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Naturally Occurring *Trichogramma* Species in an Apple Orchard in Hungary

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Simple Summary *Trichogramma* wasps are tiny insects that can be used to control pests naturally. They do this by laying their eggs inside the eggs of pest insects, preventing the pest eggs from hatching and thus protecting crops. To use *Trichogramma* wasps effectively, it is important to know which species is best suited for a particular area. We can assume that naturally occurring species are well-adapted to a specific region and can be highly effective in organic pest control. This study aimed to identify the *Trichogramma* species naturally present in Hungary and to assess their parasitism rates. From the beginning of July until the end of September 2024, we placed traps containing pest eggs in an apple orchard and counted how many of those eggs were parasitised by *Trichogramma* wasps. We also monitored temperature and air pressure to investigate their potential influence on parasitism success. Our finding indicate that cooler temperatures favour the parasitism activity of *Trichogramma* wasps. The *Trichogramma* species identified in the orchard was *T. evanescens*. This suggests that *T. evanescens* is a promising candidate for use in biological pest control programs in Hungary, and potentially in other regions with similar temperature climates, offering a way to protect crops without the need for harmful pesticides.

Abstract: The genus *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) includes insect egg parasitoids widely used worldwide as biological control agent of pests. The success of these parasitoids in pest management depends, among other factors, on their adaptation to the climatic conditions of the release area, particularly temperature. This study aimed to identify the *Trichogramma* species naturally occurring in Hungary. Parasitism was observed by trapping *Trichogramma* spp. individuals using bait cards containing *Sitotroga ceraella* eggs (provided by the AMW company) in apple orchard between July 8, 2024, and September 30, 2024. We evaluated the relationship between several variables – the rate of cards with observed parasitism, the parasitism rate, the number of host eggs and the hatching rate – and the time of release, the age of the host eggs and meteorological factors. We found that the parasitism rate significantly depends on the time of release. Pearson's correlation coefficients indicated that temperature parameters (average minimum, maximum and the mean temperatures during the exposure period of the cards) are significantly and negatively correlated with both parasitism rate and number of parasitised eggs. Air pressure showed a significant positive correlation with the number of parasitised eggs. The naturally occurring egg parasitoid species was identified as *T. evanescens*. These results suggest that these parasitoids are well adapted to the local climate and can be considered as candidates for pest management programs in Hungary and in other countries with a temperate continental climate.

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Keywords: *Trichogramma*; native; insect parasitoids; host bait cards; biological control

1. Introduction

Trichogramma wasps (Hymenoptera: Trichogrammatidae) are egg parasitoid insects that primarily parasitise the eggs of Lepidoptera, including moths. However, certain species of *Trichogramma* also parasitise eggs of insects from other orders (Coleoptera, Diptera, Heteroptera, Hymenoptera, Neuroptera) [1].

Generally, most *Trichogramma* spp. individuals are between 0.4 and 0.6 mm long, with females being slightly larger than males [2]. *Trichogramma* wasps have haplodiploid sex determination: unfertilised eggs develop into males, while fertilised eggs develop into females [3–5]. The female wasp locates host eggs with chemicals and visual clues. The chemical clues, called kairomones, are on the moth scales that the female moth leaves near the egg during the oviposition [1,5]Female parasitoids obtain sugars and certain proteins by utilising floral and extrafloral nectar, pollen, and honeydew produced by insects [6]. Parasitoid female wasps can also obtain nutrients from the host by feeding on the egg fluids that ooze from the wounds inflicted by the ovipositor [7–10]. Host feeding is important for the fecundity of the parasitoids [11–13]. The larvae undergo three developmental stages and pupates inside the host eggs. Parasitised host eggs undergo a change in coloration, becoming black, which makes them easily recognizable [1,11]. *Trichogramma* spp. overwinter via quiescence, diapause or a combination of these two strategies as immature stages within their host eggs [12].

