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Impact of natural control agents of the citrus leafminer *Phyllocnistis citrella* on lemon trees varies among seasons

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Abstract

Studies on insect natural enemies and their effects on host populations are of immense practical value in pest management. Predation and parasitism on a citrus pest, the leafminer *Phyllocnistis citrella* Stainton, were evaluated by sampling over 3 years in four locations within a world leading lemon producing area in Northwest Argentina. Both mortality factors showed seasonal trends consistent across locations, with predation exerting earlier and more sustained pressure than parasitism, which showed wider seasonal variations. The dominant parasitoids, native *Cirrospilus neotropicus* and introduced *Ageniaspis citricola*, showed different seasonal trends: *C. neotropicus* was dominant in spring whereas *A. citricola* superseded it in autumn and winter. Although parasitism rates were relatively low, the native *C. neotropicus* revealed favourable features as potential control agent, by showing density-dependence, parasitism rates comparable with those of the specific *A. citricola* during part of the cycle, and earlier synchronization with the host. The study provides highly relevant information for a sustainable management of this worldwide pest, for which biological control is considered the best long-term option.

Keywords: Predation, parasitism, *Cirrospilus neotropicus*, *Ageniaspis citricola*, density-dependent control

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Introduction

Population dynamics of herbivore insects are influenced by bottom-up and top-down forces (Hawkins, 2001; Santolamazza-Carbone *et al.*, 2014) which, acting separately or together and with spatial or temporal variations (Walker & Jones, 2001; Girardoz *et al.*, 2007), may result in natural regulation of insect pests (Gratton & Denno, 2003; Miller, 2008). In particular, top-down pressure from a plethora of natural enemies including parasitoids, predators and pathogens, can have a dramatic impact on insects (Colloff *et al.*, 2013; Calabuig *et al.*, 2014) and is the basis of biological control, a key ecosystem service (Naranjo *et al.*, 2015). As such, knowledge gained from ecological studies of insect natural enemies is of immense practical value in pest management (Kidd & Jervis, 1996).

Among herbivore insect guilds, leaf miners support the most diverse parasitoid assemblages and the highest parasitism rates, providing successful cases of classical biological control (Hawkins, 1994, Karamaouna *et al.*, 2010). The relative impact of predators and parasitoids on leafminers appears to be variable (Salvo & Valladares, 2007), changing between native (Hawkins, 1994; Eber, 2004) and alien species (Grabenweger *et al.*, 2005; Xiao *et al.*, 2007) and even

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along the growing season (Urbaneja *et al.*, 2000; Queiroz, 2002) or with environmental conditions (Rott & Ponsonby, 2000).

The citrus leafminer (CLM) *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) is a serious pest of commercial citrus production throughout the world (Hoy & Nguyen, 1997; Smith *et al.*, 1997; Mustafa *et al.*, 2014). Eggs are laid on young leaves and larvae feed within the leaf tissue in distinctive serpentine mines, finally pupating in a pupal cell at the leaf margin. CLM is a multivoltine species, with developmental time ranging from 13 to 52 days depending on temperature (Sarada *et al.*, 2014). Experience has shown that sole reliance on pesticides for the management of CLM is neither biologically nor economically feasible, leading to the current tendency to enhance chemical control by simultaneously favouring natural enemies (Garcia-Marí *et al.*, 2004; Sarada *et al.*, 2014). Thus, various studies have addressed the identification, biology and incidence of predators (Urbaneja & Jacas, 2003; Lioni & Cividanes, 2004; Xiao *et al.*, 2007) and parasitoids (Legaspi *et al.*, 2001; Hoy & Jessey, 2004; Mafi & Ohbayashi, 2004; Tsagkarakis *et al.*, 2013) on CLM populations over the expanding distribution area of this pest. Although temporal fluctuations in the impact of these mortality agents have been described (Amalin *et al.*, 2002; Karamaouna *et al.*, 2010), their seasonal trends have not been properly compared. CLM was first detected in Argentina in 1995, and has since spread to all citrus growing areas in the country, on a variety of citrus hosts (Goane *et al.*, 2008). In Tucumán province (NW Argentina), its world leading lemon production (FAO, 2012) has been threatened by CLM arrival, with severe risk to young plants. The report of citrus canker in the region 7 years later raised concerns of citrus producers since feeding galleries of CLM on leaves become contaminated with the bacterium, increasing the vulnerability and susceptibility of trees to citrus canker (Christiano *et al.*, 2007) with a consequent rise of disease incidence (Graham *et al.*, 2004) in open field areas. The exotic parasitoid species *Ageniaspis citricola* Logvinovskaya (Encyrtidae) and *Citrostichus phyllocnistoides* Narayanan (Eulophidae) were introduced in an attempt to curb the increasing expansion of CLM in the region (Willink *et al.*, 2002). Although *C. phyllocnistoides* failed to be established on CLM populations in the region, high parasitism rates were achieved by the exotic *A. citricola* (Zaia *et al.*, 2006) and low parasitism rates by native parasitoids have also been recorded (Diez *et al.*, 2006).

