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Native egg parasitoids on *Leptoglossus occidentalis* Heidemann (Hemiptera: Coreidae) in Spain: Potential biological control agents?

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Abstract

Leptoglossus occidentalis Heidemann (Heteroptera: Coreidae), commonly known as western conifer seed bug (WCSB), is an invasive pest in the Mediterranean, where it has been associated with the decline of *Pinus pinea* L. kernel production. A biological control of WCSB populations with native egg parasitoids may reduce economic losses. With the purpose to obtaining egg masses laid in situ bags with WCSB, adults were placed, for three consecutive years, in a clone bank, and for 1 year, in a pine forest in Central Spain. To estimate the impact of the parasitoid species on the WCSB population, four parasitism indexes were calculated. The WCSB activity period was estimated for two consecutive years in the clone bank through visual monitoring. Two native parasitoid species were found on the WCSB eggs: *Ooencyrtus pityocampae* (Mercet) (Hymenoptera: Encyrtidae) and *Ooencyrtus obscurus* (Mercet) (Hymenoptera: Encyrtidae). The latter parasitoid species represents a new finding for WCSB eggs. In the clone bank, *discovery efficiency* of the egg masses, *parasitism rate* of the eggs, *impact on host population* and *exploitation efficiency* of the egg masses were significantly higher for *O. obscurus* than for *O. pityocampae*. In the pine forest, parasitism index values were also higher for *O. obscurus*, but significant differences were not found by species. *Ooencyrtus obscurus* reduced the WCSB population by 6.2%, down to 19.6%, while *O. pityocampae* reduced it by 1.0%, down to 11.5%. Predated eggs were recorded in both experimental sites in low amounts. Both native parasitoid species could be proposed as biological control agents (BCA) in a biological control programme (BCP) in Europe after measuring their effectiveness with laboratory assessments.

KEYWORDS
activity period, *Ooencyrtus obscurus*, *Ooencyrtus pityocampae*, parasitism indexes, western conifer seed bug

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1 | INTRODUCTION

The western conifer seed bug (WCSB), *Leptoglossus occidentalis* Heidemann, (Hemiptera, Coreidae), is a pest of conifers, native to Western North America (Heidemann, 1910) that spread rapidly across Southern and Eastern North America (Gall, 1992; Koerber, 1963) and many other countries around the world (Olivera et al., 2020). This can be attributed to its wide host range (i.e. more than 40 conifer species) (Fent & Kment, 2011) and anthropogenic activities (Kim et al., 2020). The latest records show that the WCSB continues to move across Central and South America, Africa (Faúndez et al., 2017; Faúndez & Silvera, 2019; Kun & Masciocchi, 2019; Van der Heyden & Faúndez, 2020), Europe (Lesieur et al., 2019) and Asia (Kim et al., 2020). The phenology of the WCSB depends on environmental conditions (Barta, 2016; Tamburini et al., 2012) and is characterized by overwintering in large aggregations in sheltered locations (Blatt, 1994). Eggs are laid in rows on conifer needles, and the nymphs pass through five instars to reach adulthood (Koerber, 1963). All WCSB stages feed mainly on conifer cones at all phenological phases (Pasek & Dix, 1988) causing their abortion in the early development stages (Connelly & Schowalter, 1991) or seed damage in ripe cones, which may result in the absence of its germination (Bates et al., 2001,2002; Lesieur et al., 2014). In the Mediterranean, the recorded declines in the kernel-per-cone yield of *Pinus pinea* L. in the last decade have been associated with the WCSB invasion, which has also been reported through the analysis of kernel damages (Calama et al., 2017; Farinha et al., 2018,2021; Roversi, Strong, et al., 2011). In this regard, *P. pinea* kernels are considered a gourmet forest product with a high commercial price (Awan & Pettenella, 2017; Calama et al., 2020; Mutke et al., 2011), whose economic sector is suffering significant economic losses as a result of the WCSB adaptation to Mediterranean stone pine forests (Mutke et al., 2017; Parlak, 2017). Sustainable control methods to reduce the damage caused by this pest have yet to be described (Blatt & Borden, 1996; Blatt et al., 1998; Kegley et al., 2001). One possible control option is a biological control that allows for the reduction in the pest populations through biological control agents (BCA) such as parasitoids, predators or pathogens (Beddington et al., 1978; Orr, 2009). This option avoids the use of chemicals which are not sustainable for the environment, human health and wildlife (Baker et al., 2020). In the WSCB native area, three egg parasitoids from the genera *Anastatus*, *Ooencyrtus* and *Gryon* have been recorded on WCSB eggs, where the parasitoid species G*ryon pennsylvanicum* (Ashmead) (Hymenoptera: Platygastridae) is the predominant one (Bates & Borden, 2004; Maltese et al., 2012). This parasitoid species has been characterized in laboratory trials as a potential BCA in classical biological control programmes (BCP) for the WCSB populations in Europe (Peverieri et al., 2012, 2013; Roversi, Strong, et al., 2011). However, BCPs based on inundative or conservative methods (Eilenberg et al., 2001) could be proposed with the WCSB egg parasitoid community native to Europe, which is influenced by the environmental conditions, type of vegetation, main host populations and its densities (Costi et al., 2018). In Italy and France, different native parasitoid communities formed by the genera *Anastatus*, *Baryscapus* and *Ooencyrtus* have been associated with WCSB eggs (Lesieur & Farinha, 2021; Niccoli et al., 2009; Roversi et al., 2011). The aims of this study were (I) to survey and identify the native egg parasitoid community of the WCSB in Central Spain and (II) to estimate its impact on the WCSB population through four parasitism indexes.

2 | MATERIAL AND METHODS

2.1 | Experimental sites

Trials were conducted in two sites: the clone bank 'El Molinillo', located in Tordesillas (Valladolid, Spain; latitude: 337. 458; longitude: 4.596.035 ETRS89, UTM 30N), and in a *P. pinea* reforested pine forest (M.U.P. 17) located in Nava del Rey (Valladolid, Spain; latitude: 328.547; longitude: 4.590.594, ETRS89, UTM 30N). Two plots were selected in the clone bank, one consisting of 144 grafted *P. pinea* and the other of 138 grafted *P. pinea*. All pines were 15 years old, had a 6 × 6 m orchard density and measured 1 to 3 metres high.

In the pine forest, one plot of 100 × 100 metres was selected. Pines were 15 years old with a circumference of 3 × 6 m and a height between 1.5 and 3 metres.