Trichogramma wasps naturally occur in a wide range of terrestrial and aquatic habitats. Some of the most harmful caterpillar pests of field crops, forests, and fruit and nut trees are subject to attack by *Trichogramma* spp. However, in the majority of crop production systems, the number of caterpillar eggs destroyed by native *Trichogramma* populations is insufficient to prevent the pest from reaching damaging levels [1]. The use *Trichogramma* wasps in biological control began at the turn of the 20th century. In 1930 Flanders [13] developed a method of rearing *Trichogramma* wasps on eggs of the Angoumois grain moth *Sitotroga cerealella* [14]. Natural enemies offer several advantages over chemical pesticides: (1) Toxic residues on plants and soil are eliminated. (2) Application is simple. (3) Resistance by pests is prevented. (4) They are effective if used correctly. (5) They are harmless to natural enemies of pests. (6) The production of these items is possible in any country, including those with restricted access to traditional techniques [11]. The success of *Trichogramma* wasps in crop protection is favoured by its very short generation times, simple mass rearing systems, sustained economic efficiency and technologies adapted for commercial use [11,15]. Initially *Trichogramma* spp. are being mass-reared to control pests on corn, sugarcane, rice, cotton, soybean, sugar-beet, vegetables and pine [11] Today, the *Trichogramma* wasps became an integral part of pest management strategies to control agricultural and forest pests throughout the world, including the Americas [16,17], Europe [18,19], Africa [20–22] and Asia [23,24]

The process of parasitism by insect parasitoids comprises a series of interconnected steps, the outcome of which is influenced by a multitude of intrinsic and external factors [25,26]. The parasitism characteristics of *Trichogramma* species (parasitism, hatching and female offspring rates) are influenced by a number of abiotic (temperature, light and photoperiod) [3,27,28] and biotic factors (host species, age of individuals, age and number of female wasps, available food source) [3,29,30].

There is a growing interest in using indigenous *Trichogramma* species for pest control, because they are thought to be better suited to the local climatic conditions [31] and there are concerns about releasing exotic species [32].

Apple (*Malus domestica* Borkh) is considered to be the world's oldest fruit crop. Apple is attacked by a large number of insect pests, including moths, such as the Codling Moth, *Cydia pomonella* and leaf miners [33]. In attempts to control *C. pomonella*, five species of *Trichogramma* wasps have been used: *T. platneri* Nagarkatti [34,35] *T. minutum* Riley and *T. pretiosum Riley* [36–38], *T. dendrolimi* Matsumura and *T. cacoeciae* (Marchal) [39,40].

The results show that *Trichogramma* wasps can be used effectively against a number of pests that are prevalent also in Hungary, such as Tomato Leaf Miner (*Tuta absoluta*) [41]; Tomato Fruit Borer, (*Helicoverpa armigra*) [42]; oriental fruit moth (*Grapholita molesta*) [43] and European corn borer (*Ostrinia nubilalis*) [19]. However, information about the efficacy of *Trichogramma* species in Hungarian apple orchards is limited. *T. evanescens* Westwood and *T. cacoeciae* Marchal species have been reported to infest *C. pomonella* and leafrollers [44–46].

The area under apple cultivation in Hungary is currently around 33,000 hectares [47]. Plant protection products based on *Trichogramma* species that are currently on the market are approved for use in maize and vegetable crops, and there is no *Trichogramma* product available for apple crops in Hungary [48]. Our goal is to survey the naturally occurring *Trichogramma* species in a Hungarian apple orchard. Our results could provide with the scientific basis for the development of a *Trichogramma*-based crop protection product in Hungary and other countries with a temperate continental climate.

