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Dipartimento di Agricoltura, Alimentazione e Ambiente
Dottorato di ricerca in tecnologie fitosanitarie e difesa degli agroecosistemi (XXVIII ciclo)
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Applicative, biological and olfactometer responses of Hymenoptera Bethylidae with special attention to **Holepyris sylvanidis** ***and*** **Cephalonomia waterstoni.**

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1 Introduction

During recent years much research on stored grain has focused on biological control, which is an important component of Integrated Pest Management (Flinn et al., 1994). The studies were focused on this topic because the massive employ of insecticides could pose risks to humans, animals and environment. The main problems could derive from residues left on the food and disperse in the environment. Because a lot of pests are resistant to major insecticides, the opportunity to find organic molecules or innovative systems to control pests was taken into account by researchers (Collins et al., 1993, Herron, 1990; Muggleton, 1987; Muggleton et al., 1991).

In this context, politicians began to find solutions and rules, and in this direction a common example is the worldwide phaseout and ban of the fumigant methyl bromide (Fields and White, 2002). One of the most adopted biological control systems of stored products is represented by parasitoids, which belong almost entirely to the order Hymenoptera. Although biological control of pests using parasitoids is becoming more frequent, a lack of knowledge is still present and operators able to use this relatively new approach are very few. Parasitoids have showed useful consequence on human activities therefore they are considered of economic importance (Gauld and Bolton, 1988; LaSalle and Gauld, 1993). Even though many parasitoids are very well studied from different points of view, others are less known and Bethylidae are among them. Considering that the majority of wasps reported as promising biological control agents are in the families Pteromalidae and Bethylidae (Hangstrum and Flinn, 1992), it appears clear that more knowledge on the natural enemies belonging to Bethylidae could play an important role for the future of the Integrated Pest Management.

Bethylidae belong to the order Hymenoptera, it is a primitive family of Aculeata, which comprises about 2,200 species and has a worldwide distribution (Hawkins and Gordh, 1986). Bethylidae are idiobionts, ectoparasitoids and typically oligophagous. The morphology, behaviour and biological aspects of this family were studied for different species during recent years (Clausen, 1940; Evans, 1964; Lauzière et al., 2000). From a morphological point of view, Bethylidae appear similar among them (ant like in appearance, small to medium sized and dark coloured), but at a more detailed glance they exhibit significant structural differences (flat body, reduced wing vein) (Clausen, 1940; Evans, 1964; Lauzière et al*.,* 2000).

The biology of Bethylidae was studied accurately for some species (Finlayson, 1950a; Mayhew and Heitmans, 2000; Powell, 1938; Rilett, 1949). A common characteristic of Bethylidae is that males emerge little a prior to females and they can enter inside the cocoon of females in order to mate (Howard and Flinn, 1990; Rilett, 1949). This involves a very high inbreeding, but females compensate for it mating different times during their life with unrelated males. This is facilitated by the shorter life of males; consequently females may mate with males of several generations (Clausen, 1940, 1962). The courtship and copulatory behaviour was described for *Cephalonomia tarsalis* (Ashmead) (Hymenoptera: Bethylidae) and *Plastanoxus westwoodi* (Kieffer) (Hymenoptera: Bethylidae) (Ahmed and Khatun, 1996a; Cheng et al., 2003; Cheng et al., 2004). In both cases the male plays an active role starting the courtship and inducing sexual receptivity in the female. The copulation time of *Cephalonomia gallicola* (Ashmead) *Cephalonomia waterstoni* (Gahan) and *Holepyris sylvanidis* (Brèthes) (Hymenoptera: Bethylidae) lasted on average 2 minutes (Ahmed and Islam, 1988; Finlayson, 1950a; Itoh, 1980; Rilett, 1949). An aspect that is of interest both from a biological and applicative point of view, is that the number of hosts which are attacked and paralyzed by a female is often in excess in respect to those which receive eggs. A typical example was reported for *Prorops nasuta* Waterston (Hymenoptera: Bethylidae), which females feed on young larvae but oviposit preferably on mature larvae of *Hypothenemus hampei* (Ferrari) (Coleoptera: Scotylidae) (Clausen, 1940, 1962).

