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Research Article

Population Dynamics of Native Parasitoids Associated with the Asian Chestnut Gall Wasp *(Dryocosmus kuriphilus)* in Italy

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Native parasitoids may play an important role in biological control. They may either support or hinder the effectiveness of introduced nonnative parasitoids released for pest control purposes. Results of a three-year survey (2011–2013) of the Asian chestnut gall wasp (ACGW) *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) populations and on parasitism rates by native indigenous parasitoids (a complex of chalcidoid hymenopterans) in Italian chestnut forests are given. Changes in *D. kuriphilus* gall size and phenology were observed through the three years of study. A total of 13 species of native parasitoids were recorded, accounting for fluctuating parasitism rates. This variability in parasitism rates over the three years was mainly due to the effect of *Torymus flavipes* (Walker) (Hymenoptera: Torymidae), which in 2011 accounted for 75% of all parasitoid specimens yet decreased drastically in the following years. This strong fluctuation may be related to climatic conditions. Besides, our data verified that parasitoids do not choose host galls based on their size, though when they do parasitize smaller ones, they exploit them better. Consequently, ACGWs have higher chances of surviving parasitism if they are inside larger galls.

1. Introduction

Host shifting of native parasitoids onto alien insect pests is not always taken into account in applied entomology. This phenomenon is particularly worth understanding when classical biological control programs plan to contain alien pests by releasing exotic antagonists coming from the same native range. This strategy aims to reestablish the original host-antagonist association in the new area of distribution. However, native antagonists may also be able to play an important role in biological control, supplementing, for instance, the effect of the exotic antagonist [1, 2], or they can hinder its establishment and, thus, its effectiveness [3–5]. For example, cynipid parasitoids attack both gall formers and inquilines found inside galls; in addition they may act as facultative hyperparasitoids, attacking other parasitoids [6], including the exotic antagonist [5, 7].

Native cynipid parasitoids are able to rapidly adapt to new exotic hosts; as a result host shift events may occur and even changing in parasitoid behaviour [8] showed how galls of invading cynipid species in Britain have become the main hosts of local parasitoid populations. These kinds of phenomena may have a great impact on parasitoid communities. For example, the exploitation of a new cynipid host may cause shift in sex ratio of a parasitoid populations, as in the case of *A. quercuscalicis* in Britain [9]. In addition, the availability of a new host, with a different phenology, may promote diversification within the parasitoid community, as demonstrated by the occurrence of cryptic species of cynipid parasitoid with differing seasonal phenologies [10].

The present paper reports the results of a study on the Asian chestnut gall wasp (ACGW), *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae), and its parasitoids in Italy. *D. kuriphilus* is a hymenopteran native to China, which

induces gall formation on green chestnut organs. It has one generation per year: females lay eggs (parthenogenetically) in the buds of chestnut trees during the June/July period. After 30–40 days, larvae hatch and remain inside buds to overwinter [11, 12]. Green- or red-coloured galls develop in spring, at the time of bud burst. Each gall may host one (unilocular) or more (multilocular) larvae, which pupate after a feeding period of 20–30 days [12].

The hymenopteran is an alien invasive species in Europe. After its first recorded presence in Italy, in plant nurseries in Piedmont [13], the ACGWs rapidly spread throughout and beyond Italy; now they can be found throughout Europe wherever chestnuts grow [14, 15]. Following the severe damage caused by this pest to the chestnut market [16–18], a control system that was both effective and ecologically sustainable had to be found. In this regard, some control measures have already been attempted, but currently the introduction of an exotic parasitoid, native to China, is the most promising one. This parasitoid is *Torymus sinensis* Kamijo (Hymenoptera: Torymidae), which was used for biological control initially in Japan and the United States [19, 20], followed by Italy [21], France, Slovenia, Croatia, and Hungary [15, 22, 23]. In 2003, following the mostly successful Japanese experience, *T. sinensis* was introduced in Italy from Japan. The purpose was starting a classical biological control program against *D. kuriphilus*. The first release of the exotic parasitoid was carried out in 2005 in some chestnut woods of Piedmont, where for some time *T. sinensis* was also reared extensively in premultiplication areas. Parallel to the spread of the cynipid in the rest of Italy, local administrations cooperated for the release of the exotic antagonist which gradually covered many other Italian regions. In Tuscany, in 2009-2010 the biological control program started with a total of 5 sites for release and one premultiplication area. In 2011, release sites were increased to 20, plus three premultiplication areas. However, private suppliers soon started to sell *T. sinensis* to chestnut growers complicating the inventory of the actual release sites. On the other hand, little consideration has been given to the native parasitoids, associated with oak gall wasps, as a potential natural resource to be exploited, even if only to supplement the role of *T. sinensis*.

The ACGW is a gall inducing insect; gall induction is an adaptive phenomenon, which has been described suggesting some hypotheses. Among these, the "enemy hypothesis" states that gall structure has been selected to minimize cynipid mortality by parasitism [25]. Parasitoids can attack larvae inside galls as long as they reach them with their ovipositor. If galls are large, some larvae will be out of reach; thus large gall size increases chances of larval survival [26]. Gall size depends on gall wall thickness and multilocularity (many larval cells in a single gall); thus while larvae in peripheral position may be easily attacked, those in deeper cells are less accessible and, therefore, more protected. Thus, as stated by the "enemy hypothesis," gall characteristics may be the results of parasitoids' selective pressure [25]. More specifically, larger galls seem to function as a defensive strategy against parasitoids [27, 28].

However, this form of protection has not precluded native parasitoids from promptly adapting to the new host, constituting a variegated and numerous complex. In Italy, all native parasitoids recruited to ACGW are species associated with oak gall wasps. Nonetheless, despite the high number of species, they generally show rather low parasitism rates, which are also highly variable over time [7, 29–31]. This seems correlated to an asynchrony between parasitoid emergence and ACGW gall susceptibility [29], that is, "galls in appropriate developmental stage for attack" [32]. The present three-year study, carried out in a chestnut area of northeastern Tuscany (Italy), aimed to study parasitism activity by native parasitoids recruited by ACGW, also taking into account the establishment of *T. sinensis*. In fact, the recent release of the latter did not hinder studying host shifting by native parasitoids of oak gall wasps. Also, data concerning the parasitism rates are compared to the galls' characteristics, size in particular, to verify the "enemy hypothesis."

