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Reproductive traits in *Torymus sinensis*, biocontrol agent of the Asian chestnut gall wasp: implications for biological control success

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Abstract

Torymus sinensis Kamijo is the main parasitoid associated with the Asian chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu. It was released as biocontrol agent in Asia and many European countries in order to contain the gall wasp outbreaks. It is reported as univoltine, but it also exhibits a prolonged diapause mainly as late instar larva.

In the two-year period (2014-2015) we investigated longevity, egg load, ovigeny strategy, and body metrics of *T. sinensis* under laboratory conditions in order to assess the potential fecundity and the effects of age on the egg load, considering univoltine and diapausing adult wasps.

T. sinensis females reached a maximum age of 102 days as univoltine and 73 days as diapausing adults. Newly emerged adult wasps contained 0.5 ± 0.31 and 0.9 ± 0.60 mature eggs in the univoltine and diapausing population, respectively. The total mean egg load (mature + immature oocytes) recorded for the univoltine females at day 0 was 215 ± 2.43 whilst for the diapausing females was 240 ± 2.31. The ovigeny index calculated for univoltine wasps was 0.0023 and 0.0041 for diapausing ones, indicating for both populations an extremely synovigenic behaviour. Except for the first two weeks, diapausing females had a higher egg load that univoltine. Univoltine males, females and their eggs showed to be significantly larger than diapausing ones. A decrease in egg load with an increase in adult female age was found in both populations, probably due to egg resorption. This study demonstrates that the aging of parasitoids plays an important role concerning the egg load, influencing the effectiveness of *T. sinensis* in the context of biological control programs, and suggesting that 2-3 week-old females have to be preferred during the release stage in order to maximize parasitism. Implications of these reproductive traits for the success of *T. sinensis* as biological control agents are discussed.

Key words: egg load, ovigeny strategy, body metrics, univoltine and diapausing populations, Asian chestnut gall wasp.

Introduction

Torymus sinensis Kamijo (Hymenoptera Torymidae) is the main parasitoid associated with the Asian chestnut gall wasp (ACGW), *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera Cynipidae) (Murakami, 1981; Rieske, 2007).

T. sinensis reproduces amphigonycally, but it may reproduce by arrhenotokous parthenogenesis if there is lack of mating. It is reported as univoltine, like its host (Moriya *et al*., 1990). However, recent investigations carried out in NW Italy highlighted that a small fraction of the insect population my undergo a prolonged diapause extended for 12 months, mainly as late instar larva, probably as a response to host shortage (Ferracini *et al*., 2015a).

This parasitoid was released as biocontrol agent in Japan in 1975, and in the USA in 1977 to control the ACGW populations (Moriya *et al*., 2003; Cooper and Rieske 2007; 2011). In Europe after the report of the ACGW in 2002 in NW Italy (Brussino *et al*., 2002) and due to the importance of chestnut throughout the country for fruit and wood production, it was released on a nationwide scale. In addition to Italy, classical biological control with the release of this parasitoid was performed in France, in Croatia, Slovenia and Hungary (Quacchia *et al*., 2008; Borowiec *et al*., 2014; Matošević *et al*., 2015), and more recently in Spain and Portugal (Paparella *et al*., 2016).

The introduction of this parasitoid is widely known as one of the typical successful cases of classical biological control in Japan and Italy as well. In fact for this latter country it proved to settle successfully in the chestnut-growing areas in NW Italy, significantly reducing the gall wasp outbreaks after 10 years from its first release (Alma *et al*., 2014; Ferracini *et al*., 2015b).

Besides that, *T. sinensis* was object of other field and laboratory studies to improve knowledge on its life history and ecological traits, assessing its host range which might not be limited only at *D. kuriphilus* (Ferracini *et al*., 2017), its dispersal capability (Colombari and Battisti, 2015), and systematics deepening its relationship with closer and congeneric species (Yara *et al*., 2007; 2012; Francati *et al*., 2015; Colombari *et al*., 2016).