2. Materials and Methods

2.1. Orchards/Location

The experiment was conducted in an apple (*Malus domestica*) orchard, located in the middle of the country, at Bács-Kiskun county, near to Kecskemét (GPS: 46°54'39.3"N 19°47'04.6"E). The age of the trees was 21 years, the row and stem distance were 4.5 x 2 m. The following varieties were present in the experimental area, with the varieties for which the bait cards were placed being marked in bold: Red Rose, **Ten Rose**, Ginson Golden, Mutsu, Regal Prince, **Idared**, Jonagold, **Gloster**, **Close**, Akane, **Vista Bella**. In addition to the mowing of plant vegetation between the rows, no other agrotechnical or plant protection methods were carried out in the experimental area and no previous applications of *Trichogramma* releases have been conducted. The experiment was set up between July 8, 2024, and September 30, 2024. The meteorological data were recorded at the National Meteorological Service meteorological station (GPS: 46°54'43.9"N 19°45'34.9"E).

2.2. Bait Cards

Folded cardboard bait cards containing *Sitotroga cereaella* eggs were used to collect the *Trichogramma* wasps. The bait cards and the eggs were provided by the company Appel-Melchior-Wührer (AMW, https://www.amwnuetzlinge.de/en/about-amw/about-us/). The age of the *Sitotroga cereaella* eggs used in the experiment ranged from one to four weeks. Prior to field use, the bait cards were stored at 6 °C to prevent *S.cerealella* eggs from hatching. The eggs were placed on the cards immediately prior to reliese (within 1 hour). To protect *S. cereaella* eggs from predators, the cards were covered with a net, that still allowed the *Trichogramma* wasps to enter.

2.3. Experimental Method/Field Collection

A total of 50 cards were placed at 10 different points on a weekly basis. The GPS coordinates of each placement point were recorded. The cards were replaced weekly. Following the collection of the bait cards, they were stored at room temperature (at 22–26 °C) for a further 2–3 days. Then, on the second or third day after collection, the eggs were examined under a stereomicroscope to check for parasitism. The parasitised eggs were closed in a 2 cm diameter, 4.5 cm high glass vial until the adults hatched. The hatched adults together with the parasitised but unhatched eggs were placed into 99% alcohol and stored in a freezer (at -20 °C) until molecular identification. The number of discovered cards (i.e. the number of cards containing at least one parasitised egg), the total number of eggs on the cards, the number of parasitised eggs, and the number of hatched individuals were recorded.

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2.4. Molecular Identification

2.4.1. Polymerase Chain Reaction (PCR) of ITS2 Region

Thermo Scientific Phire Tissue Direct PCR Master Mix was used to amplify the ITS2 region using primers described by Stouthamer et al. [49]. Dilution Protocol was chosen and 5 Individuals (preserved in 99.5% Ethanol, stored at -20 °C) were placed into 20 µL of Dilution Buffer, add 0.5 µL of DNARelease Additive and mixed by vortexing the tube briefly, and spin down the solution. Incubated the reaction for 2–5 minutes at room temperature and then placed the tube into the pre-heated (98 °C) block for 2 minutes. PCR reaction was performed in 20 µl volumes containing H2O (20 µl), 2X Phire Tissue Direct PCR Master Mix (10 µl), Primer A (1 µl), Primer B (1 µl), Sample (1 µl). The PCR cycling programme was 5 min at 98 °C, followed by 40 cycles of 5 s at 98 °C, 5 s at 62.2 °C and 20 s at 72 °C, with 1 min at 72 °C after the last cycle. PCR products were visualised after electrophoresis on 1.5% agarose gel stained with Midorii green Advanced, to confirm amplification.

2.4.2. Restriction Analysis

For the PCR products restriction enzymes were used to find specific differences between the samples. *Trichogramma* wasps were identified using a proprietary method developed by AMW.

2.5. Data Analysis

Discovery rate (%) was calculated as the rate of cards detected (i.e. the number of cards containing at least one parasitised egg divided by the total number of cards released). Parasitism rate was calculated as the ratio of parasitised eggs to the total number of eggs released. Hatching rate (%) was calculated as the number of hatched eggs divided by the total number of parasitised eggs. Discovery rates (%), parasitism rates, hatching rates (%), age of host eggs (days) and the numbers of host eggs (pcs) were discretised according to Table 1.