2. Material and Methods

2.1. Study Sites. Our study was carried out in two chestnut forests near Marradi, northeastern Tuscany (Italy), where chestnut production plays a key role in the local forestry market. One chestnut forest (site A) was at Pian della Quercia (44.072143N, 11.632116E), in a steep hillside area at 478 m a.s.l., with an eastern exposure. Here, several cultivated chestnut trees were present (some of which over a hundred years old), belonging to the "Marron Buono di Marradi" cultivar complex, together with some wild chestnut trees and other broadleaves, such as oaks and hornbeams. The other forest (site B) was at La Casetta (44.059591N, 11.647478E), a chestnut forest at 612 m a.s.l., with a western exposure. In the centre of this area cultivated chestnuts are dominant ("Marron Buono di Marradi" cultivar), surrounded by both wild chestnuts and oaks. Climate is typical of submountain Tuscany, that is, with the highest mean maximum temperatures in August (mean of the 1992–2013 period = 28.6°C) and lowest mean minimum temperatures in February (1992–2013 mean = 1.7°C). Highest monthly rainfall occurs in October (1992–2013 mean = 159.97 mm), while the lowest one is recorded in July (1992–2013 mean = 28.78 mm). Climate records from the nearby Marcoiano weather station were kindly provided by the SIR (Servizio Idrografico Regionale). *T. sinensis* releases were carried out in our study area one year before our first samplings, as part of the regional biological control program.

2.2. Gall Samplings. In both study sites (A and B), samples were collected every two weeks during the 2011–2013 period. Samples consisted of ACGW current-generation galls, in which ACGW specimens were developing. In 2011, four samplings were carried out between May and June, while in 2012 and in 2013 five samplings were carried out between May and July. During each sampling, for each study site, ACGW galls were randomly collected from a total of 60 chestnut trees (divided into two management categories: 30 cultivated trees and 30 wild ones). Samples were collected within a height of four meters using a long reach pruner: two galls per tree, each on opposite sides of the crown (for a total of 120 galls per sampling at each study site) and categorized according

to chestnut management category. A total of 3,360 ACGW current-year galls were collected during this three-year study. After each sampling, collected galls were divided in two equal groups, 60 galls each (30 per study site: 15 from cultivated chestnut trees and 15 from wild ones). One group was stored at −20°C (frozen galls) and then used to analyse gall content; the other group was stored at room temperature to rear adult parasitoids (reared galls).

2.3. Analysis of Frozen Galls. After each sampling, collected galls were stored at −20°C [29]. Before that, their volume was assessed applying the ellipsoid formula ($4/3 \times \pi \times r \times r2 \times r3$); three perpendicular measurements of each gall were taken with a calliper, which were then divided by two to obtain the radiuses. Later, each gall was dissected to examine its contents, tallying the following: (1) cells with the ACGW ((i) larvae, (ii) white pupae, (iii) black pupae, and (iv) adults); (2) cells with parasitoids ((i) larvae, (ii) pupae, and (iii) adults); (3) cells with parasitoid exuviae (most parasitoid species leave the pupal exuviae inside the cell after emergence); (4) empty cells from which the ACGW had already emerged (all cells linked with an emergence hole and without pupal exuviae fell in this category, even though this overestimated slightly the number of emerged ACGW since a small percentage of parasitoids does not leave pupal exuviae in the cell, similarly to the ACGW); (5) empty cells without emergence holes. If hyperparasitism was observed, it was annotated separately. These galls were used to gather information about ACGW parasitism rate. In fact, observations on fresh galls are more precise, as cells are easily counted and examined via dissection of still moist tissues. On the other hand, fresh galls are not suitable for parasitoid identification, mainly because parasitoid development is not yet completed.

2.4. Gall Rearing. The other half of the galls was labelled, placed individually in a clear plastic cup, covered with a fine mesh, and kept at room temperature (15–25°C). Galls were checked daily for ACGW and parasitoid emergence. Emerged ACGWs were collected and counted, while each emerged parasitoid specimen was labelled according to sampling date, study site, management category, and gall number and stored to be identified later. About six months after the end of insect emergence all galls were dissected to examine their contents. At this time, galls were already withered; thus, unlike frozen galls, some observations were not possible. As a consequence, content categories were as follows: (1) cells with dead ACGW; (2) cells with dead parasitoids; (3) cells with parasitoid exuviae. For the adult parasitoids' identification, we used a key for Chalcidoidea families and genera provided by Goulet and Huber [33] as well as an unpublished one compiled by R. R. Askew (Manchester, UK), which is a basic identification tool on species level used for decades in the research of oak gall wasps' parasitoid communities. The catalogue of oak gall wasp parasitoids [34] was based exclusively on this latter key. The parasitoid specimens collected during this study are deposited at the Department of Agrifood Production and Environmental Sciences, at the University of Florence. Reared galls were used to gather information about the parasitoid species adapted to the ACGW galls, while they were not used to assess parasitism rates, since frozen galls allowed for a far more precise cell count.

2.5. Analysis of Withered Galls. After the end of ACGW emergence in our study sites, withered galls still present on the chestnut trees were collected to assess the natural mortality of both the ACGW and its parasitoids. In December 2013 and in February 2014, 130 withered galls (65 from cultivated chestnuts and 65 from wild chestnuts) were randomly collected. In the laboratory, each gall was dissected to record the number of dead ACGWs, dead parasitoids, and live parasitoids (those which overwinter inside ACGW galls). When possible adult parasitoids were identified using the methodology described in the previous paragraph and when parasitoid specimens were found alive but at the preimaginal stages of their development, they were kept in separate vials until emergence of the adults; then they were identified.

2.6. Statistical Analysis. Gall volumes and parasitism rates (i.e., parasitized cell number/total cell number × 100) were transformed using $\sqrt[3]{}$ log-formula and Freeman & Tukey's formula [35], respectively, and analysed separately via ANOVA, taking into account "year" and "site" as random factors, while "management" was considered a fixed factor. In order to standardize volume effects on parasitism, galls were sorted in three volume categories, defining the intermediate volume category (including 33% of the distribution) by the following formula: mean of transformed volumes $\pm 0.416\sigma$; the other two categories were defined accordingly. As a result, the three volume categories were as follows: small = 0.02–0.64 cm^3; medium = 0.65–1.31 cm^3; large = 1.32–15.48 cm^3. The strong positive correlations "gall volume/number of cells" [36] and "number of cells/number of parasitized cells" may conceal the real effects of gall volume on parasitism (cells deep inside bigger galls are less reachable by parasitoid ovipositors). Thus, the creation of these categories is necessary to fully understand how the volume affects parasitism rates, regardless of the number of cells. A *G*-test [35] was used to analyse parasitism rates across three volume categories, comparing (i) the three years studied, (ii) the two study sites, and (iii) the two management categories. With the same test and considering the same factors, the percentage of parasitized cells only in parasitized galls (number of parasitized cells/total number of cells of parasitized galls × 100) was also analysed. Finally, a *G*-test was also used to compare annual parasitism rate of *Eupelmus urozonus* Dalman and mortality indexes of both the ACGW and its parasitoids in reared galls with those observed in withered galls which were collected during the winter.