The reproductive biology of a parasitoid is crucial when evaluating its potential as biological control agent and developing a fitting integrated pest management (IPM) (Donaldson and Walter, 1988; Godfray, 1994; Jervis and Copland, 1996), since the number of mature oocytes in the ovaries (egg load) may influence many aspects of parasitoid biology (Eliopoulos *et al*., 2003).

The egg load as well as the time required for finding suitable hosts and ovipositing represent limitations to parasitoid reproduction (Rosenheim, 1996; 1999; Sevenster *et al*., 1998). Time limited species often have a high proportion of mature eggs or can quickly replace their egg supply, therefore their reproductive success is related to the number of hosts they are able to attack during their lifetime (Stephens and Krebs, 1986; Charnov and Stephens, 1988; Van Baalen, 2000; Diaz-Fleischer *et al*., 2015).

Feature that can determine parasitoid behavior is the ovigeny strategy which can be proovigenic or synovigenic (Jervis and Copland, 1996; Quicke, 1997). This trait is usually described as ovigeny index (OI) (Jervis *et al*., 2001) which measures the degree of early-life concentration of egg production. This index refers to proovigeny when close to 1 and to synovigeny when close to 0. While proovigenic species have a full complement of mature eggs at eclosion, synovigenic parasitoids do not have a full complement of eggs at eclosion maturing them throughout adult life, and in case of food scarcity are known to be able to reabsorb mature eggs and reallocate resources (egg resorption) using them for somatic maintenance (Jervis and Kidd, 1986; Bernstein and Jervis, 2008).

In insect, fecundity is generally a function of time, increasing after the onset of reproduction and decreasing with females' age, and is strongly correlated with body size, food quality and ambient temperatures (Kindlmann *et al*., 2001).

Both egg limitation and time limitation can lead to missed reproductive opportunities (Dieckhoff *et al*., 2014). Females are therefore predicted to balance the risk of becoming egg- or time-limited to maximize lifetime fecundity (Minkenberg *et al*., 1992; Rosenheim, 1996; Heimpel and Rosenheim, 1998; Rosenheim *et al*., 2008).

Egg limitation is mediated by oviposition, egg maturation and resorption rates (Papaj, 2000; Richard and Casas, 2012). In synovigenic insects egg maturation rate may respond to internal conditions such as egg load or external factors such as host density in ways that minimize the risk of egg limitation. Eggs are replenished as they are laid, so that consequently egg load oscillates (Dieckhoff and Heimpel, 2010).

The purpose of this study was to evaluate longevity, egg load, ovigeny strategy, and body metrics in the parasitoid *T. sinensis* under laboratory conditions in order to assess its potential fecundity and the effects of age on the egg load. Since recent investigations highlighted that the wasp may undergo an extended diapause and the presence of mature eggs was already confirmed in second year emergence individuals by Ferracini *et al*. (2015a), research was carried out comparing a univoltine and a diapausing population.

Materials and methods

Insect culture

Investigations were carried out in laboratory conditions in the two-year period (2014-2015). Galls were randomly collected during winter according to the method described by Ferracini *et al*. (2015a) in chestnut-growing areas of NW Italy with a stable population of *T. sinensis*, individually isolated in plastic vials (120 mm in length by 25 mm in diameter), and kept in outdoor conditions until *T. sinensis* emergence. Collections were performed during winter 2014 and 2015 in order to have two different populations emerging in the spring 2015: univoltine (adults emerged from galls collected in 2015) and diapausing (adults emerged from galls collected in 2014; the univoltine adults emerged during spring 2014 were not considered). 60 newly emerged females were sorted out every day for one month, generating 30 age cohorts for both populations. They were placed into plastic vials (120 mm in length by 25 mm in diameter), and fed with honey drops changed weekly. The parasitoids were maintained in climatic chamber under controlled conditions (15 ± 1 °C, 60 ± 5% RH, and a photoperiod of 16:8 L:D), and kept till dissection or natural death, this latter in order to determine the maximum longevity in captivity.