2.2 | Laboratory colony

Starting in 2014, a permanent WCSB colony was reared in the laboratory at the Department of Vegetal Production and Forest Resources (University of Valladolid, Palencia, Spain) using insects collected by forestry agents in the region and by ourselves in the clone bank. All WCSB used in the field trials were from the laboratory colony, which was kept at standard laboratory conditions (21 ± 2°C; RH 40%) and natural daylight into rectangular breeding boxes (47.5 × 47.5 × 93 cm, 160 micron mesh) (Entomopraxis, Barcelona, Spain). Ad libitum shelled *P. pinea* kernels were provided as food, while young potted *Pinus halepensis* Mill. (2–3 years old) were provided a source of water and ovipositional substrate.

2.3 | Parasitism field trials

Parasitism trials were conducted from May/early June to September for three consecutive years (2017, 2018 and 2019) in the clone bank and for 1 year in the pine forest (2019).

Two females plus one male were caged in a mesh bag (40 × 30 cm, grey polyethylene, 1 × 1 mm mesh) which was placed around a pine branch (approx. 30 cm long) to collect the egg masses laid in situ. The bags were implemented with a Velcro side opening

TABLE 1 Number of bags with two WCSB females and one male to obtain egg masses laid in situ placed in a clone bank (2017, 2018 and 2019) and in a pine forest (2019) in Central Spain

Trial	May 1st wk	May 2nd wk	May 3rd wk	May 4th wk	June 1st wk	June 2nd wk	June 3rd wk	June 4th wk	July 1st wk	July 2nd wk	July 3rd wk	July 4th wk	Aug 1st wk	Aug 2nd wk	Aug 3rd wk	Aug 4th wk	Aug 5th wk	Total/year
2017 clone bank					10				10			10			10			40
2018 clone bank						4		4			3		5	5	5			26
2019 clone bank			5	5														10
2019 pine forest					2	5		3			1	1						12
Total/month				10				28				25			25			88

to allow the search of the egg masses. Ad libitum shelled *P. pinea* kernels were provided as food. The number of bags and set dates depended on the availability of WCSB adults in the laboratory colony, varying from 22 to 40 per year (Table 1). Bags were checked weekly, dead adults were replaced, and egg masses were identified and dated. Egg masses were exposed to the parasitoids by removing the bag to avoid any physical barriers (Costi et al., 2018). The removed bags were placed on another branch of the same tree. The egg masses were exposed for 1 week. Then, they were collected and placed in a glass tube (diameter 1.5 cm, 10 cm long) which was closed with a cotton wool cover, kept at laboratory conditions and was exposed to natural daylight. Parasitoids found on the surface of the egg masses laid in situ without parasitoid emergence holes were collected to identify them. Each collected egg was codified and categorized under a stereomicroscope (Nikon SMZ 745T) (Figure S1). *Fertile eggs* were characterized by a smooth chorion, and light brown colour when they were freshly laid or more intense reddish-brown colour depending on the nymph development. *Hatched eggs* were also included in this category (Figure S1b). *Non-fertile eggs* were characterized by a dried/dehydrated or collapsed chorion, which was a pale yellow colour, and also by the absence of nymph development. *Aborted eggs* were distinguished by a deep red colour, and in some cases, by the presence of red dots, lines or the nymph carcass under the chorion. *Predated eggs* were characterized by the absence of lumen and by a partial and chewed chorion. *Parasitized eggs* showed a characteristic ashen colour, stalks on the chorion (tubes that connect the parasitoid ovipositor with the lumen of the host egg (Maple, 1947)), and black dots and lines and/or detritus under the chorion depending on the parasitization development. Hatched nymphs were collected and added to the WCSB laboratory colony. Parasitoid emergences were checked daily in the laboratory. A sample of parasitoid specimens with different morphological characteristics was sent to The Natural History Museum (NHM, London, UK) to determine the species. All parasitoids were identified according to Mercet (1921) under a stereomicroscope. Parasitoid specimens were stored in the NHM and in the Department of Vegetal Production and Forest Resources (University of Valladolid, Palencia, Spain).

Parasitized eggs without parasitoid emergence holes were kept at laboratory conditions and were exposed to natural daylight for 45 days to test the parasitoid diapause period (Anderson & Kaya, 1974). These eggs were then dissected and classified into parasitized eggs without parasitoid development but with either a granulated translucent mass (Maple, 1947) or dead larvae or dead parasitoid.

2.4 | WCSB activity period

The WCSB field population was monitored weekly in the two clone bank plots for two consecutive years, from early June to late October in 2018 and from mid-May to late September in 2019. The WCSB observation consisted of a visual inspection of all lower cones and branches (max. height 2 m) of all trees (2018:274 trees; 2019:280

trees), except for those used in the parasitism trials. WCSB instars were recorded, and naturally occurring egg masses were collected, kept and categorized as described for the egg masses laid in situ.

2.5 | Data analysis

Egg masses were classified into unparasitized and parasitized, while eggs were grouped in unhatched (non-fertile plus aborted eggs), viable (fertile plus parasitized eggs) and non-viable eggs (unhatched plus predated eggs), all per week and trial (Table 2).

Parasitized egg masses and eggs were sorted by species according to that of the emerged parasitoids. Parasitized eggs without parasitoid emergence were considered parasitized by the same species as the eggs that were parasitized within the same mass from which parasitoids emerged. This was considered because no egg masses parasitized by both parasitoid species were simultaneously recorded.

To estimate the impact of the native egg parasitoids on the WSCB population, we used four parasitism indexes (Colazza & Bin, 1995; Costi et al., 2018): *Discovery efficiency index*, or the ability of the parasitoid species to find egg masses in field, was determined as the number of parasitized egg masses divided by the total number of egg masses; *Parasitism rate* was determined as the number of parasitized eggs divided by the total number of eggs; *Impact on host population index* (modified), or the ability of the parasitoid species to reduce the pest population, was determined as the number of parasitized eggs divided by the number of viable eggs, that is eggs capable of producing host populations; and *Exploitation efficiency index*, or the ability to completely or partially parasitize an egg mass, was determined as the number of parasitized eggs divided by the total number of eggs within the parasitized egg mass. The four indexes were expressed in percentages by parasitoid species and by trial.

Parasitoid *sex ratio* (% females) was calculated by dividing the emerged females by the emerged males. The *emergence ratio* was calculated by dividing the total emerged parasitoids by the number of parasitized eggs with parasitoid emergence holes. Both ratios were determined by parasitoid species and by trial.

The *unhatched eggs rate* and the *predation rate* were calculated regarding the total number of eggs and were expressed in percentage by trial.