To verify the effect of spring climatic variables on the ACGW development and then on its parasitism rates, we considered the percentages of ACGW (pupae and adults) on the total number of observed specimens observed inside galls. For the ACGW development, we chose three variables: (1) adult %; (2) adult% + black pupa%; (3) adult% + white and black pupa%. The cumulative fashion of these percentages seems to better describe the ACGW development during the spring season. For parasitism, another two variables were considered: parasitoid presence/absence inside galls and the

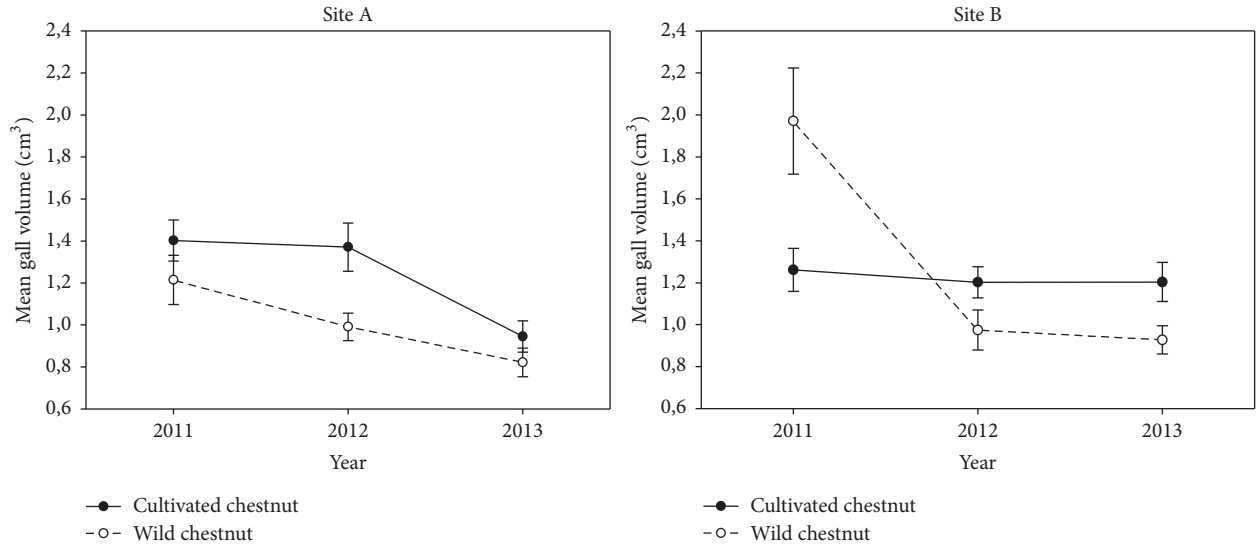


Figure 1: *Dryocosmus kuriphilus* gall size. Samples collected from cultivated and wild chestnuts in the two study sites (site A = 478 m a.s.l.; site B = 665 m a.s.l.) at Marradi (Italy) during the three-year period. Bars represent standard error.

percentage of parasitized cells in each gall. All five variables are binomial. Finally, the following independent variables were considered: (1) sampling date (as Julian date); (2) "site"; (3) "management"; (4) number of cells per gall; (5) GDD_{10} = Growing Degree-Days with the minimum threshold of 10°C for the *D. kuriphilus* development [37], from the 1st of April to each sampling date of each year, calculated with the following averaging method:

$$\begin{aligned} &GDD_{10} \\ &= \left[\frac{(\text{daily maximum temp.} + \text{daily minimum temp.})}{2}\right] \\ &\quad - \text{ACGW minimum developmental threshold} \\ &= \text{average daily temperature} - 10. \end{aligned} \quad (1)$$

For each dependent variable, owing to the nested nature of the independent variables, we chose a Generalized Linear Mixed Models (GLMM) approach [38]. More specifically, all samples collected on a specific day are nested into their date; the "site" variable is the same for all samples coming from a site; each "management" variable includes all samples pertaining to it; finally, GDD_{10} values regard all samples collected on a specific day. All these variables had to be treated as random factors. The LR test and the AIC (Akaike Information Criterion) were used to choose the best models. These were, therefore, those with the minimum AIC value and for which the LR test provides a significant result ($P < 0.01$) for all the variables considered. The model was validated by visual inspection of residuals [38], which were also checked for homogeneity of variance. All the analyses were carried out using the R programming language (version 3.1.2; R Development Core Team 2014), and specifically for GLMM the lme4 package [39] was used.

3. Results

3.1. Gall Volume and ACGW Phenology. Mean gall volumes decreased significantly from 2011 to 2013 (ANOVA, df1 = 2, df2 = 1308, $F = 23.742$, $P < 0.001$). Interaction among the variables "year," "site," and "management" was also significant (ANOVA, df1 = 2, df2 = 1308, $F = 4.324$, $P < 0.05$). In fact, galls from cultivated chestnuts were always larger than those from wild chestnuts at site A. This was confirmed also at site B for years 2012 and 2013, but in 2011 it was the opposite (Figure 1).

A total of 7,733 larval cells were examined in three years. 85.18% of these cells were occupied by live ACGW specimens and 0.25% by dead ACGW specimens, and from 1.34% of the cells AGCW adults had already emerged. Furthermore, 9.44% of cells were parasitized (from 2.33% of these cells adult parasitoids had already emerged). Finally, 3.79% of cells were empty without any signs of emergence holes.

By comparing ACGW data, distinguishing between each sampling date, we can observe remarkable differences among the three study years. Pupae were observed starting from the end of May (second sampling) in 2011 and from mid-June in 2012 and 2013 (Figure 2). Thus, adults appeared earlier in 2011 (third sampling, June 10th) than during the two following years (fourth sampling). Indeed, in the third sampling in 2011 larvae were almost completely absent, pupae were predominant, and some adults were present (Figure 2). On the contrary, in 2012 and 2013 no adult was observed at the third sampling, while larvae were still numerous.