Here, we evaluated the relative importance of predators and parasitoids as top-down control agents on CLM populations in NW Argentina, from 3-year field samplings performed in lemon orchards. Considering the usually dominant role of parasitoids in controlling leaf miner insects (Salvo & Valladares, 2007) and the introduction of *A. citricola*, parasitoids could be expected to be more important than predators in this system. However, generalist predators tend to be earlier colonizers of introduced insect pests in comparison with the usually more specialized parasitoids (Ehler, 1998), thus they could exert strong pressure on the studied populations given their relatively short exposure time (about 10 years). We also asked whether seasonal variations may obscure general trends or, on the contrary, reveal possible associational effects of parasitoids and predators on CLM. Finally, since successful biological control has been associated with density-dependent responses of natural enemies to pest species (Speight *et al.*, 2008, but see Matsumoto *et al.*, 2004), we have also analyzed this attribute for parasitoids and predators of CLM.

Materials and methods

Sampling and study area

The study was carried out in four lemon (*Citrus limon* [L.] Burn) orchards within the commercially producing area of Tucumán province, in NW Argentina. Orchards were located at La Ramada (26°41′15″S, 64°56′51″W), La Granja (26°43′34″S, 65°10′23W), Famaillá (27°03′14″S, 65°24′17″W) and Alberdi (27°35′13″S, 65°37′15″W), thus covering the northern, central and southern areas of the province and reflecting the gradient of environmental conditions prevailing in the lemon producing area. Annual rainfall is lower in the north (700–900 mm), with up to 200 mm water deficit between winter and spring and a frost risk period extending from June to August. Rainfall increases towards the central and southern areas (900–1700 mm), without soil water deficit and with very low or null frost risk (Zuccardi & Fadda, 1985). Each orchard sustained between 30 and 100 ha of 8–10 year-old lemon trees. Throughout the study period, orchards remained under conventional management consisting of copper oxychloride sprays combined with mancozeb and/or citrus mineral oil. In the orchard from Alberdi, two annual applications of insecticide (abamectin) targeted to CLM control were additionally made.

At each orchard, one shoot with ten new leaves was randomly taken from the middle or upper region of each of ten plants (i.e., 100 leaves per orchard). Plants were randomly chosen from different rows in the middle region of the orchard, and samples were placed in plastic bags and transported to the laboratory. Samplings were performed every two weeks in Alberdi and on a weekly basis in the other orchards, from September 2002 to October 2005.

