rates: ordination and pattern**

If there were strong local effects on parasitism rates (e.g., due to environmental conditions or local population genetics), we would expect to see parasitism rates grouping together according to their spatial proximity. To explore this possibility, we measured whether the temporal trend in parasitism rates (expressed as a site × site distance matrix obtained from a site × year matrix in which cells represented parasitism rates) was most similar when sites were spatially clustered (i.e., when they occurred in the same sub region of New Zealand) or when they had received released parasitoids in the same year. To determine this, we used Non-Metric Multidimensional Scaling (NMDS) implemented in the additional "vegan" package. NMDS is useful when datasets cannot be presumed to consist of an assumed probability distribution as in this case. Moreover, the NMDS technique graphically depicts similarity and/or dissimilarity within or between the assemblages of clusters. We selected the most suitable distance matrix (i.e., Jaccard dissimilarity index) that best separates the sub regions using the rank orders of correlations of standardized environmental variables to their unit variance via the '*rankindex*' function. We then selected the NMDS model with the lowest *stress* (i.e., the best model fit) using the '*metaMDS*' function.

To classify the New Zealand sub regions into different clusters, we specifically used a dendrogram generated by hierarchical clustering with the distance between cluster centroids (i.e., single linkage method) as the preferred method to correctly reproduce the actual estimated distance within the dendrogram (i.e., cophenetic distance). To optimize the classification for a given number of clusters (i.e., number of classes) we tested the K-means clustering using 100 iterations and random starts, optimized with Hellinger transformation as the standardization (60). The resulting classes were added "manually" to the ordination graph.

To test which of the abiotic or biotic variables were related to the clusters of sites identified by their ordination according to New Zealand sub regions, we used the '*envfit*' function with 10000 permutations and then fitted significant vectors (with $P \leq 0.05$) which were overlaid onto the ordination.

**Field experiment: influence of pasture type on parasitism rate**

Previous evidence suggests that the predominant plant species used in pastures may have played a part in the observed reduction in parasitism. In a preliminary study (35), we observed in the field that parasitism of *L. bonariensis* by *M. hyperodae* was significantly higher in plots comprising a less-common grass species (*L. multiflorum*) than in plots compromising the commonly-grown *L. perenne*; further laboratory experiments confirmed that parasitism rates differed according to the host-plant present (34). These findings contrasted with unpublished data obtained from the same experimental laboratory conditions in the 1990s, which suggested that at that time parasitism rates did not differ in the presence of the two grass types. Combined, these results implied that the loss of parasitoid efficacy in the intervening years may be specific to the *L. perenne* plants, but less so in the *L. multiflorum* plants. From this it follows that, if plant species is an important mediator of evolved resistance, the weevil would not have had the opportunity to effectively adapt to *L. multiflorum* (34).

Here we extended the field experiment of Goldson, Tomasetto and Popay (35), with an additional year of sampling specifically to explore potential temporal variability in the mean parasitism rates. Detailed methods can be found in Goldson, Tomasetto and Popay (35), but the experiment is briefly summarized here. The experimental plots were sown in 2013 on the AgResearch Lincoln Research Farm (-43.631788, 172.464938) and comprised *L. multiflorum* (cv. Lush AR37 fungal endophyte) and the *L. perenne* (cv. Samson endophyte free) plots. Previous experiments had found no effect of *L. perenne* cultivars and endophytes on field parasitism levels of Argentine stem weevils (35). The treatments comprised two sets of 35 × 50 m plots that were sown on September 2013 and established well. The plots were set-stocked with lambs throughout the winters. Nitrogen was applied as urea on four occasions (in spring 2013, in summer/autumn 2014, in early December 2014 and early January 2015).

The *L. multiflorum* and *L. perenne* plots were sampled fortnightly from October 8, 2014 until May 19, 2016 using a modified leaf-blower vacuum (Echo 21cc) to suck pasture litter into a removable net recessed in the inlet pipe (61). Collections were made by dragging the machine across each plot for 15 minutes. The weevils were then removed from the litter and dissected to determine parasitism rates. A minimum sample size of weevils for dissection was set at 14 individuals (35).

We tested for statistical significance between the mean parasitism rates in the *L. multiflorum* vs. *L. perenne* plots. Because the normality assumption for a traditional one-way analysis of variance (ANOVA) was not met, we used non-parametric complete random permutation tests (n cycles = 10,000) for a one-way ANOVA via the additional package "lmPem". In addition to dealing with violation of the normality assumption, this approach provides a flexible and intuitive methodology for statistical analysis and implements the methods for permutation tests described by Kabacoff (62).

Moreover, the multiple time-series data obtained were analyzed using a cross-correlation analysis built in the additional package "tseries". This analysis measured the extent of similarity of two series (Cross-correlation = 1) as a function of the lag of one relative to the other. This allowed estimation of the extent to which temporal trends in parasitism rates differed in *L. multiflorum* vs. *L. perenne*.

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