Parasitoids also showed differences in their dynamics during the three-year period. In 2011, parasitoid specimens were recorded inside galls from the start of samplings, with a peak at the second sampling (end of May) and then lower occurrences until the end of June (fourth sampling) (Figure 3). Samplings in 2012 and 2013 showed a different picture: not only were parasitoids extremely less abundant,

Table 1: *Dryocosmus kuriphilus* galls collected from cultivated and wild chestnuts in the two study sites (site A = 478 m a.s.l.; site B = 665 m a.s.l.) at Marradi (Italy) during the three-year period. Nonsignificant P values are indicated by n.s.

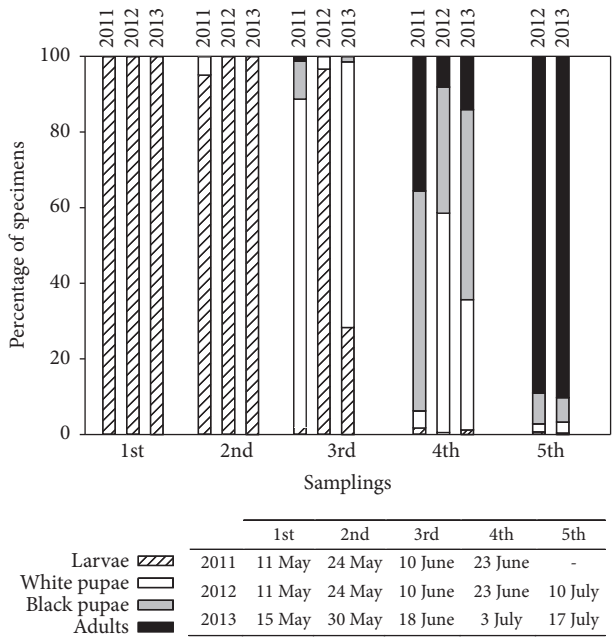
(a)

Year	Total galls collected	Parasitized galls (%)						
		Total	Site A			Site B		
			Total	Cultivated	Wild	Total	Cultivated	Wild
2011	480	63.13	30.21	13.75	16.46	32.92	16.88	16.04
2012	600	7.17	4.67	1.83	2.83	2.50	1.67	0.83
2013	600	6.67	3.67	1.00	2.67	3.00	1.33	1.67

(b) G-test results

Year	Site			Management[a]					
				Site A			Site B		
	d.f.	χ^2	P	d.f.	χ^2	P	d.f.	χ^2	P
2011	1	1.514	n.s.	1	2.952	n.s.	1	0.296	n.s.
2012	1	4.296	n.s.	1	1.428	n.s.	1	1.787	n.s.
2013	1	0.429	n.s.	1	5.076	n.s.	1	0.237	n.s.

[a]Cultivated versus wild chestnuts.



	1st	2nd	3rd	4th	5th
2011	11 May	24 May	10 June	23 June	-
2012	11 May	24 May	10 June	23 June	10 July
2013	15 May	30 May	18 June	3 July	17 July

Figure 2: Stages of *Dryocosmus kuriphilus* specimens inside frozen galls collected from cultivated and wild chestnuts in the two study sites (site A = 478 m a.s.l.; site B = 665 m a.s.l.) at Marradi (Italy) during the three-year period.

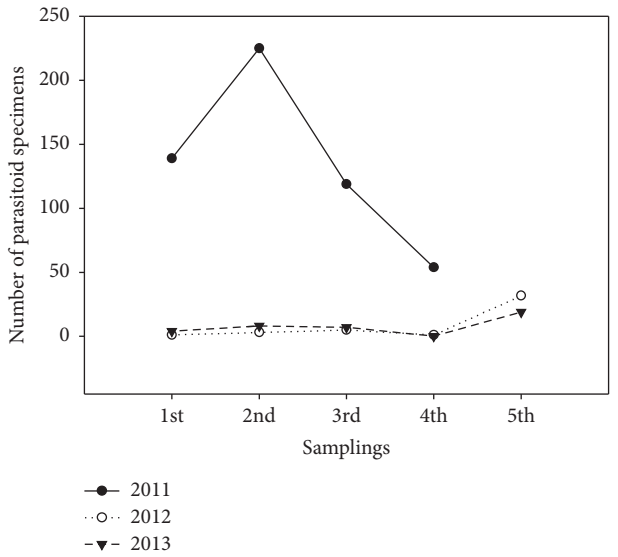


Figure 3: Quantities of parasitoid specimens inside frozen galls collected from cultivated and wild chestnuts in the two study sites (site A = 478 m a.s.l.; site B = 665 m a.s.l.) at Marradi (Italy) during the three-year period.

but also they showed similar low numbers during the first four samplings (late June-early July period). Moreover, in 2012 and 2013 the parasitoid peak was during the fifth sampling (mid-July).

3.2. Gall Parasitism. The percentage of parasitized galls varied significantly during the three study years (G-test, df = 2, $\chi^2 = 575.615$, $P < 0.001$). This percentage was calculated taking into account both the parasitoid specimens observed inside galls (in all developmental stages) and the exuviae left behind by parasitoids that had already emerged by the time of sampling. More than half of the galls were parasitized in 2011, while only a few parasitized galls were found in 2012 and 2013 (Table 1). Finally, no difference was distinguishable between the two study areas (Table 1).

Upon dividing sampled galls into three size categories, we compared parasitized galls rates from different size categories. A significant difference was observed only at site B in 2011: on cultivated chestnuts a higher percentage of smaller galls (about 90%) were parasitized compared to the other two size categories, under 65% (Table 2).

Table 2: Galls of *Dryocosmus kuriphilus,* collected from cultivated and wild chestnuts in the two study sites (site A = 478 m a.s.l.; site B = 665 m a.s.l.) at Marradi (Italy), sorted by volume categories (small = 0.02–0.064 cm^3; medium = 0.065–1.31 cm^3; large = 1.32–15.48 cm^3). Nonsignificant *P* values are indicated by n.s.