Twenty adults randomly chosen (ten males and ten females) were killed upon emergence with ethyl acetate and the scutellum width (maximum width), the right hind tibia length, and the ovipositor sheath length (mm) were recorded, comparing univoltine and diapausing *T. sinensis* (figure 1A-B-C). Measurements were taken using a Leica MZ16A stereomicroscope (50 × magnification) with the software LAS version 3.7.0.

Identification of *T. sinensis* specimens

For every age cohort of each population (univoltine and diapausing) ten *T. sinensis* adults randomly chosen (five males and five females) were submitted to DNA extraction and then sequenced for the cytochrome oxidase I (COI) gene following Kaartinen *et al*. (2010) to confirm their morphological identification.

Egg load quantifying and ovigeny strategy

Ten females for each age cohort were collected daily and killed with ethyl acetate. The specimens were placed on a microscope slide (25 × 75 × 1 mm) into a phosphate buffered saline (PBS) solution for ovary dissection. Egg load was assessed by dissecting each female under a stereomicroscope at 25 × magnification. The abdomen was separated from the rest of the body using a couple of needles. Ovaries were gently removed from the abdomen by means of micro-pin needles and spread on a slide for egg load counts. In order to distinguish the development stage of eggs in the ovaries we classified them into two stages based on yolk deposition. When the yolk deposition was complete into the egg chamber and the egg was provided with a clear apical pedicel and no nurse cells were present, it was defined as mature egg. When the yolk deposition was incomplete or not clearly visible into the oocyte and the egg shape did not show the pedicel and the nurse cells were still present, it was defined as immature (figure 1D-E-F). The number of mature and immature eggs was counted under microscope at 80 × and 115 × magnification, respectively.

Ovigeny strategy was determined on twenty females following the criteria described by Jervis *et al*. (2001) by recording lifetime potential fecundity (FEC), meant as the total oocyte load (mature + immature oocytes) of newly emerged females (within 12 hours) and the initial egg load (IEL) to calculate ovigeny index (OI).

In both univoltine and diapausing populations, egg size was also measured under a microscope by the measure module of the software LAS version 3.7.0. Maximum length and width of thirty eggs were measured.