Identification of CLM mortality causes

All CLM immatures present in the sampled leaves were examined in the laboratory under a stereoscopic microscope within 24 h from field collection, in order to identify and record CLM developmental stage (from first larval instar, till pupa), alive and dead specimens, and causes of mortality. A CLM was considered preyed upon when an empty mine or pupal chamber showed a small hole or when a mine containing visible remains of the CLM larva was perforated or torn (Amalin *et al.*, 2002; Zappalà *et al.*, 2007). A CLM was considered parasitized when endoparasitoid prepupae or pupae were found within CLM exoskeleton or pupal chambers; e.g., *A. citricola*, a koinobiont species attacking eggs and early instar larvae of the host (at which the parasitoid is not visible without dissection) and killing it in the pupal chamber. A CLM was also considered parasitized when ectoparasitoid eggs, larvae or pupae were found inside a mine or pupal chamber (e.g., *Cirrospilus neotropicus*, an idiobiont species parasitizing latest instars of CLM and killing it at the same stage). When death cause was uncertain, CLM were classified as 'dead by unknown causes'. Deaths caused by 'host feeding' were not identified and were possibly included within the last item, since this behaviour was at least proven for the native *C. neotropicus* (Foelkel *et al.*, 2009). CLM immatures showing ongoing parasitism signs were isolated in hermetic bags (Ziploc® Thai GRIPTECH, Bangkok 10150, Thailand) at 22 ± 4°C, to obtain parasitoid adults. The latter were identified using taxonomic keys.

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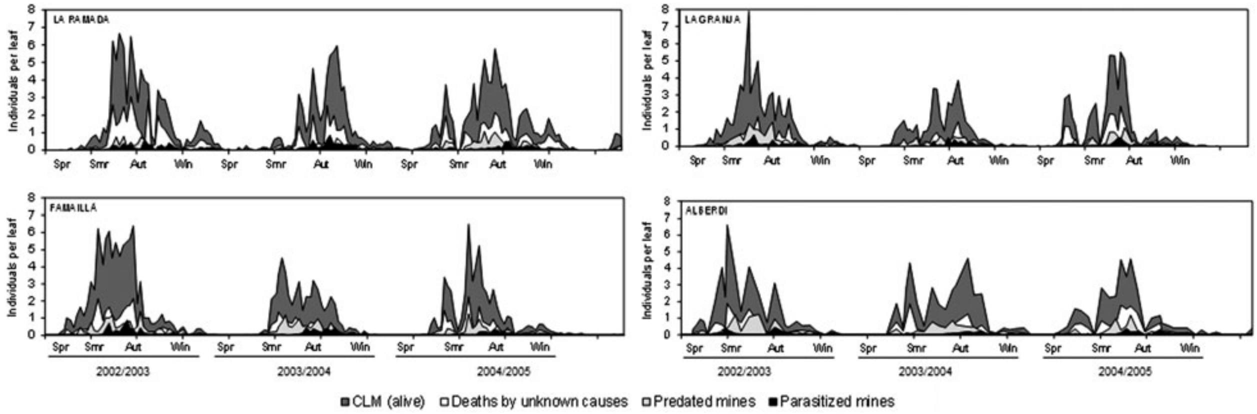

Fig. 1. Fluctuations (numbers in 100 leaves) of CLM population and main mortality factors (CLM preyed, parasitized and dead from unknown causes) throughout 3 consecutive years in four locations from NW Argentina. Seasons are indicated as: Spring (Spr), Summer (Smr), Autumn (Aut) and Winter (Win).

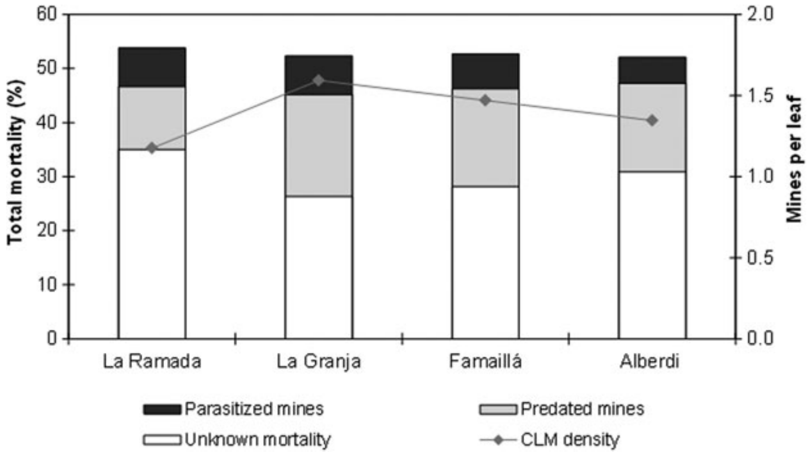

Fig. 2. Annual mean density (number of mines per leaf) and total mortality (as % of all mines) of CLM in four locations from NW Argentina. Relative rates (as % from all dead individuals) of each mortality factor are represented within each column.