Year	Gall volume categories						d.f.	χ^2	*P*
	Small		Medium		Large				
	Gall number[a]	Parasitized galls (%)	Gall number[a]	Parasitized galls (%)	Gall number[a]	Parasitized galls (%)			
Site A									
Cultivated chestnut									
2011	23	65.22	37	45.95	60	56.67	2	2.276	n.s.
2012	46	6.52	50	8.00	54	7.41	2	0.078	n.s.
2013	63	3.17	56	5.36	31	3.23	2	0.415	n.s.
Wild chestnut									
2011	37	72.97	44	56.82	39	69.23	2	2.610	n.s.
2012	55	7.27	59	16.95	36	8.33	2	3.007	n.s.
2013	72	12.50	52	7.69	26	11.54	2	0.791	n.s.
Site B									
Cultivated chestnut									
2011	29	89.66	37	54.05	54	64.81	2	10.952	0.01
2012	40	7.50	56	5.36	54	7.41	2	0.254	n.s.
2013	46	6.52	57	1.75	47	8.51	2	2.856	n.s.
Wild chestnut									
2011	26	61.54	37	54.05	57	71.93	2	3.221	n.s.
2012	77	1.30	39	5.13	34	5.88	2	2.179	n.s.
2013	70	10.00	51	3.92	29	3.45	2	2.392	n.s.

[a]Total number of galls collected per each category.

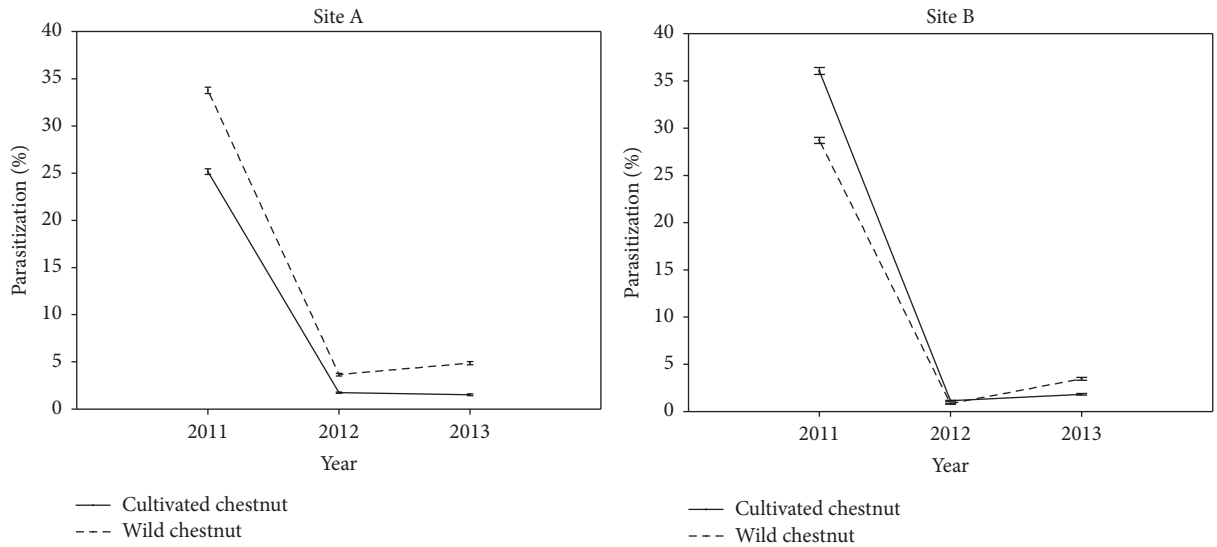


Figure 4: Mean percentage of parasitized *Dryocosmus kuriphilus* cells. Samples collected from cultivated and wild chestnuts in the two study sites (site A = 478 m a.s.l.; site B = 665 m a.s.l.) at Marradi (Italy) during the three-year period. Bars represent standard error.

We observed significant differences in parasitism rates (parasitized cell number/total cell number × 100) during the three years of study, also in terms of study site and management. Again, the year factor was statistically significant (ANOVA, df1 = 2, df2 = 1668, $F = 336.407$, $P < 0.001$), with parasitism rates over 25% in 2011, which plunged to below 5% in the following two years (Figure 4). Furthermore, the interaction among year, management, and site was also statistically significant (ANOVA, df1 = 2, df2 = 1668, $F = 6.702$, $P < 0.001$). As regards hyperparasitism, it was not observed inside frozen galls in 2011 and 2012, but only in two cases in 2013, when two parasitoid specimens were found hyperparasitized. In summary, two hyperparasitoids were observed in frozen galls during three years on 1680 galls.

Table 3: Parasitized cells within *Dryocosmus kuriphilus* galls collected from cultivated and wild chestnuts in the two study sites (site A = 478 m a.s.l.; site B = 665 m a.s.l.) at Marradi (Italy) during the three-year period. Nonsignificant *P* values are indicated by n.s.

(a)

Year	Parasitized gall number	Total cell number	Parasitized cells (%)						
			Total	Site A			Site B		
				Total	Cultivated	Wild	Total	Cultivated	Wild
2011	303	1725	36.35	39.57	36.44	42.41	33.62	38.29	30.65
2012	43	278	18.71	21.14	18.42	23.23	14.56	13.70	16.67
2013	40	157	32.48	30.00	26.92	31.25	35.82	29.73	43.33

(b) *G*-test results

Year	Site (A versus B)			Management (cultivated versus wild chestnuts)					
				Site A			Site B		
	d.f.	χ^2	*P*	d.f.	χ^2	*P*	d.f.	χ^2	*P*
2011	1	6.549	0.01	1	2.948	n.s.	1	5.772	n.s.
2012	1	1.900	n.s.	1	0.602	n.s.	1	0.147	n.s.
2013	1	0.591	n.s.	1	0.167	n.s.	1	1.332	n.s.

Table 4: Parasitized cells within *Dryocosmus kuriphilus* galls collected from cultivated and wild chestnuts in the two study sites (site A = 478 m a.s.l.; site B = 665 m a.s.l.) at Marradi (Italy) sorted by volume categories (small = 0.02–0.064 cm^3; medium = 0.065–1.31 cm^3; large = 1.32–15.48 cm^3). Nonsignificant *P* values are indicated by n.s.