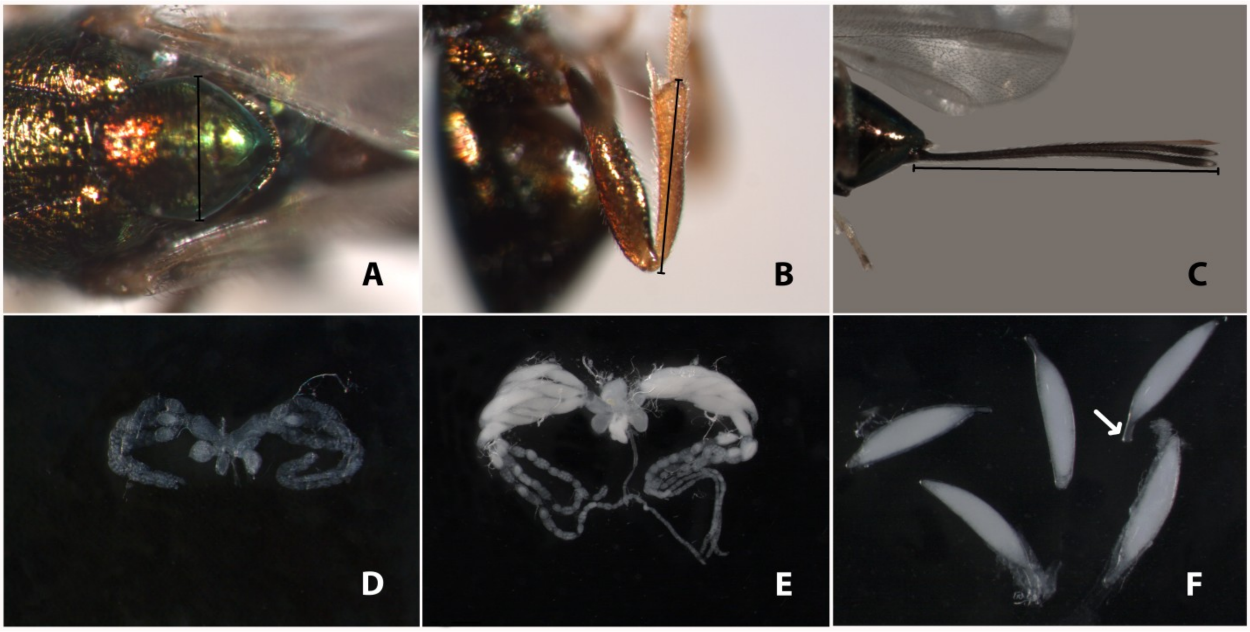

Figure 1. Morphometric measurements and ovaries dissection of *T. sinensis* specimens: (**A**) scutellum width, (**B**) hind tibia length, (**C**) ovipositor sheath length, (**D**) dissected ovaries at day 0, (**E**) dissected ovaries at highest egg load, and (**F**) individual mature eggs with pedicel (white arrow indicating the pedicel).

Statistical analysis

After testing for homogeneity of variance (Levene test, $P < 0.05$), data were analyzed by T test independent samples ($P < 0.05$) to compare records obtained in different populations. All analyses were performed using the software SPSS version 20.0 (SPSS, Chicago, IL, USA).

Results

All the morphologically analyzed univoltine and diapausing specimens were identified as *T. sinensis*. The cytochrome oxidase I gene obtained submitted to molecular identification was sequenced and sequences were compared with those in the National Center for Biotechnology Information (NCBI) sequence database. In all cases, a minimum of 99% similarity with *T. sinensis*-related sequences was observed.

Longevity of univoltine population reached a maximum of 102 days while the longevity of diapausing population reached 73 days; that is why 11 weeks were considered in order to compare the two population mean egg mature number (figure 2A).

Microscope diagnosis of egg production over lifetime of the two populations showed that the newly emerged specimens lack almost completely mature eggs, with an average of 0.5 ± 0.31 in univoltine and 0.9 ± 0.60 mature eggs in diapausing, at day 0. In the following days the number of mature eggs increased rapidly reaching the highest average number, 25 ± 1.37 at day 8 in univoltine with a maximum value per individual of 30 eggs, and 25 ± 0.84 at day 16 in diapausing with a maximum value per individual of 37 eggs (figure 2B).

On average the total egg load (mature + immature oocytes) recorded for the univoltine females at day 0 was 215 ± 2.43 whilst for the diapausing females was 240 ± 2.31.

The egg load trend over lifetime was obtained in laboratory conditions. No significant difference was observed in the mean number of mature eggs between univoltine and diapausing populations, 12.2 and 5.34 respectively were found in the first week ($t = -1.861$, $df = 8.993$, $P = 0.096$) and 6.42 and 7.60 respectively ($t = 0.812$, $df = 4$, $P = 0.462$) in the 11th week. Conversely, from the second to the tenth week significant differences were found (figure 2A).

The OI calculated for univoltine wasps was 0.0023 and 0.0041 for diapausing ones.