Discovery efficiency index, *parasitism rate*, *impact on host population index* and *exploitation efficiency index* were analysed using a generalized linear mixed model with binomial distribution and logit link function with the event/trial syntax (GLIMMIX procedure in the SAS software). The egg masses were the experimental units for the first three indexes, and the parasitized egg masses were the experimental units for the final one. The parasitoid species was the within-subject factor, and the trials (2017 clone bank, 2018 clone bank, 2019 clone bank and 2019 pine forest) were the levels of the between-subject factor. The comparison between parasitoid species and trials was performed using a *t* test at a 0.05 significance level for the estimated logits in the model. An unstructured random covariance matrix (UN)

was used in the models. Generalized chi-square tests were used to test the main effects and the interaction effect in the model.

Predation rate and *unhatched eggs rate* were also analysed using a generalized linear mixed model with binomial distribution and logit link function with the event/trial syntax. Only the trial factor was considered, and comparisons were also performed with *t* tests. The *emergence ratio* was analysed with a linear mixed model (MIXED procedure in the SAS software) with a between-subject factor (trials) and a within-subject factor (species). The compound symmetry (CS) variance structure was used, and all the comparisons were performed with a *t* test at a 0.05 significance level.

A statistical analysis was performed with the software SAS 9.4 from SAS Institute Inc., Cary, NC, USA (http://www.sas.com, Statistical Analysis System, RRID:SCR_008567).

3 | RESULTS

3.1 | Parasitism field trials

Two native parasitoid species of WCSB eggs were identified: *Ooencyrtus obscurus* (Mercet) (Hymenoptera: Encyrtidae) and *Ooencyrtus pityocampae* (Mercet) (Hymenoptera: Encyrtidae).

The number of WCSB egg masses and eggs varied by week and by trial (Table 2). In the clone bank, the mean percentage (95% CL) of the total parasitized egg masses (*discovery efficiency index*) was 26.2% (23.1, 29.5) in 2017, 9.1% (6.6, 12.5) in 2018 and 9.7% (5.6, 16.2) in 2019. At the egg level (*parasitism rate*), the mean percentage (95% CL) was 15.1% (12.9, 17.6), 3.3% (2.2, 4.8) and 3.8% (1.9, 7.5), for the three consecutive years. The statistical analysis showed significant differences for both parasitism levels between 2017 and 2018 but not between 2018 and 2019 (egg mass level: 2017 vs. 2018: *t-value* = 6.37 with *p-value* = <.0001; 2017 vs. 2019: *t-value* = 3.84 with *p-value* = .0001; egg level: 2017 vs. 2018: *t-value* = 7.52 with *p-value* = <.0001; 2017 vs. 2019: *t-value* = 3.98 with *p-value* = <.0001). In the pine forest (2019), the overall mean percentage (95% CL) of the egg masses was 9.9% (5.2, 18.2) and of the eggs was 5.4% (2.7, 10.4). In that year, no significant differences were found for both overall indexes by sites.

The mean percentages (95% CL) of the four indexes by parasitoid species and by trial are given in Table 3. Considering all trials in the clone bank, the mean percentage (95% CL) of the egg masses (*discovery efficiency index*) parasitized by *O. obscurus* was significantly different from those parasitized by *O. pityocampae*: 27.3% (23.6, 31.3) and 6.1% (4.0, 9.3) respectively (*t-value* = 6.67 with *p-value* = <.0001). Comparing by trial, the *discovery efficiency index* was significantly higher for *O. obscurus* compared with *O. pityocampae* in the clone bank (2017: *t-value* = 3.72 with *p-value* = .0002; 2018: *t-value* = 3.56 with *p-value* = .0004; 2019: *t-value* = 4.63 with *p-value* = <.0001). In the pine forest, *O. obscurus* continued to have a higher index value but significant differences were not found. Comparing this index by parasitoid species and site in 2019, significant differences were only found between sites for the parasitoid species *O. obscurus*

TABLE 2 Number of egg masses and eggs laid *in situ* by category and week obtained in a clone bank (2017, 2018 and 2019) and in a pine forest (2019) in Central Spain

Weeks	N egg masses				N eggs								
					N viable				N non-viable				
									N unhatched				
	N unparasitized	N parasitized by *O. obscurus*	N parasitized by *O. pityocampae*	Total	N fertile	N parasitized by *O. obscurus*	N parasitized by *O. pityocampae*	Total viables	N non-fertile	N aborted	N predated	Total no viables	Total
2017 clone bank trial													
July wk 1	3	0	0	3	25	0	0	25	9	1	0	10	35
July wk 2	8	0	0	8	24	0	0	24	12	0	2	14	38
July wk 3	20	7	0	27	87	12	0	99	58	2	2	62	161
July wk 4	16	7	0	23	61	13	0	74	21	2	4	27	101
Aug. wk 1	13	15	0	28	72	44	0	116	20	2	0	22	138
Aug. wk 2	11	10	0	21	54	19	0	73	18	4	2	24	97
Aug. wk 3	6	3	3	12	51	7	4	62	2	0	0	2	64
Aug. wk 4	10	24	13	47	47	73	37	157	53	3	1	57	214
Aug. wk 5	19	12	9	40	59	43	28	130	78	1	1	80	210
Sept. wk 1	4	13	13	30	18	35	37	90	26	0	0	26	116
Sept. wk 2	4	0	9	13	16	0	39	55	3	2	0	5	60
Sept. wk 3	2	0	0	2	15	0	0	15	1	1	0	2	17
Sept. wk 4	1	0	0	1	0	0	0	0	5	0	0	5	5
Total	117	91	47	255	529	246	145	920	306	18	12	336	1256
2018 clone bank trial													
June wk. 4	1	0	0	1	10	0	0	10	0	1	0	1	11
July wk. 1	5	0	0	5	34	0	0	34	3	2	1	6	40
July wk. 2	5	0	0	5	35	0	0	35	3	1	0	4	39
July wk. 3	13	0	0	13	65	0	0	65	10	0	0	10	75
July wk. 4	10	0	0	10	62	0	0	62	1	1	17	19	81
Aug. wk 1	20	2	0	22	79	2	0	81	63	2	21	86	167
Aug. wk 2	16	3	1	20	144	12	1	157	18	3	2	23	180
Aug. wk 3	23	7	1	31	102	13	6	121	66	8	4	78	199
Aug. wk 4	27	16	6	49	115	36	11	162	80	22	18	120	282
Aug. wk 5	16	4	0	20	43	13	0	56	47	7	4	58	114
Sept. wk 1	11	3	0	14	35	8	0	43	28	6	7	41	84
Sept. wk 2	10	0	2	12	10	0	5	15	38	0	0	38	53
Sept. wk 3	7	0	0	7	7	0	0	7	15	0	0	15	22
Total	164	35	10	209	741	84	23	848	372	53	74	499	1347
2019 clone bank trial													
June wk. 3	7	0	0	7	48	0	0	48	3	4	0	7	55
June wk. 4	13	0	0	13	52	0	0	52	7	1	7	15	67
July wk. 1	12	5	1	18	74	9	1	84	2	5	0	7	91
July wk. 2	14	5	1	20	69	9	2	80	10	6	4	20	100
July wk. 3	2	3	0	5	3	8	0	11	5	0	0	5	16