Year	Gall volume categories						d.f.	χ^2	*P*
	Small		Medium		Large				
	Cell number[a]	Parasitized cells (%)	Cell number[a]	Parasitized cells (%)	Cell number[a]	Parasitized cells (%)			
Site A									
Cultivated chestnuts									
2011	30	66.67	75	46.67	271	30.26	2	19.131	<0.001
2012	10	30.00	24	20.83	42	14.29	2	1.383	n.s.
2013	6	50.00	12	25.00	8	12.50	2	2.447	n.s.
Wild chestnuts									
2011	69	63.77	107	46.73	239	34.31	2	20.095	<0.001
2012	9	55.56	65	21.54	25	16.00	2	5.250	n.s.
2013	19	52.63	19	21.05	26	23.08	2	5.565	n.s.
Site B									
Cultivated chestnuts									
2011	47	85.11	87	36.78	229	29.26	2	52.286	<0.001
2012	12	25.00	19	15.79	42	9.52	2	1.833	n.s.
2013	9	44.44	3	33.33	25	24.00	2	1.295	n.s.
Wild chestnuts									
2011	29	79.31	80	48.75	462	24.46	2	49.308	<0.001
2012	2	50.00	9	22.22	19	10.53	2	1.940	n.s.
2013	17	41.18	9	55.56	4	25.00	2	1.155	n.s.

[a]Total number of cells recorded on sample galls per each category.

By considering only parasitized galls, we could focus on how much the galls were being exploited by parasitoids. In fact, in 2011 the percentage of parasitized cells in parasitized galls was higher (parasitized cell number/total cell number × 100) (*G* test, df = 2, $\chi^2 = 36.378$, $P < 0.001$). Furthermore, in 2011 cell parasitism was significantly higher in site A than in site B (Table 3). Finally, small galls had a higher percentage of parasitized cells (Table 4). This was true only for 2011, but it was observed in all sites, on both cultivated and wild chestnut trees (Table 4).

3.3. Parasitoid Complex Composition. Parasitoids that emerged during this study are Chalcidoidea hymenopterans belonging to six families (Table 5). Torymidae was the most represented family (four species and 345 specimens), followed by Pteromalidae (four species and 51 specimens).

Table 5: Number of insect specimens from *Dryocosmus kuriphilus* galls collected at Marradi (Italy). Percentages of dead specimens inside galls are also reported, as well as the estimated number of parasitoids that emerged before the sampling date.

Family	Species	Primary or secondary parasitoid	Parasitoid n. gen/yr	Ecto- or endo-parasitic	2011		2012		2013	
					Total number	Dead (%)	Total number	Dead (%)	Total number	Dead (%)
Parasitoids	*Dryocosmus kuriphilus*				1645	67.9	2621	78.7	1714	82.1
Torymidae	*Torymus flavipes* (Walker)	Primary	2	Ecto	330	23.9	2	0	0	-
	Torymus formosus (Walker)	Primary	2	Ecto	4	0	0	-	0	-
	Torymus auratus (Muller)	Primary	2	Ecto	5	20	2	0	2	0
	T. sinensis (larvae inside galls)	Primary	1	Ecto	0	-	2	0	7	0
Megastigmidae	*Bootanomyiadorsalis* (Fabricius)	Primary	2	Ecto	3	0	4	0	2	0
Eupelmidae	*Eupelmus urozonus* Dalman[a]	Primary/secondary	2	Ecto	5	20	25	20	9	44.4
Pteromalidae	*Mesopolobus tibialis* (Westwood)	Primary (rarely secondary)	2-3	Ecto	34	35.3	2	0	0	-
	Mesopolobus tarsatus (Nees)	Primary (rarely secondary)	2-3	Ecto	9	22.2	0	-	0	-
	Mesopolobus fuscipes (Walker)	Primary (rarely secondary)	2-3	Ecto	3	33.3	0	-	0	-
	Mesopolobus sericeus (Forster)	Primary (rarely secondary)	2-3	Ecto	0	-	0	-	3	0
Ormyridae	*Ormyrus pomaceus* (Geoffroy)	Primary	2	Ecto	3	0	0	-	2	0
Eurytomidae	*Sycophila biguttata* (Swederus)	Primary	2	Endo	1	0	7	0	0	-
	Sycophila flavicollis (Walker)	Primary	2	Endo	1	0	0		0	-
	Eurytoma brunniventris Ratzeburg	Primary/secondary	2	Ecto	1	0	2	0	0	-
Unidentified					53	100	13	100	20	100
Emerged before the sampling date					142	-	5	-	9	-
Total parasitoids					594	25.1	64	28.1	54	44.4

[a]Subsequent to our study, further species discrimination within the *E. urozonus* complex has come about [24], resulting in five new *Eupelmus* species. Thus, within our *E. urozonus* figures, we might well have some of these newly described species, for example, *E. confusus* [24] and *E. gemellus* [24], both of which had been reared from *D. kuriphilus* galls.

Table 6: Results of the linear generalized mixed models: for each model (row) we present the variables entered with their corresponding significance LR test. All independent variables are treated as random factors, except for the number of cells per gall.

Dependent variables	Sampling date	GDD_{10}	Management	Site	Year	Number of cells/gall	AIC
% adults + % white and black pupae	* * *	* * *	*			* * *	1647.4
% adults + % black pupae	* * *	* * *	* * *				1419.2
% adults	* * *	* * *	* * *				779.5
Parasitized galls	* * *				* * *	* * *	871.1
Parasitized cells	* * *	* *			* * *		1851.8

* * * = $P < 0.001$; * * = $P < 0.01$; * = $P < 0.05$; no asterisks are shown for nonsignificant LR test results.

Bootanomyia dorsalis (formerly, *Megastigmus dorsalis*) (Fabricius), formerly accounted among Torymidae, was here listed as belonging to the new family Megastigmidae according to a recent review of Torymidae [40]. Three species of the Eurytomidae family were also found, though with a low number of specimens (only 12). As regards Eupelmidae, *Eupelmus urozonus* Dalman was the only species identified; however, it accounted for a considerable number of specimens (39 in total). All indigenous species recruited to the new host (ACGW) are idiobiont parasitoids mainly associated with oak gall wasps. Furthermore, *T. sinensis* was also observed, in low amounts, starting from 2012, and with increasing numbers in 2013. *T. sinensis* had been released in our study area in 2010.