The adult metrics showed significant differences among females and males of the same population and in different population (table 1). Univoltine females are significantly larger than the males of the same population (tibia $t = 4.738$, $df = 48$, $P < 0.001$; scutellum $t = 3.960$, $df = 48$, $P < 0.001$) as well as for the diapausing specimens (tibia $t = 3.769$, $df = 48$, $P < 0.001$; scutellum $t = 4.167$, $df = 48$, $P < 0.001$). Moreover univoltine females and males were significantly larger than diapausing ones (females: tibia $t = -7.174$, $df = 48$, $P < 0.001$; scutellum $t = -3.337$, $df = 48$, $P = 0.002$; ovipositor sheath $t = -3.690$, $df = 48$, $P = 0.001$ and males: tibia $t = -3.920$, $df = 48$, $P < 0.001$; scutellum $t = -3.278$, $df = 48$, $P = 0.002$).

Eggs from univoltine females were significantly larger than from diapausing ones (length: $t = -2.163$, $df = 52$, $P < 0.035$; width: $t = -5.002$, $df = 52$, $P < 0.001$) (table 1).

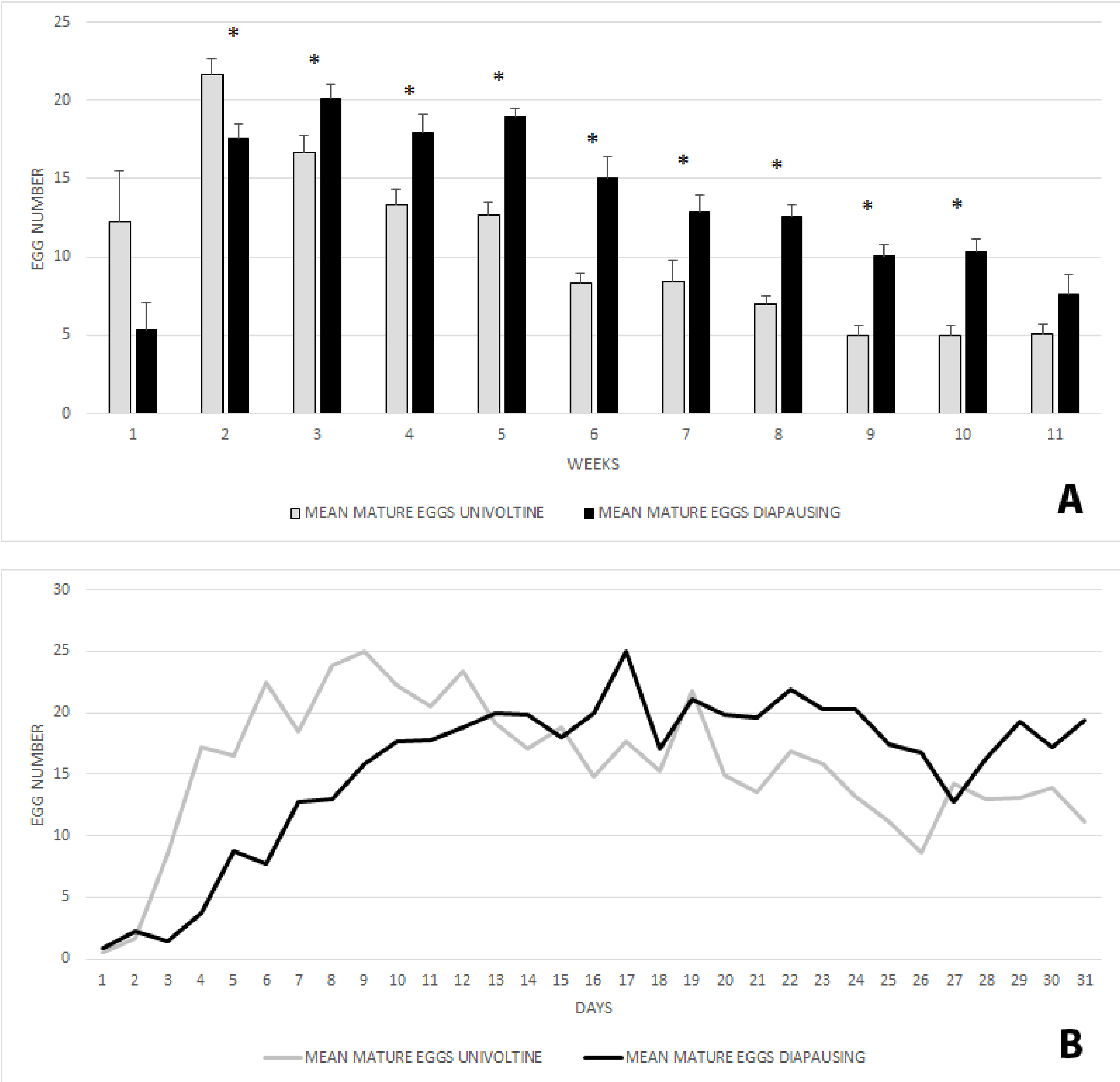

Figure 2. Mean number (± SE) of mature eggs between univoltine and diapausing *T. sinensis* populations expressed on a weekly (**A**) and daily scale (**B**). Bars topped by the asterisk represent means that are statistically different (T test independent samples; P < 0.05)

Table 1. Average (± SE) hind tibia length, scutellum width, and ovipositor sheath length (mm) (N = 25), and egg size (N = 30) in univoltine and diapausing *T. sinensis* populations.

Gender	Population	Ovipositor sheath length (mm) ± SE	Scutellum width (mm) ± SE	Hind tibia length (mm) ± SE	Egg size	
					Maximum length (mm) ± SE	Maximum width (mm) ± SE
Male	Univoltine	-	0.347±0.0074**	0.629±0.0160***		
Male	Diapausing	-	0.312±0.0073**	0.551±0.0121***		
Female	Univoltine	1.672±0.0455***	0.393±0.0089**	0.731±0.0142***	0.479±0.006*	0.123±0.003***
Female	Diapausing	1.475±0.0279***	0.355±0.0070**	0.608±0.0094***	0.457±0.008*	0.104±0.002***