Some studies were conducted to evaluate the number of eggs laid by Bethylidae and the results were reported as follow: *Perisierola emigrata* Rohwer (Hymenoptera: Bethylidae) laid 236 eggs in 44 days, *C. gallicola* laid 158 eggs on 76 hosts during 36 days, mated females of *C. tarsalis* deposit ca. 85 eggs, whereas virgin females produced only 50 eggs (Kearns, 1934a, 1934b; Willard, 1927). Clausen (1940, 1962) reported that the way in which eggs are released on the host referring a difference between solitary and gregarious species. According to his studies solitary Bethylidae lay eggs longitudinally with the anterior end directed caudal whereas the gregarious species lay eggs transversally. Eggs can hatch at different moments according to environmental conditions. Regarding this aspect, some data were reported in the case of *C. gallicola* for which eggs required 13 days to hatch when temperature was of 22.6°C and *Parascleroderma berlandi* Maneval (Hymenoptera: Bethylidae) from 1 to 4 days with an extreme of 7 days. The new larva starts to feed immediately after it is completely free from the eggshell. As reported by Clausen (1940, 1962), the larva makes only one feeding puncture during its life. The duration of larval stage is generally different; the mean is 5 days but some examples reported interesting differences: in *Goniozus* and *Cephalonomia* it is completed in 2-3 days and a longer period was registered for *Bethylus cephalotes* (Förster) (Hymenoptera: Bethylidae) for which 10 days were reported. After Bethylidae larvae have finished the feeding period, they are able to move some centimetres away in order to spin a cocoon.

A relevant behaviour reported for Bethylidae is the maternal care (Clausen, 1940, 1962), which range from attention for only few days after egg laying as reported for *C. gallicola,* to the end of larval period as reported for *Scleroderma macrogaster* Ashmead (Hymenoptera: Bethylidae). Other examples of maternal care were reported for *Goniozus nephantidis* (Muesebeck) (Hymenoptera: Bethylidae) and for *Sclerodermus harmandi* (Buysson) (Hymenoptera: Bethylidae). The female of the second Bethylidae remains with the offspring until pupation (Hu et al., 2012), defending young offspring more than the older (Goubault et al., 2007), while the female of the first natural enemy remains with the offspring until the adulthood (Hardy et al., 1999). Another characteristic regarding the biology of Bethylidae is the regulation of brood size. On this aspect Clausen (1940, 1962) reported a habit consisting in eggs eating. Mayhew (1997a) evaluated the ovicide aspects and clutches competitions in the gregarious parasitoid *Laelius pedatus* (Say) (Hymenoptera: Bethylidae), concluding that the wasp can destroy eggs belonging to other female and that the secondary clutch suffers high mortality when in competition with the primary clutch.

The sex allocation strategy can represent behaviour of interest especially when rears of Bethylidae are set up. Mayhew and Godfray (1997) investigated the sex allocation strategy for the gregarious *L. pedatu*s on different hosts size, reporting relations among the number of eggs laid in a different sized host, the sex of offspring and the size of the host. Different studies have evaluated the reproductive aspects of Bethylidae, with regard to aspects like offspring development mortality, sex ratio (Pérez and Hardy, 1999), relationship between mean sexual dimorphism and sex ratio (Hardy and Mayhew, 1998), primary and secondary sex ratios (Khidr et al., 2012). It is of interest to report the superparasitism stated by Ahmed et al. (1997) for *H. sylvanidis* and contest and agonistic behaviours reported by Hardy et al. (2013) for *Goniozus legneri* Gordh (Hymenoptera: Bethylidae) and *G. nephantidis* female.

Similarly to other idiobiont parasitoids, Bethylidae paralyze hosts before laying eggs and the hatching larva will develop as ectoparasitoid (Howard et al., 1998). To get host paralysis the female attacks the host as follows: initially she creeps over the host larva and curves the abdomen beneath the thorax, then inserts her ovipositor either in the throat or in the intersegmental membrane of the host abdomen injecting the venom. During the attack the larva starts to roll itself, as signal that wasp female is stinging and injecting the venom (Hu et al., 2012). After the struggle is finished and the paralysis of the host is completed, the Bethylid female transports the prey, dragging it along the surface, to a crevice or a cavity where oviposition takes place (Finlayson, 1950a, Powel, 1938).

The major Bethylid species found in stored grain ecosystems are in the genera *Cephalonomia*, *Holepyris* and *Laelius* (Howard et al., 1998). During the recent decade interest in biological control methods for stored products has grown considerably and it has been mostly focused on parasitoids belonging to Pteromalidae, Braconidae and Ichneumonidae (Schöller et al. 2006; Suma et al., 2014). During last years, applied entomologists have focused the attention on Bethylidae because some species have been indicated as promising candidates in biological pest control programs. The importance of considering Bethylidae as biocontrol agents is due to the fact that their hosts are widely reported as pests of crops and stored products (Pérez and Hardy, 1999) and they are also biologically different if compared to parasitoids belonging to other families (Evans, 1964). In order to determine if a parasitoid is suitable for a biological pest control program, evaluations on its biology are essential. Only a deep knowledge of the biological characteristic of a natural enemy can furnish a good start point to develop valid applicative solutions. Here, a revision of the main biological and applicative characteristic of Bethylidae as biological control agents is reported (Tab. 1).