Table 1. Results of GLMMs analysing mortality rates due to predation and parasitism (mortality factor) on CLM populations, considering variations among seasons and locations.

Variable	Source of variation	df	*F*	*P*-value
Mortality Rates	Season	3	49.735	<0.001
	Location	3	0.007	0.999
	Mortality factor	1	24.722	<0.001
	Season × mortality factor	3	11.618	<0.001
	Location × mortality factor	3	1.144	0.331
	Season × mortality factor × location	9	1.579	0.193
	Error	561		

Data analysis

A first general description of the relative importance of each mortality factor was provided by estimating the annual rates of parasitism, predation and deaths by unknown causes, as percentages of total CLM immatures recorded at each location.

In order to analyse in further detail the impact of predators and parasitoids on CLM populations and their seasonal variations, predation and parasitism rates were adjusted to the precise resource exploited in each case. Thus, parasitism percentage was recalculated considering only the CLM stages at which parasitoids could be observed (third instar larvae and pupae), whereas for predation, second instar larvae were also included. For gregarious species like *A. citricola*, the presence of several parasitoids in a single host was recorded as a single parasitism event. Parasitism and predation rates thus calculated were compared by generalized linear mixed models (GLMMs), in which parasitism and predation rates (gamma distribution, log link function) were the dependent variables, with season, location and mortality factor (parasitism vs. predation) as fixed factors. Interactions among fixed factors were also included. A variance component structure was estimated to control for data dependence due to successive measures being taken at the same location. Contrasts were performed to look for differences between means. Similar GLMMs were carried out for a more detailed analysis of parasitism rates of the two main parasitoid species in the studied locations and seasons.

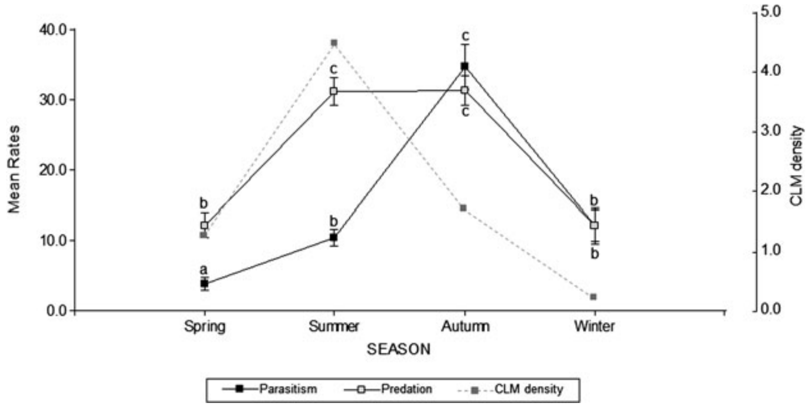

Fig. 3. Seasonal predation and parasitism rates (% of miners ± SE) on CLM, from 3-year sampling in NW Argentina. Data of the four locations were averaged and vertical bars denote SE (location effects were not significant according to GLMM, see table 1). Means accompanied by different letters are significantly different (GLMM contrast, $P < 0.05$). Average CLM density is also included for illustration.

Table 2. Results of GLMMs analysing parasitism rates caused by *A. citricola* and *C. neotropicus* on CLM considering variations among season and locations.

Variable	Source of variation	df	*F*	*P*-value
Parasitism Rate	Season	3	49.135	<0.001
	Location	3	0.031	0.993
	Parasitoid species	1	14.825	<0.001
	Season × parasitoid species	3	22.315	<0.001
	Location × parasitoid species	3	1.701	0.166
	Season × parasitoid species × location	9	0.558	0.831
	Error	552		

Finally, simple linear regressions were performed to analyse density dependence of predation and parasitism in this system, with the average number of CLM individuals per leaf as independent variable and proportion of parasitized or preyed leafminers as dependent variable. Density data were log transformed (log10 $(x + 1)$), whereas square-root arcsine transformation was used for percentage data. Statistical analyses were performed using the software R 2.11.0.