TABLE 2 (Continued)

July wk. 4	3	5	0	8	16	8	0	24	2	0	0	2	26
Aug. wk 1	1	6	0	7	18	13	0	31	16	1	0	17	48
Aug. wk 2	6	7	0	13	20	13	0	33	15	10	5	30	63
Aug. wk 3	12	4	1	17	46	9	3	58	13	10	0	23	81
Aug. wk 4	3	3	0	6	5	10	0	15	11	0	0	11	26
Aug. wk 5	2	2	0	4	11	4	0	15	5	0	1	6	21
Sept. wk 1	3	0	0	3	6	0	0	6	1	0	0	1	7
Sept. wk 2	3	0	0	3	2	0	0	2	5	1	0	6	8
Sept. wk 3	1	0	0	1	0	0	0	0	2	0	0	2	2
Total	82	40	3	125	370	83	6	459	97	38	17	152	611
2019 pine forest trial													
July wk. 1	3	0	2	5	14	0	5	19	0	1	19	20	39
July wk. 2	2	0	0	2	11	0	0	11	0	0	0	0	11
July wk. 3	0	2	0	2	2	6	0	8	1	0	0	1	9
July wk. 4	6	0	0	6	31	0	0	31	1	2	1	4	35
Aug. wk 1	6	1	0	7	18	6	0	24	2	1	2	5	29
Aug. wk 2	3	1	0	4	15	2	0	17	0	2	0	2	19
Aug. wk 3	10	1	0	11	39	2	0	41	8	4	1	13	54
Aug. wk 4	2	1	1	4	10	1	3	14	2	0	0	2	16
Aug. wk 5	2	0	0	2	6	0	0	6	0	0	0	0	6
Total	34	6	3	43	146	17	8	171	14	10	23	47	218

(t-value = 2.20 with p-value = .0285). Taking the egg as the unit (*parasitism rate*), the overall mean amount (95% CL) in the clone bank was also significantly higher for *O. obscurus*, 12.0% (10.0, 14.3), compared to *O. pityocampae*, 2.7% (1.8, 4.2) (t-value = 6.13 with p-value = <.0001). Comparing by trials, the *parasitism rate* for *O. obscurus* was significantly higher than for *O. pityocampae* in all clone bank trials (2017: t-value = 2.88 with p-value = .0042; 2018: t-value = 2.93 with p-value = .0032; 2019: t-value = 3.47 with p-value = .0006), but not in the pine forest trial. In 2019, no significant differences were found for this index comparing by parasitoid species and sites. The *impact on host population* caused by *O. obscurus* was significantly higher than that caused by *O. pityocampae* in the clone bank for all years (2017: t-value = 2.80 with p-value = .0054; 2018: t-value = 2.81 with p-value = .0051; 2019: t-value = 3.30 with p-value = .0010). In 2019, no significant differences were detected when comparing the two sites by species. The *exploitation efficiency index* was also significantly higher for *O. obscurus* than for *O. pityocampae* in all clone bank trials (2017: t-value = 2.77 with p-value = .0061; 2018: t-value = 2.82 with p-value = .0052; 2019: t-value = 3.41 with p-value = .0008), but not in the pine forest trial. Significant differences were also not found when we compared by parasitoid species and by site in 2019.

The parasitoid activity period on WCSB eggs was estimated through the number of WCSB parasitized eggs by species and by week (Figure 1). With all trials in the clone bank, the parasitoid activity period on WCSB eggs of *O. obscurus* was recorded from early July to early September and from early July to mid-September for *O. pityocampae*. In the pine forest, the parasitoid activity period on

WCSB eggs was intermittent for both species; for *O. obscurus*, this was recorded from mid-July to late August, while for *O. pityocampae* it was recorded from early July to late August. In the 2017 trial, parasitoid activity peaks on WCSB eggs were reported in the 1st, 4th and 5th weeks of August for *O. obscurus*, while for *O. pityocampae* the parasitoid activity on WCSB eggs was focussed from the 4th week of August to the 2nd week of September. In the 2018 trial, the parasitoid activity period peak on WCSB eggs for both species was reported in the 4th week of August. In the 2019 trials, the parasitoid activity on WSCB eggs of both species was less intensive. For *O. obscurus*, it was focussed in the two first weeks of August in the clone bank and in the 3rd week of July and the 1st week of August in the pine forest, while for *O. pityocampae* it was focussed in the 3rd week of August in the clone bank and the 1st week of July in the pine forest.

Several parasitoids were collected while perched on the WCSB egg masses laid in situ in the clone bank in 2017 and 2018 (Figure 1). No parasitoids were collected on egg masses with parasitoid emergence holes. All parasitoids were females except for one for both *O. obscurus* collected in the 3rd week of August 2018.

No diapause period was detected in WCSB eggs for any parasitoid species. The number of parasitoids emerged, their *sex ratio* and *emergence ratio* varied by parasitoid species and by trial (Table 4). The maximum *emergence ratio* was five individuals/eggs for *O. obscurus* and four for *O. pityocampae*. For this ratio, no significant differences were found between species by trial.

The mean percentage (95% confidence limits) of *unhatched eggs rate* in the clone bank was 25.8% (23.5, 28.3) in 2017, 31.6%

(29.2, 34.1) in 2018 and 22.1% (19.0, 25.7) in 2019, being significantly different in 2017 compared with 2018 (t-value = −3.22 with p-value = .0013) and in 2018 compared with 2019 (t-value = 4.15 with p-value = <.0001). In the pine forest, this percentage was significantly lower (11.0% (7.5, 16.1)) than in the clone bank for the same year (2019) (t-value = 3.37 with p-value = .0008). The amount of parasitized eggs without emergence holes for the total of parasitized eggs was 10.7% (42 eggs) in 2017:22 eggs in egg masses with other eggs parasitized by *O. obscurus*, and the rest in egg masses parasitized by *O. pityocampae*. The dissection of these eggs showed 12 aborted parasitoids, 28 aborted larvae and four eggs with a granulated translucent mass associated with parasitism (Maple, 1947). In 2018, only three eggs were dissected (2.8%): two with a granulated translucent mass and one egg with one aborted larva; all inside egg masses parasitized by *O. obscurus*. In 2019, four eggs had one aborted larva and another egg had a granulated translucent mass (5.6%); all within egg masses parasitized by *O. obscurus*. Finally, in the pine forest (2019), two eggs had one aborted larva each (8.0%). One was recorded in an egg mass parasitized by *O. obscurus* and the other in an egg mass parasitized by *O. pityocampae*.