From the daily data of mean, maximum and mean temperatures (°C), mean relative humidity (%), mean wind speed (m/sec) and mean air pressure (hPa), we calculated their averages in relation to the time intervals of the cards released.

We used a Chi-square test to find out whether parasitism rate is independent from the age of the eggs, the time of release, the number of the host eggs. Having significant result, we used adjusted standardised residuals (AdjRes) to detect under- and over-represented cases, with the criteria of the values being above 1.96 or below -1.96, respectively.

We used IBM SPSS Statistical software (v 29, Armonk, New York, 2023).

Table 1. Categorisation of data for statistical analysis.

	Group Category Values					
Group Category	0	1	2	3	4	5
Discovery rate* (%)	0	1–5	6–15	>16		
Parasitism rate†	0	0.001–0.1	0.101–0.4		-	-
Hatching rate (%)‡	0	0–95	96–99	100		-
Date of the placement	-	08.07.2024–13.08.2024	14.08.2024–17.09.2024	18.09.2024–01.10.2024	-	-
Age of host eggs (days)	-	1–7	8–14	>14	-	-
Number of host eggs (pcs)	-	1–100	101–200	201–300	301–400	>400

* the number of cards containing at least one egg parasitised by *Trichogramma* wasps divided by the total number of cards released (%). † the ratio of parasitised eggs to the total number of eggs released. ‡ the number of hatched eggs divided by the total number of parasitised eggs.

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3. Results

First, we tested whether the placement points have a spatial block effect on parasitism rate or the hatching rate. Chi-square test indicated that both the parasitism and the hatching rates were independent from the placement of the cards ($\chi^2(9)=6,221$; $p=0.718$; $\chi^2(27)=15.591$; $p=0.960$, respectively).

3.1. The Dependence of Discovery Rate and Parasitism Rate on the Time of Release

Chi-square test indicated that the discovery rate was significantly dependent on the time of release (Fisher's test value=10.49; $p<0.05$). According to the adjusted residuals, medium discovery rates (between 6–15%) were significantly more frequent between 21 August and 17 September 2024 (AdjRes >2) while high discovery rates (above 15%) were significantly more frequent between 24 September and 1 October 2024 (AdjRes >3).

The Chi-squared test showed that the detection rate was significantly dependent on the time of release ($\chi^2(4)=18.549$; $p<0.001$). According to the adjusted residuals, the low parasitism rates (between 0.001–0.1%) were significantly more frequent between 8 July 2024 and 13 August 2024 (AdjRes >2), while the high parasitism rates (between 0.101% and 0.4%) were significantly less frequent in this period (AdjRes <-2). In autumn, between 18 September and 1 October 2024, the high parasitism rates (between 0.101 and 0.4%) were significantly more frequent (AdjRes >2), while the zero-parasitism rate (0%) was significantly less frequent (AdjRes <-2).

3.2. Relationship Between Parasitism Rate and the Number and Age of the Host Eggs

Chi-square test indicated that the parasitism rate is independent from both the number and the age of the host eggs ($\chi^2(8)=4.617$; $p=0.798$; $\chi^2(4)=5.511$; $p=0.24$, respectively).