Results

CLM density and mortality: general trends

A total of 42,963 CLM immatures were collected throughout the study. CLM density fluctuated widely along the citrus growing season, from nearly null presence in late winter to about eight mines per leaf in summer–autumn, depending on the year and the location (fig. 1). These fluctuations resulted in an annual average of slightly over one mine per leaf, in all locations (fig. 2). Immature stages including egg, larvae and pupae were collected in virtually all sampling dates, even in winter months.

In all the studied locations, about half of CLM larvae did not reach complete development (fig. 2). The dominant mortality factor was represented by unknown causes (30.1% ± 1.5), explaining on average more than half of total mortality and followed by predation (16.2% ± 1.3), which was in turn higher than overall parasitism (6.4% ± 0.5). The fluctuations depicted in fig. 1 also show the number of preyed mines generally increasing from early summer and declining at the end of autumn, with a tendency for parasitoid presence to increase from late summer to autumn.

CLM predation and parasitism: temporal and spatial variations

Mortality rates inflicted by predators and parasitoids on CLM populations showed important temporal rather than spatial variations, with no significant effects of location being detected (table 1). There were significant differences between both mortality factors (table 1), with higher overall predation (21.68 ± 2.88%) than parasitism rates (15.26 ± 3.22%), and with a strong interaction between mortality factor and season (table 1, fig. 3). Thus, predation in summer and autumn was about three times higher than in spring and winter, whereas parasitism showed a pronounced peak in autumn (a ten-fold increase from spring), reaching values similar to those of predation; predation led to higher mortality than parasitism in spring and most strongly in summer when CLM density was highest (fig. 3).

The 2392 adult parasitoids reared from CLM belonged to two families: Eulophidae (three native species) and Encyrtidae (the introduced species *A. citricola*). The two most abundant parasitoid species were *A. citricola* and *C. neotropicus* Diez and Fidalgo, together accounting for over 99% of the specimens, while *Elasmus phyllocnistoides* Diez, Torrens & Fidalgo (0.75%) and *Galeopsomyia fausta* LaSalle (0.05%) were scarcely represented.

Parasitism rates of CLM by the two dominant species were similar in all the studied locations, but differed among seasons and between species, with a highly significant interaction between both factors (table 2). Parasitism by *A. citricola* (10.37 ± 2.76%) was on average higher than *C. neotropicus* (4.89 ± 0.67%) when means for all seasons were combined. However, both species varied along seasons in a different way, as indicated by the species x season interaction, revealing dominance of *A. citricola* parasitism in autumn and winter months, whereas *C. neotropicus* was responsible for most of the scarce parasitism recorded in spring (fig. 4).

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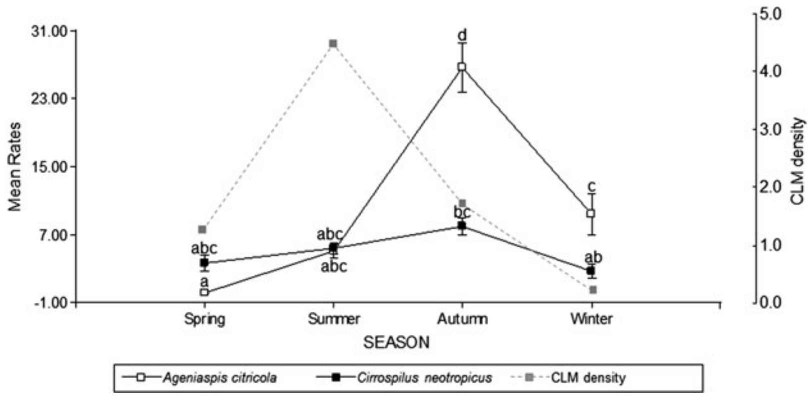

Fig. 4. Seasonal parasitism rates (%) by *A. citricola* and *C. neotropicus* on CLM, from 3-year sampling in NW Argentina. Data of the four locations were averaged and vertical bars denote SE (location effects were not significant according to GLMM, see table 2). Means accompanied by different letters are significantly different (GLMM contrast, $P < 0.05$). Average CLM density is also included for illustration.