1.1 *Cephalonomia gallicola*

C. gallicola is a cosmopolitan ectoparasitoid, which female was redescribed by Lim et al. (2007). This natural enemy is characterized by the presence of three different forms of adult: apterous female, wingless male and winged male (Kearns, 1934a, 1934b). The body length of apterous female ranges from 1.94 mm to 2.68 in length, the body, the antennae and the compound eyes are dark brown; the antennae are twelve segmented and the five distal segments are black (Kearns, 1934a, 1934b; Kuwahara, 1984). The female has a rectangular-shaped head, a thorax with pronotum bell-shaped, abdominal segments well developed and postabomen with a reduced sting (Kearns, 1934b; Lee et al., 2014). The wingless male is from 1.5 to 2 mm in length and it is paler than female, the winged male is from 1.3 mm to 2 mm. Both males show a dark transverse band upon the caudal half of each abdominal segment. The wingless male presents a more ovate-shaped head than the winged male (Kearns, 1934a, 1934b).

C. gallicola is an arrhenotokous, synovigenic, gregarious and external parasitoid of larvae and pupae of Coleoptera infesting the stored products (Kearns, 1934a, 1934b; Lee et al., 2014; Lim et al., 2007; Yamasaki, 1982). This natural enemy can undergo four or five generations per year in the absence of heating (Tanioka, 1982). The pre-oviposition period is 4.9 days in mated female and 25.8 days in unmated female (Yamasaki, 1982). The egg is crystalline white, elliptical, 0.82 mm in length and 0.66 mm in width (Kearns, 1934a). Eggs are laid on the venter of the host larvae, but when the female parasitoid oviposits upon pupae, the eggs are laid on the inter-segmental grooves of the dorsum. Being a gregarious parasitoid, *C. gallicola* female can lay from one to nine eggs upon each host (Kearns, 1934a). This involves consequence on the developing time of the offspring and when the number of eggs laid upon a host is more than five the width of the head capsule of the offspring decreases (Yamasaki, 1982). *C. gallicola* female lays an average of 4.4 eggs on a host (Kearns, 1934a; Lim et al., 2007; Yamasaki, 1982) at intervals of 2.1 days. Tanioka (1982) reported different rates of oviposition when different temperatures were tested. According to the author, this natural enemy produces: 33 offspring at 25°C, 68 at 30°C, 115 at 32.8°C, and a peak of 146 at 32.8°C was also registered. During a period of 36 days and 76 hosts available *C. gallicola* female laid 158 eggs (Lim et al., 2007). The egg laying of *C. gallicola* is influenced by the density of hosts, it means that if a female perceives a scarcity of host larvae, she is stimulated to lay the maximum number of eggs upon one host (Kearns, 1934a). Eggs are attached upon the host by a dense fluid (Kearns, 1934a) and they hatch within 2 to 4 days when temperatures range from 20°C to 26°C. The female sometimes opens the skin of the host before laying eggs. This behaviour offers to the newly hatched larvae an easy way to feed on the host, especially during their first stages (Kearns, 1934a). The newly hatched larva is white, slightly transparent, with undefined segmentation and form. Immediately after hatching it starts to feed from the host haemolymph, and continues for 2-3 days after which it becomes yellow (Kearns, 1934a). The larval stage lasts for 6 to 9 days when temperatures varied from 20°C to 26°C. At the end of the feeding period, the larva shows a definite segmentation and a pyriform body shape (Kearns, 1934a), and as soon as the host is completely devoured, it starts to spin a cocoon. In this sense *C. gallicola* has shown some differences compared to other Bethylidae reported as biological control agents of stored products pests, because the parasitoid larva can pupate inside the empty cocoon of host *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae) (Kearns, 1934a). The pupal stage can last for 7 to 18 days depending on the sex and the adult form. So, the pupal stage of the female lasts for 11 to 18 days, that of the winged males from 9 to 15 days and that of the wingless males from 7 to 12 days, when temperatures range from 20 to 26°C. Consequently, the entire developmental time may change according to temperatures requiring

34 days at 22.6°C and 64 days at 18.4°C (Kearns, 1934a, 1934b). Itoh (1980) evaluated the developmental period under natural conditions reporting 60 days during the spring and from 20 to 30 days in summer.