Concerning the *predation rate*, the mean percentage (95% CL) varied from 1.0% (0.5, 1.7) in 2017 to 5.5% (4.4, 6.9) in 2018 and 2.8% (1.7, 4.5) in 2019 in the clone bank, being significantly different between all years (2017 vs. 2018: t-value = −5.67 with p-value = <.0001; 2017 vs. 2019: t-value = −2.86 with p-value = .0044; 2018 vs. 2019: t-value = 2.57 with p-value = .0103). In the pine forest, this percentage was higher (10.6% (7.2, 15.6)) and significantly different than that of the clone bank in 2019 (t-value = −4.30 with p-value = <.0001).

3.2 | WCSB activity period

The number of WCSB nymphs and adults recorded in 2018 (nymphs: 525; adults: 183) was higher than in 2019 (nymphs: 257; adults: 53). The beginning of the WCSB activity was recorded in late May (2018) and early June (2019). Eggs, all nymph instars and adults were recorded in different amounts along the weeks (Figure 2).

In 2018, four naturally occurring egg masses were collected of which two were parasitized: one with eight hatched eggs and three parasitized eggs (27.3%) by *O. obscurus* (4th week of August 2018), and another with one non-fertile egg and two parasitized eggs (66.7%) by *O. obscurus* (5th week of August 2018). In 2019, three naturally occurring egg masses were collected, of which none were parasitized.

4 | DISCUSSION

4.1 | Parasitism field trials

To our knowledge, this is the first native parasitoid study conducted on WCSB eggs in Spain. Two parasitoid species have been found in the 3 years surveyed (2017–2019) and in both experimental sites:

TABLE 3 Mean percentage (95% confidence limits) of discovery efficiency index, parasitism rate, impact on host population index and exploitation efficiency index by species and trial

Trial	Discovery efficiency index (%)		Parasitism rate (%)		Impact on host population index (%)		Exploitation efficiency index (%)	
	O. obscurus	O. pityocampae	O. obscurus	O. pityocampae	O. obscurus	O. pityocampae	O. obscurus	O. pityocampae
2017 clone bank	35.7 (30.0, 41.8)a	18.4 (14.1, 23.7)b	19.6 (16.0, 23.8)a	11.5 (8.7, 15.1)b	26.7 (21.8, 32.3)a	15.8 (11.8, 20.8)b	35.3 (29.7, 41.4)a	20.8 (15.8, 27.0)b
2018 clone bank	16.8 (12.3, 22.5)a	4.8 (2.6, 8.7)b	6.2 (4.3, 9.0)a	1.7 (0.8, 3.5)b	9.9 (6.8, 14.3)a	2.7 (1.2, 5.8)b	25.1 (18.2, 33.5)a	6.9 (3.3, 13.9)b
2019 clone bank	32.0 (24.4, 40.7)a	2.4 (0.8, 7.2)b	13.6 (9.5, 19.1)a	1.0 (0.2, 4.0)b	18.1 (12.5, 25.5)a	1.3 (0.3, 5.8)b	38.8 (28.7, 50.0)a	2.8 (0.6, 11.5)b
2019 pine forest	14.0 (6.3, 28.0)a	7.0 (2.2, 19.8)a	7.8 (3.4, 16.9)a	3.7 (1.1, 12.0)a	9.9 (4.2, 21.7)a	4.7 (1.2, 16.0)a	32.7 (15.7, 55.9)a	15.4 (4.3, 42.4)a

Note: Means in the same row for index followed by the same letter are not significantly different (t test; p-value <.05).

O. pityocampae and *O. obscurus*. This latter parasitoid species constitutes a new host–parasitoid association.

Both *Ooencyrtus* species are native to Spain and are generalists (Mercet, 1921). *Ooencyrtus pityocampae* is recorded in 50 species belonging to 9 families and 2 orders, and its primary host is the moth *Thaumetopoea pityocampa* Denis & Schiffermüller (Lepidoptera: Notodontidae). This parasitoid species emerges 1 month before its main host (Battisti, 1989), at the end of July in the clone bank

(L. Ponce, personal observation), when it can parasitize secondary hosts (Battisti et al., 1988). *Ooencyrtus obscurus* has fewer known hosts than *O. pityocampae* (4 species of 4 families and 2 orders) (Huang & Noyes, 1994), and to our knowledge, its main host has not yet been defined. In Spain, this species was recorded in eggs of *Hipparchia statilinus* Hufnagel (Lepidoptera: Nymphalidae) (García-Barros, 1989), butterfly species that oviposits on the grass of pine and holm oak forests (Aguado Martin, 2007).
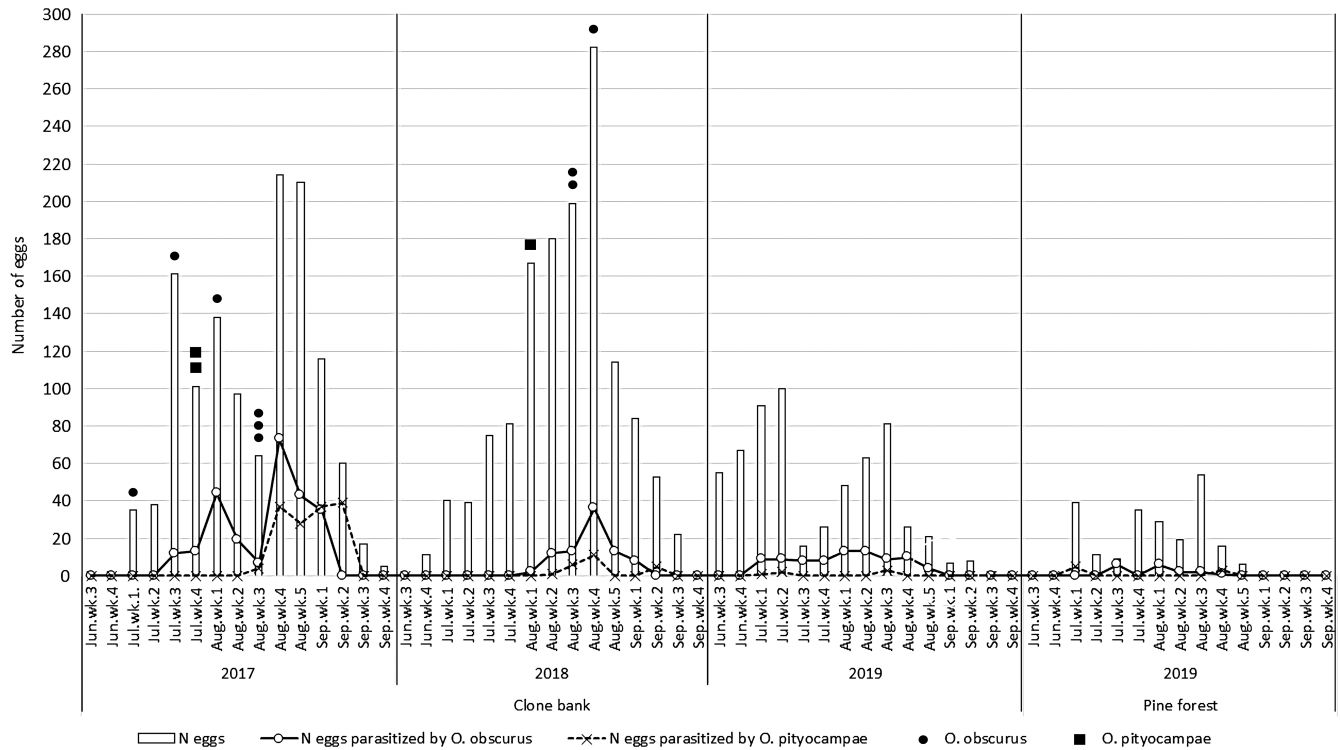
FIGURE 1 Parasitoid activity period of *O. obscurus* and *O. pityocampae* in 2017, 2018 and 2019 in the clone bank and in 2019 in the pine forest experimental sites, estimated through the number of parasitized eggs by *O. obscurus* and by *O. pityocampae* compared with the total number of WCSB eggs laid in situ (bar) per week, and natural population recorded on egg masses laid in situ by species *O. obscurus* (●) and *O. pityocampae* (■)