Student's t tests were performed on data referred to males and females separately; within the same column values followed by the asterisk are significantly different (*P < 0.05, **P < 0.01, ***P < 0.001)

Discussion

Reproductive traits of parasitoids are crucial when developing biological or IPM programs.

Many factors commonly contribute to the success of biological control plans in particular searching efficiency, mobility, longevity, and fecundity. In particular this last plays an important role, and parasitoid species can be divided into two fundamental physiological categories, depending on the availability of ready mature oocytes at birth or on their formation during adult life.

The results presented in this research showed that *T. sinensis* egg load is strongly < 1 into early females being the ovigeny index calculated, 0.0023 for univoltine and 0.0041 for diapausing wasps, indicating for both populations an extremely synovigenic behaviour. Parasitic hymenoptera rarely show a proovigeny, except within Cynipoidea, as they are considered primarily synovigenic (Jervis *et al*., 2001; 2008). This characteristic is known to be connected to the adult need of feeding during the adult life (Jervis *et al*., 2001). Parasitoid wasps usually feed on various sources in the field: floral nectar, homopteran honeydew, pollen and so on. The food is meant to be used for storage, ovigenesis, somatic maintenance and locomotion (Clarke and Smith, 1967; Rivero and Casas, 1999a; 1999b). This adaptive strategy implies the ability of adapting egg availability to variation in oviposition opportunities (Bernstein and Jervis, 2008). Furthermore synovigeny, in the presence of sugar-rich foods, could increase pest suppression since the time spent searching for nourishment should not limit the daily number of hosts (Mills and Wajnberg, 2008).