3.3. Relationship Between Hatching Rate and Time of Release and Age of the Host Eggs

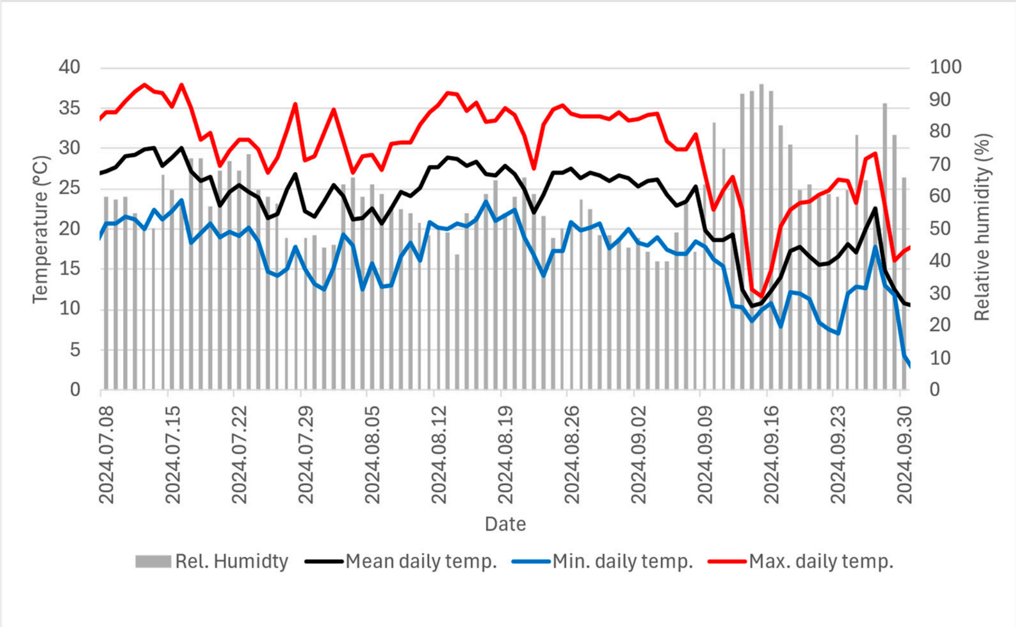
Cases where parasitism was 0 were excluded. Chi-square test indicated that the hatching rate is not independent from the time of release ($\chi^2(6)=26,816$; $p<0.001$). According to the adjusted residuals, the low hatching rate (0%) was significantly more frequent between 8 July 2024 and 13 August 2024 (AdjRes >2) while later, between 17 September and 1 October 2024, high hatching rate (95–99%) was significantly more frequent (AdjRes >2).

Chi-square test indicated that the hatching rate is independent from the age of the host eggs ($\chi^2(6)=4.926$; $p=0.553$).

3.4. Relationship Between Parasitism, the Number of Parasitised Eggs and Meteorological Factors

The results of the correlation analysis between meteorological factors (Figure 1) and the parasitism rates with the number of parasitised eggs (Figure 2) are presented in Table 2.