Reared galls showed a high insect mortality in general and a higher parasitism rate in 2011 than in the two following years. High mortality was observed in both sites and for all years of study. ACGW mortality was particularly high, ranging from about 68% to about 82% during the three study years (Table 5). The overall mortality of parasitoids was less severe, varying from about 25% to about 44%. In 2011, the mean number of parasitoids per gall (dead, alive, and emerged) was 1.24, while in 2012 and 2013 it was 0.11 and 0.09, respectively (ANOVA, df1 = 2, df2 = 1665, $F = 288.504$, $P < 0.001$). This severe decrease was caused mainly by *T. flavipes* (Table 4). Indeed, 75% of observed parasitoids in 2011 were torymids, mostly *T. flavipes*. The abundance of this species decreased considerably in the following years, completely disappearing in 2013 (Table 5). In addition, other parasitoid species were also less abundant in 2012/2013, except for *E. urozonus*, which slightly (but significantly) increased, growing from 0.01 specimens per gall in 2011 to 0.04 and 0.02 in 2012 and 2013, respectively (*G* test, df = 2, $\chi^2 = 81.045$, $P < 0.001$).

During the May-July sampling period, not all parasitoid species were present at the same time. For example, every year, *B. dorsalis* emerged only from galls recorded in the last two sampling dates (late June-July). Similarly, 97% of *E. urozonus* specimens emerged from galls collected during the last sampling date. On the other hand, early species, like *M. fuscipes*, emerged only from galls recorded during the first two samplings. As expected, the percentage of parasitoids already emerged by the time of sampling increased over time, ranging from 0 during the initial stage of the survey (first two sampling dates) to 23.39% at the third sampling date and increasing up to 73.16% at the last sampling date.

3.4. Late ACGW Mortality. To compare ACGW natural mortality (parasitism excluded) with that recorded in reared galls, a mortality index (MI) was used (MI = number of dead ACGWs inside galls/total number of cells × 100). Since counting the number of cells inside withered winter galls is far less reliable, their total number for each year was estimated by multiplying the number of sampled galls by the average number of cells observed in frozen galls dissected during the same year (4.70 and 3.06, resp., for 2012 and 2013). We recorded a total number of 144 dead ACGWs in withered galls, with an MI of 1.98 and 35.95 in 2012 and 2013, respectively. The MI for reared galls was 73.37 in 2012 and 76.56 in the next year. After the *G*-test, both values were higher than the MI for whither galls (chi-squared test, df = 1, $\chi^2 = 1178.58$, $P < 0.001$ for 2012 and df = 1, $\chi^2 = 603.97$, $P < 0.001$ for 2013).

A similar index was used to compare parasitoid natural mortality with that recorded in reared galls [parasitoid mortality index (PMI) = number of dead parasitoids inside galls/total number of cells × 100]. In withered galls, the PMI was 0.33 in 2012 and 1.36 in 2013. More specifically, we tallied 19 parasitoid specimens (12 of which were still alive: 2 *T. sinensis*, 2 *E. urozonus*, 1 *E. brunniventris*, and 7 unidentified). However, PMI values in reared galls were 0.64 and 1.31 in 2012 and 2013, respectively. Thus, no significant difference was recorded in both years (in 2012 and 2013, resp., *G*-test, df = 1, $\chi^2 = 1.06$, P = ns and df = 1, $\chi^2 = 0.88$, P = ns).

3.5. Climate Effect on the ACGW and Its Parasitoids. In early April 2011, during chestnut bud burst, we observed higher average daily temperatures than in the following two years, with differences of 7–12°C for several consecutive days. In fact, during the first two weeks of April in 2011, temperatures climbed to about 18°C whereas in 2012 and 2013 they never exceeded 12°/14°C.

The GDD_{10} factor highly affects all the dependent variables, except the number of parasitized galls. Among the factors which affect ACGW development rate, such as "chestnut management" and the number of cells in each gall, the positive significant effect of the GDD_{10} factor was assessed. As regards parasitism, the number of parasitized cells per gall was significantly different among the three years and highly affected by the GDD_{10} factor (Table 6). Therefore, climate, as expected, affects ACGW development rate and, in addition, parasitism rate.

4. Discussion

The "enemy hypothesis" refers to the evolutionary mechanisms of gall formation in gall insects. According to this theory, gall formation is the result of a defence strategy against natural enemies, particularly predators, parasitoids, and pathogens [28]. Also, multilocular galls seem to be a response to the pressure of natural enemies, especially chalcid wasps [27]. Gall size may be an important evolutionary factor, helping gall wasps to escape parasitism, since host specimens inside larger galls suffer less parasitism than those in smaller ones [27, 28].

Our data show that parasitoids do not choose galls based on their size, though when they do parasitize smaller ones, they exploit them better. In fact, percentages of parasitized galls were similar in all size categories; however, the percentage of parasitized cells was significantly higher in smaller galls. This was particularly true in 2011, when an overall higher parasitism highlighted this difference. This result is in accordance with the "enemy hypothesis," as well as other studies which have found a negative correlation between gall size and parasitism rate [28, 41]. In fact, parasitoids need to reach cynipid larvae inside galls with their ovipositor to parasitize them, and clearly in larger galls some larvae are out of reach [26]. This apparently obvious result underlines an important aspect: ACGWs have higher chances of surviving parasitism if they are inside larger galls.

Furthermore, gall size also affects the parasitoid community composition. For example, some species with short ovipositor could be disadvantaged when galls are bigger. In our study, *E. urozonus* parasitism rates were lower in 2011 compared to the following years and this could in part be related to the bigger gall size of that year. However, other factors may have been involved, for example, the competition with *T. flavipes*, which was very abundant in that year, or the different climatic condition of 2011, which could have negatively affected *E. urozonus*, unlike what happened to the other species.

ACGW gall size is quite variable, and it depends on many factors: in our study, the average size of galls varied significantly depending on the sampling year and the management category of the chestnut trees. In 2011, we observed significantly larger galls than in the following two years; this fluctuation was confirmed by other studies [41, 42]. In addition, cultivated chestnuts have larger galls than wild chestnuts, as previously reported by others [28, 36, 42], exception being made for site B in 2011. Other factors affect gall size, such as the habitat [43], and ACGW population density: at high infestation levels, the number of available buds decreases, leading to more than one female ovipositing in the same bud [44], thus increasing gall size. Finally, climate conditions are also correlated with the galls' size [45]. However, in our study the gall size was highly variable across the different sampling years, sites, and management categories.