TABLE 4 Number of emerged parasitoids (males, females and total) from WCSB eggs parasitized in field

Trial	Species	N parasitoids emerged			Sex ratio	Emergence ratio
		Males	Females	Total		
2017 clone bank	*O. obscurus*	69	295	364	1:4	1.6
	O. pityocampae	5	202	207	1:40	1.6
2018 clone bank	*O. obscurus*	32	130	162	1:4	1.7
	O. pityocampae	0	39	39	--	1.9
2019 clone bank	*O. obscurus*	20	111	131	1:6	1.5
	O. pityocampae	0	7	7	--	1.2
2019 pine forest	*O. obscurus*	7	31	38	1:4	2.3
	O. pityocampae	0	11	11	--	1.3
Total	*O. obscurus*	128	567	695	1:4	1.7
	O. pityocampae	5	259	264	1:40	1.5

Note: Sex ratio (males/females) and emergence ratio (*N* parasitoids emerged/egg) by species and trial.

In the WCSB native area, the WCSB egg parasitoid community is composed of *G. pennsylvanicum*, *Anastatus pearsalli* Ashmead (Hymenoptera: Eupelmidae) and *Ooencyrtus johnsoni* (Howard) (Hymenoptera: Encyrtidae) (Bates & Borden, 2004; Maltese et al., 2012). In Europe, the genera *Anastatus* and *Ooencyrtus* have also been associated with the WCSB in egg observations conducted in Italy and France (Lesieur & Farinha, 2021; Niccoli et al., 2009; Roversi, Santini, et al., 2011), where the WCSB egg parasitoid community varies by experimental site. In Italy, the generalist parasitoid species *O. pityocampae* and *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae) were recorded together with *Baryscapus servadeii* Domenichini (Hymenoptera: Chalcidoidea), a parasitoid species of *Thaumatopoea* spp. eggs (Noyes, 2021), in a *Pinus pinaster* Ait forest (Roversi, Santini, et al., 2011). In a *Pinus nigra* J.F. Arnold forest from the same country, only the parasitoid species *A. bifasciatus* was recorded (Niccoli et al., 2009). In a French *P. nigra* forest, *A. bifasciatus* and *O. pityocampae* were also reported together with the parasitoid *Ooencyrtus telenomicida* Vassiliev (Hymenoptera: Encyrtidae) (Lesieur & Farinha, 2021). It is remarkable that in nearby Mediterranean countries, the parasitoid community varies with the environmental conditions (Costi et al., 2018) and that only the generalist parasitoid species *O. pityocampae* has been recorded in all observation sites, including Central Spain stone pine forests.

In terms of parasitism, the *parasitism rate* values of the parasitoid community in Central Spain were lower than those recorded in sentinel egg masses in the WCSB native area, which varied from 6%