A



B

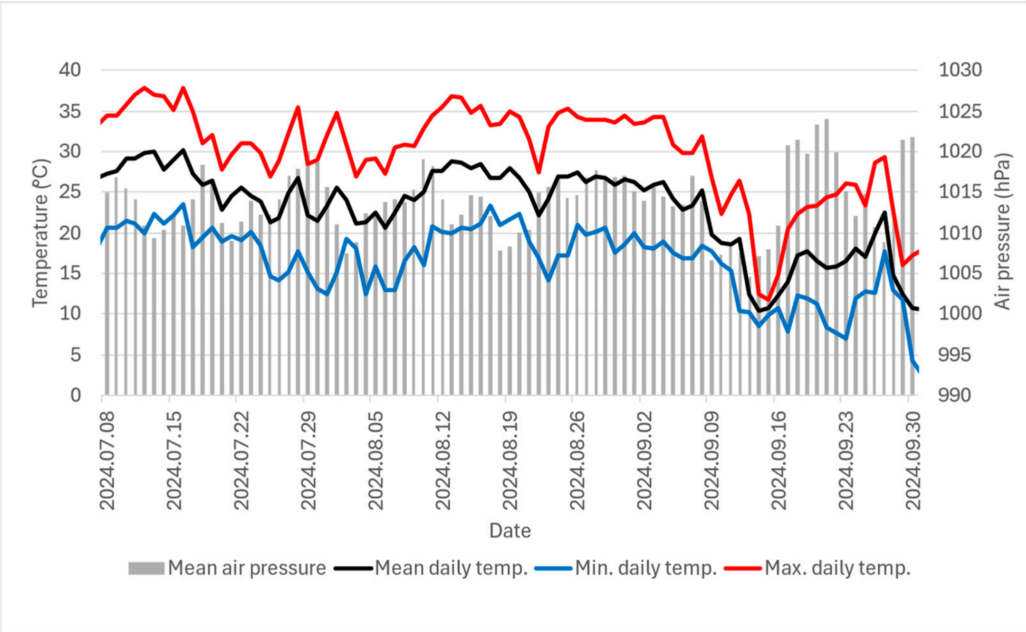


Figure 1. Meteorological data (mean daily temperature (°C); minimum daily temperature (°C); maximum daily temperature (°C); relative humidity (%) (A) and mean barometric pressure (hPa) (B) during the experimental period (8 July – 30 September 2024); Source: https://www.met.hu/rolunk/tevekenysegek/adattar/.

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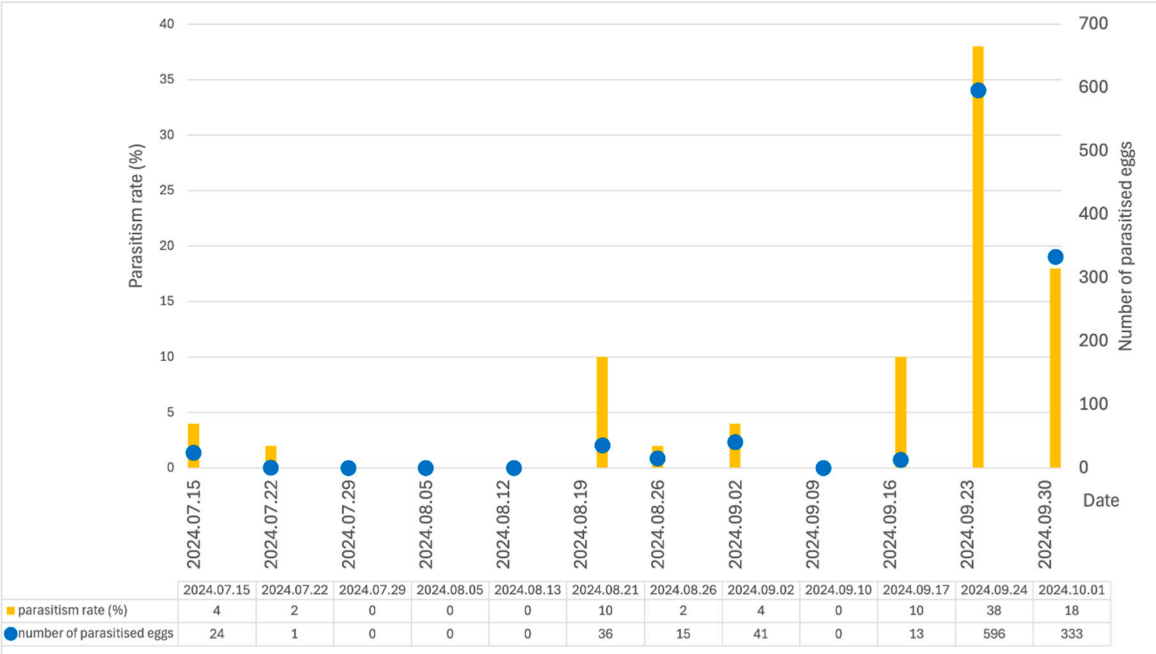

Figure 2. Parasitism rate (%) and number of parasitised eggs plotted at collection time (15 July – 1 October 2024).

Table 2. Correlation between parasitism rate (%), the number of parasitised eggs and the average meteorological parameters during the time of release: Pearson's correlation coefficient (R), test value of t-test of the significance of R with degree of freedom (t; df) and significance value (p). The only positive significant correlation is highlighted in bold.