Parasitism and predation rates vs. CLM density

Direct and highly significant relationships were detected when parasitism and predation rates were analyzed with regard to CLM density, indicating density-dependence for both mortality factors (fig. 5).

When parasitoid species were separately analyzed, only percentage of parasitism by the native species (*C. neotropicus*, *E. phyllocnistoides* and *G. fausta*) was significantly correlated with CLM density, whereas parasitism exerted by the introduced species *A. citricola* was independent of host density (table 3).

Discussion

Knowledge of the mortality factors affecting *P. citrella*, with emphasis on its natural enemies, is a basic step towards a sustainable management of this worldwide pest, for which biological control is considered the best long-term option (Sarada *et al.*, 2014). Here, by exploring temporal and spatial variations on populations of this pest on lemon plants in NW Argentina, we found that approximately half of CLM immatures failed to reach the adult stage, that predation was higher than parasitism having both a remarkably consistent impact across locations but showing different seasonal trends, and that the native parasitoid *C. neotropicus* revealed some favourable features as a potential biological control agent, such as density-dependence and earlier synchronization with the host in comparison with the introduced *A. citricola*.

Death from unknown causes accounted for more than half of the total mortality of CLM immatures.

Unexplained mortality is frequently high on leafminers (e.g., Amalin *et al.*, 2002; Lomeli-Flores *et al.*, 2009) and may include bottom-up effects linked to host plant defences, but also diseases, some degree of inadequacy of the species to local environmental conditions, intraspecific competition, unsuccessful parasitism and also 'host-feeding' by parasitoids (Auerbach *et al.*, 1995; Eber, 2004; Gripenberg & Roslin, 2008). Host-feeding behaviour could increase parasitoid impact on CLM populations (Zhang & Liu, 2008), and has actually been demonstrated for *C. neotropicus* in Brazil (Foelkel *et al.*, 2009). Thus, the impact of parasitoids on CLM populations in NW Argentina might be higher than shown by our parasitism data, an interesting possibility that deserves further study.

Predation rates were higher than those of parasitism in NW Argentina. Similar trends, with predation representing a stronger mortality factor than parasitism, have been observed in Spain and USA after relatively recent introductions of the CLM (Amalin *et al.*, 2002; Xiao & Fadamiro, 2010), in contrast with the lower predator action often associated with leafminers (Salvo & Valladares, 2007; Gripenberg & Roslin, 2008). Although a more accurate assessment of the true impact of each mortality factor would require a life-table approach (Lioni & Cividanes, 2004), adjusting the mortality rates to the specific resource density in each case, as explained in the methodology section, provides an adequate approximation for a species with multiple co-occurring generations. In this study, ants and lacewings were observed eating larvae and pupae of CLM, but the separate impact of these predators was not quantified.

The relatively low rates of parasitism here recorded are not in agreement with common trends for leafminers, in which parasitism often exceeds 50% (Langor *et al.*, 2000; Salvo & Valladares, 2007). The present results may be explained by the relatively short CLM exposure time to local parasitoids, given that native leafminers are usually heavily parasitized (Hawkins, 1994; Eber, 2004) while alien ones may be instead more preyed than parasitized (e.g., Grabenweger *et al.*, 2005; Xiao *et al.*, 2007). However, some invasive leafminer pests have shown remarkably high parasitism levels (Van der Walt *et al.*, 2009). It must be noticed that sampled orchards remained under conventional management, including applications of insecticides, as described in Materials and methods section. Insecticide applications appear to be ineffective to reduce CLM populations in the region (Diez *et al.*, 2006), but detrimental effects on predators and parasitoids cannot be ruled out, and such effects could underlie the low parasitism rates reported here.