The contribution of feeding to survival and fecundity is well documented for a diverse range of parasitoids reared in laboratory conditions and several of this species are known to be synovigenic (Jervis and Kidd, 1986; Heimpel and Collier, 1996; Jervis *et al*., 2001). It is also well documented that some parasitoid species are able to reabsorb mature eggs and redirecting energy towards other physiological needs like survival, host seeking and new eggs maturation. Egg resorption may also serve to maintain a supply of freshly mature eggs (Collier, 1995; Rivero-Lynch and Godfray, 1997; Irvin and Hoddle, 2009). Furthermore it was demonstrated for other parasitoid wasps that the combination of honey drops availability and the absence of host for deposition might induce egg resorption to occur, allowing additional energy supplies and increasing longevity (Irvin *et al*., 2007; Irvin and Hoddle, 2009). In our experimental conditions the egg maturation trend, which shows an egg load decline after 8-16 days (univoltine and diapausing specimens respectively), together with the recorded long life expectancy suggest the likelihood of oosorption as a strategy to reallocate resources in order to carry on egg maturation until death.

If oosorption does exist in *T. sinensis* it might be important to make it able to carry on egg maturation till death even if through the life-time the mature eggs number tends to become lower. Moreover the considerable maximum age reached by the two populations, 102 and 73 days for univoltine and diapausing females respectively, in laboratory conditions might be explained by the combination of egg resorption and honey drops used to feed the adults during their life together with the absence of suitable host for ovideposition. Life duration also corroborates the ovigeny strategy detected in *T. sinensis* as typically synovigenic species are longer-lived than proovigenic ones, and ovigeny index and lifespan are negatively correlated across parasitoid taxa, suggesting that the reproductive effort concentrated in early adult life may penalize lifespan (Jervis *et al*., 2001).

Other traits showed differences between the two populations: the body morphometry, and the egg size. The morphometric parameters showed that diapausing adults were smaller than univoltine ones in both sexes, confirming preliminary data by Ferracini *et al*. (2015a). The diapausing specimens seem to penalize size, both for body and eggs, and life expectancy in favour of the average egg maturation number which is almost the same in both populations, 135.18 and 148.44 for univoltine and diapausing wasps respectively. Considering egg size, univoltine females showed significative larger eggs than diapausing ones.

The age of female parasitoid is known to influence the parasitism rate of various pests (Lashomb *et al*., 1987; Amalin *et al*., 2005). In our research *T. sinensis* females showed the highest egg load in the first weeks of their lifespan. On average, in the females of the univoltine population the highest number of mature eggs was reached in the second week and then decreased steadily, while for diapausing ones the highest egg load was recorded in the third week and this high level in the egg load was observed from the second to the fifth week.

Piao and Moriya (1992) carried out similar laboratory experiments on *T. sinensis*, but rearing the insects constantly at 20 °C, and their research showed a different trend in the egg development. They recorded an initial egg load of 1 to 7 eggs at day 0 and a maximum egg load at day 4 with an average of 24.4 mature eggs, being the highest egg number detected of 37, immediately followed by a slow decline until the 15^{th} day (the last day recorded) when the egg number reached an average of 13.6. These differences might be explained by some different environmental conditions in which the specimens were kept. It is known that adults of gall forming wasps and their parasitoids spend several days after eclosion inside chambers within galls to maturate eggs (Ôtake, 1980; Kato and Hijii, 1999; Zhi-yong, 2009). Therefore, during this posteclosion to preemergence period they could start their egg development and ovarian maturation (Graziosi and Rieske, 2014). Although we cannot evaluate the ovarian development through the total lifespan in each specimen, we can assume that at highest temperature insects might be maturing eggs faster showing some mature eggs already at emergence and reaching the maximum egg load earlier than specimens kept at lower temperature.

This data could have a potential for improving biological control effectiveness in chestnut-growing areas affected by the gall wasp, suggesting that younger females (2-3 week old) have to be preferred during the release stage in order to maximize parasitism. Moreover, even if until now a low incidence of extended diapause was reported (Ferracini *et al*., 2015a), a higher egg load in diapausing *T. sinensis* females than univoltine ones highlights the need of preserving the diapausing population to favour the establishment of the parasitoid as well.

Therefore, this finding should be considered to optimize the releases, especially in a view of large scale mass production by insectaries or private companies.

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