		Average mean temperature* (°C)	Average minimum temperature* (°C)	Average maximum temperature* (°C)	Average relative humidity* (%)	Average wind speed* (m/sec)	Average air pressure* (hPa)
Parasitism rate (%) †	R	-0.64	-0.60	-0.75	0.46	-0.19	0.49
	t (df=10)	2.64	2.38	3.59	1.62	0.60	1.78
	p	0.025	0.039	0.005	0.136	0.559	0.106
Number of parasitised eggs	R	-0.61	-0.65	-0.70	0.33	-0.16	**0.67**
	t (df=10)	2.46	2.74	3.06	1.09	0.50	2.83
	p	0.033	0.021	0.012	0.301	0.625	0.018

† the ratio of parasitised eggs to the total number of eggs released; * average values are calculated from the daily data of mean, maximum and mean temperatures (°C), mean relative humidity (%), mean wind speed (m/sec) and mean air pressure (hPa), in relation to the time intervals of the cards released.

Pearson's correlation coefficients indicated that the three temperature parameters (minimum, maximum and average temperatures during the time of the release of the cards) are significantly negatively correlated with both parasitism rate and the number of parasitised eggs. Meanwhile, air pressure has a significant positive correlation with the number of parasitised eggs. For relative humidity and wind speed, no significant correlation was found with either parasitism rate or the number of parasitised eggs.

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4. Discussion

In the order Trichogrammatidae, there are about 620 described species worldwide [23,25].

The natural occurrence of the *Trichogramma* species has been demonstrated in many locations worldwide, such as *T. bourarachae* Pintureau and Babault, *T. cordubensis* Vargas and Cabello, *T. pretiosum* and *T. cacociae* from Egypt [50], *T. bourarache*, *T. cordubensis*, *T. evanescens*, *T. pintoi* Voegele, and *T. turkestanica* from Portugal [51]. In the studies of Souza et al. [52] in total, 2,242 specimens of *Trichogramma* species were obtained, belonging to the species *T. pretiosum* Riley, *T. manicobai* Brun, Moraes & Soares, *T. marandobai* Brun, Moraes & Soares, and *T. galloi* Zucchi in Brazil.

In our studies, natural parasitism of *Trichogramma* species was observed by trapping with bait cards containing host eggs. The egg parasitoid species was identified as *T. evanescen*s.

T. evanescens has been shown to occur naturally in many places around the world, including northern Moldova [53] , Portugal [51] and Russia [53]. In Europe Bírová [54,55] studied the natural parasitism of European Corn Borer (*Ostrinia nubilalis*) eggs by *Trichogramma* wasps in Slovakia as early as the late 1950s and early 1960s.

Bírová observed 65.4% parasitised European Corn Borer eggs in 1956, 49.8% in 1957 and 15.7% in 1958 and found very low parasitism in 1980–1985 [56]. The results of Cagán et al. [57] show that the percentage of natural parasitism of *O. nubilalis* eggs in south-western Slovakia is generally low, but can reach 20% or more depending on the year. Depending on the habitat, *T. cacoeciae, T. embryophagum, T. cephalciae, T. minutum* species have been identified in Czechoslovakia [58]. *Trichogramma evanescens* was observed by Ram [53] also in the eggs of *O. nubilalis* in Slovakia. The occurrence of four native species, *T. cacoeciae, T. daumalae* Dugast and Voegelé, *T. evanescens* and *T. principium* Sugonjaev and Sorokina were mentioned in a French vineyard by Barnay et al. [59]. In Germany [60], the survey of naturally occurring egg parasitoids using "trap cards" showed that only *Trichogramma evanescens* was present.

There are few literatures available on naturally occurring *Trichogramma* species in Hungary. Dudich [61] studied naturally occurring *Trichogramma* species on European Corn Borer (ECB) in and found no parasitism of *Trichogramma* spp. on ECB eggs. Nagy [62] confirmed his result and found no more than 1% parasitism in the region 30 km south-west of Budapest in 1976–1982.