Both predation as well as parasitism rates showed consistent seasonal patterns among locations distant up to 129 km from each other. Predation, with high levels during summer and autumn, provided earlier and more sustained pressure on CLM populations than parasitism, which instead showed wider seasonal variations, spanning an order of magnitude between its lowest values in spring and its peak in autumn. Slow building up of parasitoid populations, with highest parasitism

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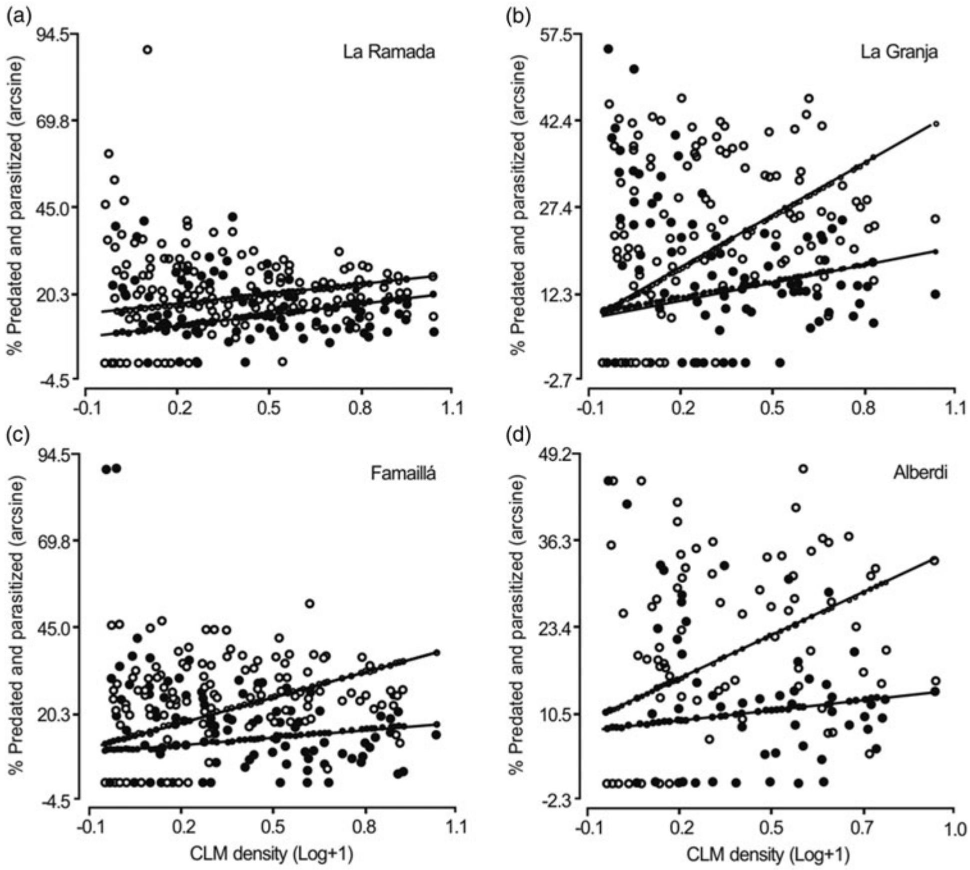

Fig. 5. Regression analysis between preyed (white points) and parasitized (black points) mines to the CLM density. Data from three consecutive citrus growing seasons: La Ramada (parasitism $y = 7.88 + 11.96x$, $R^2 = 0.10$; predation $y = 14.12 + 10.68x$, $R^2 = 0.05$), La Granja (parasitism $y = 9.06 + 10.06x$, $R^2 = 0.04$; predation $y = 8.74 + 32.27x$, $R^2 = 0.29$), Famaillá (parasitism $y = 8.91 + 7.86x$, $R^2 = 0.03$; predation $y = 11.15 + 25.25x$, $R^2 = 0.25$) and Alberdi (predation $y = 10.41 + 24.47x$, $R^2 = 0.20$).