(Maltese et al., 2012) to 32.7% (Bates & Borden, 2004), except in the 2017 clone bank trial. In that area, the amount of parasitized eggs laid in situ was also higher than in all Central Spain trials, reaching 57% (Maltese et al., 2012). In Italy, no parasitized WCSB eggs laid in situ were found (Maltese et al., 2011) and the *parasitism rate* levels on WCSB sentinel eggs were lower (< 1% sentinel eggs hatched) than those recorded in all trials from Central Spain (Roversi, Santini, et al., 2011). In France, the global *parasitism rate* values on naturally occurring egg masses (2016:8.2%; 2017:5.9%) were higher than those recorded in Central Spain in 2018 in the clone bank and in 2019 in both experimental sites, while the global *parasitism rate* values on sentinel egg masses (2016:1.6%; 2017:1.3%) were lower than in all Spain trials. For egg masses, the *discovery efficiency index* values of the parasitoid community in Central Spain were higher than in France for sentinel egg masses (2016:5.6%; 2017:5.7%) but not for natural egg masses (2016:24.8%; 2017:11.4%), excluding the 2017 clone bank trial (Lesieur & Farinha, 2021). The use of natural, sentinel or laid in situ egg masses may influence the trial results. Several egg parasitoids are attracted by the volatiles of the adult host, such as *G. pennsylvanicum*, which is attracted by the adult kairomones of *Leptoglossus australis* (F.) (Hemiptera: Coreidae) (Yasuda & Tsurumachi, 1995), and many *Ooencyrtus* species that are attracted by adult host pheromones (Battisti, 1989; Leal et al., 1995; Peri et al., 2011). Additionally, the parasitism levels in the observation sites may be influenced by the presence of the parasitoid main host and its abundance (Costi et al., 2018), the density dependence host–parasitoid relations (Van den Bosch et al., 1982), and the fact that the
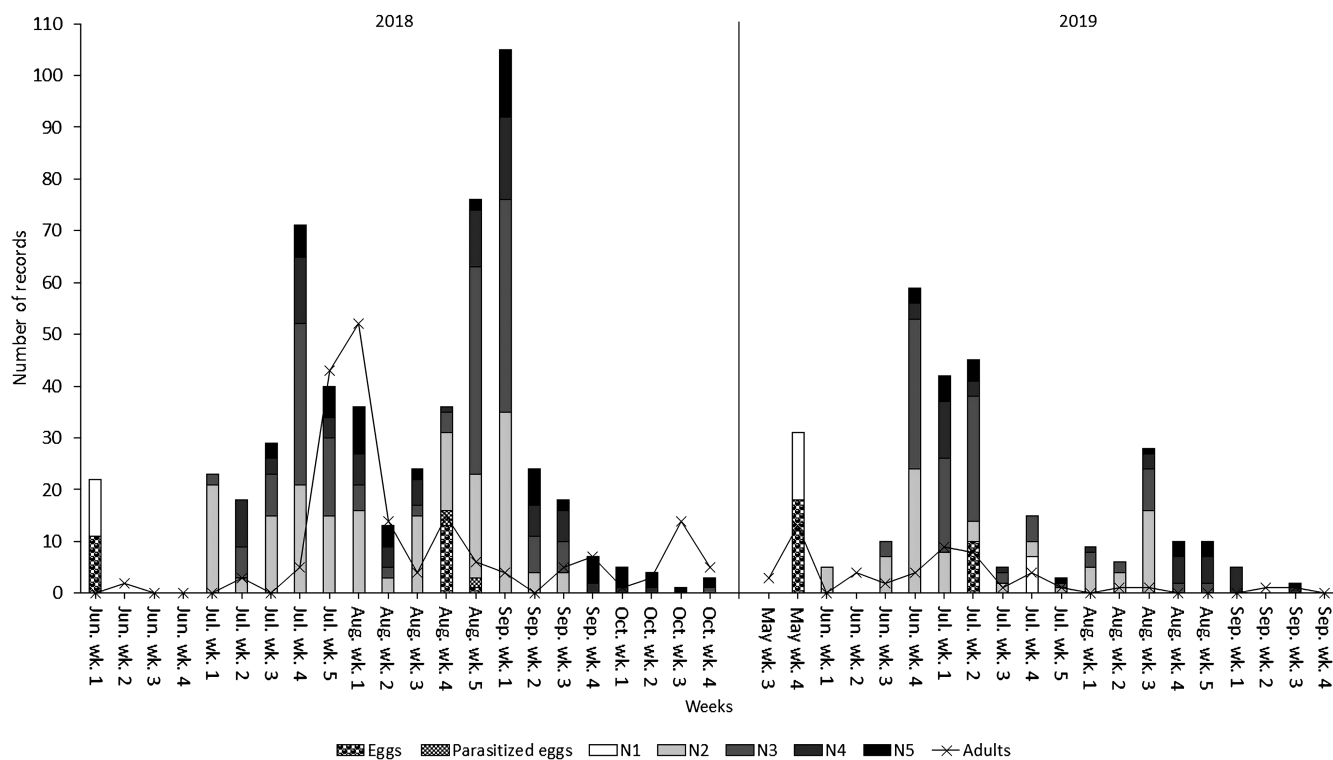

FIGURE 2 Records of WCSB eggs, parasitized eggs, nymphs per instar (N1, N2, N3, N4 and N5), and adults in low branches and cones than were visually monitored weekly in two plots of a clone bank in Central Spain in 2018 and 2019

impact of the native egg parasitoids in invasive species populations is frequently low (Cornell & Hawkins, 1993). This is the case of both parasitoid species recorded in our trials, whose *parasitism rate* values were lower than that recorded for *G. pennsylvanicum* in eggs laid in situ in the WCSB native area (44%). However, in the 2017 clone bank trial, the *parasitism rate* of *O. obscurus* was higher than those recorded for the secondary parasitoid species in eggs laid in situ in the native area: *A. perseally* (12.1%) and *O. johnsoni* (14.2%). In terms of *exploitation efficiency index*, in no trial did either of the parasitoid species reach the values recorded for *G. pennsylvanicum* in eggs laid in situ (90%) in North America (Maltese et al., 2012) and in laboratory trials (75%) (Peverieri et al., 2013).

According to Europe WCSB parasitoid species, the *parasitism rate* values of *O. obscurus* and *O. pityocampae* were higher than that recorded for the entire WCSB egg parasitoid community (*A. bifasciatus*, *B. servadeii* and *O. pityocampae*) in sentinel eggs in Italy (<1%) (Roversi, Santini, et al., 2011). In France, *A. bifasciatus* and *Ooencyrtus* spp. parasitized 31 and 38 natural egg masses, respectively (*discovery efficiency*: 7.2% and 8.9%), with these amounts being lower than in Central Spain for both *Ooencyrtus* species in all trials. The amount of natural eggs parasitized in France by *A. bifasciatus* (176; *parasitism rate*: 4.8%) was lower than the values recorded in the 2017 clone bank and the 2019 pine forest trials, whereas the percentage of natural eggs parasitized by *Ooencyrtus* spp. (50; *parasitism rate*: 1.3%) was lower than those reported in our trials for *Ooencyrtus* for both species (Lesieur & Farinha, 2021).

Focussing on the trials in Central Spain, index values for the parasitoid species *O. obscurus* were significantly higher than for *O. pityocampae* in the clone bank trials but were not significant in the pine forest trial. Moreover, when comparing the index values by species and by experimental site in 2019, the values were higher for *O. obscurus* in the clone bank and for *O. pityocampae* in the pine forest. Predated eggs, with a portion of the chorion with chewing damage, according to Bates and Borden (2005), were also recorded in all trials, with this number being significantly higher in the pine forest than in the clone bank in 2019. As mentioned above, these experimental site differences could be influenced by the management, the environmental conditions (Costi et al., 2018), despite the proximity of the experimental sites, and/or by the smallest number of egg masses laid in situ ($n = 43$) in the pine forest comparing with the clone bank ($n = 125$) in the 2019 trials.

The onset and finish dates of the parasitoid oviposition period on WCSB egg masses laid in situ were recorded every year in the clone bank trials. However, the first WCSB egg masses and parasitized eggs were found simultaneously. In France, no parasitized egg masses were recorded before July but were detected in a longer timeframe when compared to Spain (Lesieur & Farinha, 2021). To define the synchronization between the WCSB and the native egg parasitoids (Stiling, 1993), it is necessary to properly define the parasitoid activity period by surveying egg masses of their primary hosts, which could be more attractive for them, causing a decrease in impact on the WCSB population.