In our studies, the activity of *Trichogramma* by trapping bait cards containing *Sitotroga cereaella* eggs was observed from July until September. *Trichogramma* wasps showed the greatest activity in the second part of September. When Barnay et al. [59] studied the spontaneous occurrence of *Trichogramma* wasps in a French vineyard, the results showed that April–May, July and September were the periods when *Trichogramma* individuals were the most active. Sengonca and Leisse [63,64] found similar results in Germany, where *T. semblidis* Auriv. was observed more frequently from mid-May to mid-June and, after a decline, egg parasitism increased again in September and October. In Germany, however, Kot [65] found that the *Trichogramma* population in an orchard was very high in July, August and the first half of September compared with the spring population. Similarly, in 1962, Bírová [55] observed maximum occurrence of the *Trichogramma* parasitism in Slovakia during the second and the fourth pentad of July.

As suggested by Kot [65], the degree of infestation of cards may not accurately reflect the number of *Trichogramma* wasps. If natural host eggs are abundant, *Trichogramma* wasps may not be eager to parasitise factitious hosts. Cagán et al. [57] found differences between parasitism rates in different years. In 1993, when no eggs were parasitised, there was very hot and dry weather in May, which probably affected the development of the parasitoid. These data suggest that extremely dry weather can have a negative impact on egg parasitoid populations.

In our studies, *Trichogramma* wasps showed the greatest activity in the second half of September. The results can be explained by the correlation between parasitism and meteorological data. We found that Pearson's correlation coefficients indicate that the three temperature parameters (minimum, maximum and the average temperature during the time of release) are significantly negatively correlated with both parasitism rate and number of parasitised eggs. Air pressure has a significant positive correlation with the number of parasitised eggs. For relative humidity and wind

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speed, no significant correlation was found with either parasitism rate or number of parasitised eggs. Between 08 July and 17 September, the parasitism rate was low (0–0.1%). During this period, the daily maximum temperature exceeded 34 °C and in some weeks even 37.5 °C. The temperature decreased between 10 and 17 of September, when the maximum temperatures dropped by about 10 °C. In the following two weeks, totally 596 and 333 parasitised eggs were found. Our results are consistent with those of Zouba et al. [66], who investigated the effect of the temperature sensitivity of four *Trichogramma* species. All *Trichogramma* species were able to develop and survive from 25 °C to 35 °C , but not at 40 °C. Results showed that temperature affects the lifespan and fertility of the female progeny, with both decreasing as temperature rises from 25 °C to 35 °C. In their laboratory tests, Schöller and Hassan [67] established that the parasitism of the *T. evanescens* decreased with increasing temperature, but *T. evanescens* was still able to parasitise hosts at 35 °C. These results support the results of our study, where we found 24 parasitised eggs per week even when the daily average maximum temperature reached almost 38 °C. In contrast to these results, Mohammad et al. [68] found that no development could be observed for *T. evanescens* at 35 °C . They reported that the optimum temperature for the development of *T. evanescens* ranged between 22 and 27 °C. Based on the results of Metwally et al. [69], we can estimate the optimum temperature for rearing *T. evanescens* as 30 °C.

In our results, air pressure has a significant positive correlation with the number of parasitised eggs. Fournier et al. [70] evaluated the effect of barometric pressure changes on flight initiation of female *T. evanescens*. The *Trichogramma* wasps did not respond to stable or slow changes in barometric pressure, but rapid barometric changes significantly reduced the flight initiation.

Information on naturally occurring species is needed for planning biological control projects. Natural populations of *Trichogramma* species could be observed in apple orchard in Hungary. However, their natural pest control potential will not be sufficient in itself to protect the crop, as their natural parasitism rate is low during the hot summer months when the average daily temperature is close to 29 °C. Nevertheless, the fact, that *T. evanescens* occur naturally in Hungary suggests that these parasitoids are well adapted to the local climate. Consequently, *T. evanescens* may be considered as potential candidates for inclusion in integrated pest management programs.

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Conflicts of Interest: The authors declare no conflicts of interest.

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