In addition to *T. sinensis*, we found a number of indigenous parasitoid species had shifted onto the new invasive host, which attack ACGW at different times. The exotic *T. sinensis* had been released in our sampling sites one yeas before our study began (2010), and we confirmed its establishment. However, we observed quite low parasitism rates (0.07% and 0.4%), in accordance with values recorded few years after its release in other areas of Italy [21]; conversely, in other countries, parasitism rates were very high even in the first year after release [23, 25]. The adaptation of native parasitoids to the ACGW in our study agrees with other researches ([7, 41, 46] and others). The majority of parasitoid species are bivoltine and associated with oak gall wasps [34]. These species attack the ACGW at different times of its development, as each natural enemy has a specific time window for effective exploitation of their main host, that is, the oak cynipid wasps [25].

This poses a problem in choosing the best time for gall samplings. In fact, an early sampling could lead to underestimating parasitism, because some of the parasitoid species attack the galls later. Conversely, a late sampling allows a more precise assessment of parasitism, as already emerged parasitoids can be counted (with minor underestimating) because of the exuviae left in the gall cells. However, late samplings make parasitoid species identification almost impossible because the exuviae alone are not enough to accurately identify already emerged adults. Thus, a single sampling may be misleading, while multiple samplings throughout the spring/summer period allow a more precise assessment of parasitism rates and parasitoid species. Furthermore, assessing parasitism only by observing insect emergence from reared galls may be misrepresentative. In fact, once brought to the laboratory, galls rapidly desiccate, with ensuing hardening of gall tissues [36]. This causes a higher mortality of the insects inside the gall, especially ACGWs [36, 42]. Our data confirm this for reared galls, as the ACGW mortality index rises from a maximum of 35.95 in withered galls to a maximum of 76.56 in reared galls. On the contrary, no significant difference was observed for parasitoid mortality index. It is worth noting that the parasitoid mortality index cannot be considered as a mortality rate in our study, because the total number of cells is generally higher compared to the total number of parasitoids inside galls. On the other hand, the ACGW mortality index may be considered as a mortality rate in our study (parasitism excluded), because the total number of cells reflects the potential total number of ACGWs. A similar number of dead ACGWs inside withered galls were reported by Cooper and Rieske [28], while the die-off in reared galls is highly variable [42]. In our study, the ACGW death rate was very high, probably due to the early samplings, which affected the survival of insects inside the galls. Thus, in order to accurately assess parasitism, dissection of the galls is necessary, and the number of emerged ACGWs must not be taken into account, as it could lead to incorrect results.

In our study, a remarkable parasitism rate was observed in 2011, especially compared to the other two years. In fact, 63% of the galls were parasitized in 2011, with a total parasitism rate of 36%, which dropped below 5% in the following years. In 2010, at the same sites, parasitism was as low as in 2012 and 2013, with a maximum parasitism rate of only up to 5% [42]. This is mainly due to *T. flavipes*, which accounted for 75% of all reared parasitoid specimens in 2011. In the following

year, its parasitism rate abruptly diminished and completely disappeared in 2013. Similar results were reported by Santi and Maini [46] and Francati et al. [18] during the same years in Emilia-Romagna, 20–30 km north from our study sites. In fact, 2011 parasitism rates in both studies were higher than in the following years. This fluctuation of native parasitoids' effect over time was confirmed also in Japan [47]. The higher number of parasitoid species observed in 2011 is also worth mentioning: they were 12 in 2011 and then seven and five in 2012 and 2013, respectively. In 2010, Panzavolta et al. [42] in the same study sites observed six parasitoid species in ACGW galls.

T. flavipes is the dominant ACGW native parasitoids in our study area and in other Italian regions [18, 46, 48]; therefore it has higher probability than the other species of interacting with the introduced *T. sinensis*. In addition, it has a great variety of hosts [49], it is closely related to *T. sinensis,* and no other native parasitoid (in Italy) has reached its parasitism rates. Also, *T. flavipes* emergence period may partially overlap with that of *T. sinensis* [48, 49]. Thus, it is most likely to interact with *T. sinensis* in various ways; for example, it could compete with it in case of low ACGW density, or interbreed with it, as already occurred in Japan with *T. beneficus* [50]. Native parasitoids may interact with *T. sinensis* in further ways, for example, through hyperparasitism.

Hyperparasitism in frozen galls was observed in 2013 at a very low level, though it was most likely underestimated. In fact, on 130 withered galls, 10 live parasitoids other than *T. sinensis* were found. Probably, they all acted as hyperparasitoids; in fact, as stated by Cooper and Rieske [5], live *D. kuriphilus* are no more present in desiccated galls, while they can host *T. sinensis*, which overwinters inside withered galls. Thus, facultative hyperparasitoids probably attack *T. sinensis* prior to its emergence. To confirm this hypothesis among the 10 parasitoids found in withered galls, two species are already reported as *T. sinensis* parasitoids, that is, *E. urozonus* and *E. brunniventris* [7].

The methodology adopted in this paper may lead to accurate results when studying the effectiveness of biological control programs with *T. sinensis*. In fact, gall dissection proved to be the most precise methodology to assess parasitism, while the number of emergence instances from reared galls is always misleading. Furthermore, by recording *E. urozonus* and *E. brunniventris* in withered galls, our study is a reminder of how hyperparasitism may affect *T. sinensis* effectiveness. Thus, before considering biological control programs with *T. sinensis*, a preliminary survey of ACGW galls should be carried out to verify the presence and the abundance of parasitoid species that, like the two mentioned above, may behave as hyperparasitoids. A high density of potential hyperparasitoids may hinder or even undermine biological control programs as it supposedly happened in some Japanese areas where *T. sinensis* efficacy was very low [4].

5. Conclusion

In our study, the year 2011 was notable for several reasons: larger galls, earlier ACGW development, higher parasitism rates, and higher number of parasitoid species recruited by the ACGW. All of these may be linked to climate, as in April 2011 higher temperatures, as well as lower rainfalls, compared to the following years, were recorded. These may have affected both the timing of ACGW development and the galls' size. Furthermore, native parasitoids, in contrast to *T. sinensis*, are not perfectly adapted to the ACGW, so it is likely that galls available earlier in the season have positively affected parasitism, in terms of both parasitism rates and species number. In fact, the number of parasitoid species observed in ACGW galls in 2011 was higher than both in 2010 [42] and in the subsequent two-year period (2012-2013). The increase in parasitism rates in 2011 was particularly noticeable for *T. flavipes*. This was confirmed by other studies [18, 46], which reported high parasitism by *T. flavipes* during the same year in areas only a few dozen kilometres away from our study sites. The dominant role of *T. flavipes* in parasitoid assemblages of ACGW was similarly observed in Slovenia and Croatia [30, 31].

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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