The parasitoid species *O. obscurus* and *O. pityocampae* do not overwinter in WCSB eggs and, according to Lesieur and Farinha (2021), the egg masses are not parasitized simultaneously by different parasitoid species. The *sex ratio* had a strong female bias for both parasitoid species, although *O. obscurus* males emerged in all trials. However, for *O. pityocampae*, males only emerged in the 2017 clone bank trial and in low numbers. This fact could be due to the reproductive strategy; *Ooencyrtus pityocampae* is thelytoky (Tunca et al., 2019), that is only females emerge from eggs not fertilized by males (Heimpel & Boer, 2008). To our knowledge, no reference is available for *O. obscurus*. However, we could define it as arrhenotoky (L. Ponce, personal laboratory trial observation), that is females emerge from fertilized eggs and males from unfertilized eggs (Heimpel & Boer, 2008).

The mean number of parasitoids emerged by egg (*emergence ratio*) was higher than one parasitoid for both species in all trials, reaching a maximum of five parasitoids/egg for *O. obscurus* and four for *O. pityocampae*. This is unlike *G. pennsylvanicum*, whose offspring consists of one parasitoid for one parasitized egg of *Leptoglossus phyllopus* (L.) (Hemiptera: Coreidae) (Abudulai et al., 2001). This fact could be due to the smaller body size of *O. obscurus* (1.05 mm) and *O. pityocampae* (1.10 mm) (Mercet, 1921) compared with the body size of *G. pennsylvanicum* (1.6–1.8 mm) (Komeda et al., 2020). In this regard, the *Ooencyrtus* species can produce more offspring parasitizing fewer WCSB eggs than *G. pennsylvanicum*, possibly decreasing its impact on the host population.

In all trials, the number of WCSB egg masses and eggs varied weekly and depended on the number and fecundity of the bagged WCSB females in field. The *unhatched eggs rate* in the clone bank trials differs from that for sentinel egg masses recorded in France (8.4%–11.3%) (Lesieur & Farinha, 2021) and in British Columbia (2%–16%), where this rate was related to female infertility, embryonic death, or hot and dry weather (Bates & Borden, 2005). The high *unhatched eggs rate* recorded in Spain could also be due to the lack of field mating or the unsuccessful parasitization of the eggs. Host abortion can be caused by mechanical damages on the egg or the embryo by the parasitoid ovipositor, or by alterations in the egg ooplasm by the introduction of substances during the oviposition event (Cusumano et al., 2012). When the host egg is parasitized before the beginning of the nymph development and the parasitization fails, no internal (detritus) or external (stalks) parasitism signs appear in the egg and it looks like a non-fertile egg when it is actually parasitized. Detecting this type of eggs can avoid underestimating the impact of the parasitoid on the host population (Abram et al., 2016). In this study, the number of unhatched eggs was similar throughout the WCSB laying period and focus was not on the peaks of the parasitoid activity period on WCSB eggs, and, as such, the impact of the native parasitoids on the WCSB eggs has apparently not been underestimated. The number of parasitized eggs from which no parasitoid emerged also varied by species and trial, reaching the highest values in the 2017 clone bank trial, which agrees with the highest parasitism values recorded. The amount of this type of parasitized eggs was lower in all

trials than that recorded by Maltese et al. (2012) in eggs laid in situ that were parasitized by *G. pennsylvanicum* (13%) in the native area.

4.2 | WCSB activity period

The first WCSB overwintered adults, egg masses and N1 instar nymphs were recorded in late May and early June, agreeing with Barta (2016) in Slovakia, whereas the last nymphs in the later instars of development and adults were recorded in late October. The number of WCSB generations depends on the study site conditions (Barta, 2016; Tamburini et al., 2012). Our visual monitoring data are not sufficient to define the number of WCSB generations and their onset in Central Spain because of the difficulty to detect the WCSB population in the pine branches and the possible shifting of the WCSB adults between the clone bank and the surrounding mature pine forest. As such, properly defining the WCSB activity period is necessary to estimate its synchronization with the native egg parasitoids (Stiling, 1993). To this end, an effective monitoring method must be developed, for example based on chemical attraction (Blatt & Borden, 1996).

4.3 | Native *Ooencyrtus* spp. biological control agent of WCSB populations

Ooencyrtus species have already been used successfully as BCA in classical BCPs (Huang & Noyes, 1994; Noyes, 1980; Noyes & Hayat, 1994). *Ooencyrtus* spp. recorded in WCSB egg masses in Central Spain are not specific to this pest but their ability to find the egg masses (*discovery efficiency*) and to parasitize their eggs (*exploitation efficiency*) allowed for the reduction in the WCSB population by 3.3%, down to 15.1% (*parasitism rate*). *Ooencyrtus obscurus* parasitized from 6.2% to 19.6% of eggs, while these values for *O. pityocampae* were from 1.0% to 11.5%. Discarding the non-viable WCSB eggs, these values were from 9.9% to 26.7% for *O. obscurus* and from 1.3% to 15.8% for *O. pityocampae*. The *Ooencyrtus* species´ ability to parasitize most of the eggs within the egg mass (*exploitation efficiency*) can be reduced by their body size because up to 5 parasitoids can emerge from a single WCSB egg. As mentioned above, this is dissimilar to the main WCSB egg parasitoid in the native area, *G. pennsylvanicum* (Abudulai et al., 2001) which was proposed as BCA for a classical BCP in Italy. This parasitoid has not yet been introduced in Europe despite its low negative effects on non-target European host species (Roversi et al., 2014). Inundative or conservative biological control programmes (Eilenberg et al., 2001) using WCSB native egg parasitoids as BCA have not been proposed so far. Additionally, it is necessary to define the synchronization (Stiling, 1993) and the density dependence relation between the WCSB and their egg parasitoid species (Van den Bosch et al., 1982) in the biological control area. Finally, the parasitization ability and population growth parameters of these parasitoid species may be measured on WCSB eggs in laboratory conditions (Peverieri et al., 2012). The ability of *O. pityocampae* to parasitize WCSB eggs has already been tested by Binazzi

et al. (2013) in laboratory trials. A laboratory assessment needs to be developed to measure the effectiveness of *O. obscurus* as a BCA for WCSB populations in Central Spain, which is apparently higher than the effectiveness of *O. pityocampae*.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

LPH and JAPA secured funding. LPH and JAPA conceived and designed the study. LPH and AOF contributed with material. LPH conducted the data analyses, and VPF conducted the statistical analyses. LPH, AOF, VPF and FMAS wrote and approved the manuscript.

DATA AVAILABLE STATEMENT

The data that support the findings of this study are available in Ponce Herrero (2021).

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