Table 3. Results of regression analyses of parasitism rates (%) by introduced (*A. citricola*) and native parasitoids vs. CLM density.

Regression analysis	a^1	b	df	F	P	R^2
A. citricola parasitism						
La Ramada	3.44	7.67	162	10.38	0.0015	0.06
La Granja	4.75	3.75	161	1.51	0.22	0.01
Famailla	6.47	5.04	161	2.23	0.137	0.01
Alberdi	4.39	2.11	88	0.3	0.5842	0.003
Native parasitoids						
La Ramada	4.39	9.53	162	19.96	<0.0001	0.11
La Granja	3.99	11.81	161	21.47	<0.0001	0.12
Famailla	2.13	9.16	161	27.59	<0.0001	0.15
Alberdi	2.72	8.22	88	7.6	0.0071	0.07

[1] $y = a + bx$, where y = parasitism percentage, a = linear coefficient, b = quadratic coefficient, x = CLM density.

levels in autumn, when CLM populations begin to decrease, have been frequently recorded (Peña *et al.*, 1996; Ateyyat, 2002; Elekçioğlu & Uygun, 2013). Parasitism and predation in winter, even with extremely low populations of CLM, suggest high host finding capacity of the natural enemies (Zappalà & Hoy, 2004).

Despite the widely known advantages of host-specific natural enemies of the CLM (Sarada *et al.*, 2014), our results show a relevant performance of the native generalist parasitoid *C. neotropicus*. This species presented highly favourable features as a possible biological control agent, such as density-dependence, a trait often linked to population regulation capabilities (Hixon *et al.*, 2002). Another favourable attribute of *C. neotropicus* is its early activity, parasitizing CLM larvae in spring, at the onset of the annual cycle of CLM, whereas the introduced *A. citricola* showed a delayed population growth. Diez *et al.* (2006) also recorded an early activity of *C. neotropicus* on CLM populations. Moreover, relatively late action of *A. citricola* has also been observed in other regions (Amalin *et al.*, 2002), suggesting a delayed response to the increasing host density (Schowalter, 2006). Being a specific parasitoid of the CLM, *A. citricola* would find no alternative hosts during the unfavourable season, suffering a temporary population breakdown which takes a couple of generations to recover, resulting in delayed growth (Pomerinke & Stansly, 1998). Instead, the polyphagous native species *C. neotropicus* can survive on alternative hosts during the winter months. This situation also emphasizes the importance of adjacent vegetation near citrus crops as reservoirs of native parasitoids (Vivan *et al.*, 2003) and suggests a disadvantage

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of *A. citricola* specificity (Berti Filho & Ciociola, 2002; Symondson *et al*., 2002).

Significant relationships between CLM density and the proportion of preyed and parasitized individuals suggested certain degree of density-dependence, a desirable trait for natural enemies as regulatory agents (Auerbach *et al*., 1995). Nonetheless, such relationships were strongest and most consistent for predation, suggesting this factor could play an important regulatory role for the pest in the studied region. The high mortality rates caused by predation in comparison with parasitism reinforce this possibility. Relatively high correlation between the action of predators and CLM population was also observed by Amalin *et al*. (2002).

Our results provide new and needed information about natural control of the CLM, with an approximation to the effect of natural enemies on the pest and to their spatial and temporal fluctuations. From this assessment, predation emerged as an important top-down factor affecting the CLM, suggesting that predator presence should be encouraged in citrus orchards in the region. Also, from a management perspective, delayed action by *A. citricola* on CLM populations could be overcome by early augmentative releases in spring, although cautious studies about possible competition with native *C. neotropicus* would be needed. The role of the latter species deserves further study within the current trend favouring augmentative biological control with indigenous species (van Lenteren, 2011). Being well adapted to the ecological conditions prevailing in the region and having alternative hosts during the unfavourable season, *C. neotropicus* appears as an interesting candidate on which the possibility of mass-rearing for inundative releases should be evaluated. Finally, our study highlights the possibility of a complementary role for natural control agents with different phenology, which could prove advantageous in pest management programs.

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