The behaviour of this species has been less studied compared to those of other Bethylids. Before the emergence, the adult remains from one to three days enclosed in the cocoon. The male emerges little in advance in respect to the female and to mate it enters into the female cocoon (Kearns, 1934a). The females fed with constant supply of fresh larvae survived 94 days (Kearns, 1934a; Yamasaki, 1982).

C. gallicola is a candidate for biological control of stored products pests and in this direction some studies evaluating its characteristics have been reported (Tab. 1). This wasp can be released in the presence of light traps, because it is not attracted by light (Kearns, 1934a). One interesting characteristic of *C. gallicola* is its capacity to enter into cracks and crevices and to penetrate deeply into substrate when looking for hosts (Kearns, 1934a). *Cephalonomia gallicola* is a parasitoid of a large number of hosts *i.e.* the anobiids *L. serricorne* and *Stegobium paniceum* (L.) (Kuwahara, 1984; Lim et al., 2007), *Araecerus fasciculatus* (Degeer) (Coleoptera: Anthribidae), *Niptus hololeucus* (Faldermann), *Ptinus fur* (L.) and *P. tectus* Boieldieu (Coleoptera: Ptinidae) (Lim et al., 2007). Itoh (1980) reported that *C. gallicola* was able to parasitize larvae of the ptinid *Gibbium psylloides* (De Czepinski) (Coleoptera: Ptinidae) under laboratory conditions. Itoh (1980) collected the wasp in stored oil cakes infested by *S. paniceum* and based on his biological studies, he concluded that the wasp showed the best vigorous reproductive level during the period between July and September in Japan.

C. gallicola shows a lot of interesting potential as biocontrol agent, but on the other hand, this parasitoid has been reported as an insect, which causes dermatitis to humans especially during spring and summer (Lee et al., 2014; Kuwahara, 1984). *C. gallicola* may sting humans at: bare-arms and legs, trunk and neck. The symptoms are redness around the stung place, swelling, pain and pruritic erythematous papules (Lee et al., 2014; Matsuura, 1981; Yamasaki, 1982). This aspect has been evaluated only from a medical point of view and further studies are absolutely needed to fully understand the conditions in which the female stings and if it is a real obstacle during pest management programs based on the use of natural enemies.

1.2 *Cephalonomia tarsalis*

This species is a predator and an ectoparasitoid of the sawtoothed grain beetle, *Oryzaephilus surinamensis* L. and the merchant grain beetle, *Oryzaephilus mercator* (Fauvel) (Coleoptera: Silvanidae) (Lord, 2001; Powel, 1938). The female is black, with brownish yellow mandibles and brown antennae. The tarsi are pale yellow and the wings are hyaline. Females are slightly longer than males, having a 2 mm body whereas the males are 1.7 mm long. The male is similar to the female but its antennae are entirely dark (Richards, 1939).

C. tarsalis female mates from one to three times and the proportion of female offspring will be reduced gradually after 1.5 weeks and after 3 weeks the offspring will be male biased likely due to sperm depletion (Cheng et al., 2003; Cheng et al., 2004). A single male can mate with many females (Cheng et al., 2004; Powel, 1938) and he is able to enter in a female cocoon and to mate with a pre-emergence female. It involves that males can identify the presence of female inside a cocoon, it is also demonstrated by the fact that males spend long period of time walking upon the female cocoon and antennating them (Cheng et al., 2004; Powel, 1938).

C. tarsalis female forms her offspring according to the haplodiploid sex determination. As indicated by Cheng et al. (2003) a mated female can lay either a single egg or a pair of eggs on a host. The 80% of eggs laid by a mated wasp are female and when two eggs are laid on a single host one becomes male and the other one female. The first is typically laid on the prothorax of the host whereas the second on the mesothorax (Cheng et al., 2003; Powel, 1938). The mating can influence the number of eggs laid, considering that a virgin female lays an average of fifty eggs whereas a mated female yields an average of eighty-five eggs (Cheng et al., 2003).

The eggs hatch within 24 h after oviposition and males emerge 1-2 days before the female

(Powell, 1938). The larval period of *C. tarsalis* starts when contractions and expansions of the digestive tract, due to the ingestion of food, are visible. The young larva is 0.5 mm in length and after hatching it will feed as a semiectoparasitoid consuming completely its host in 4 days (Cheng et al., 2003; Powell, 1938). The number of eggs laid on the host can influence the feeding time. When two eggs are deposited, the host larva is consumed in two-third of the time required by a single larva. After the end of feeding, the larva spins a cocoon in about six hours. The cocoon plays an important protective role against parasitic mites (Powell, 1938).

Among the preimaginal stages of *C. tarsalis*, the pupal phase is the longer and typically the abdomen starts to change in dark, and after one day the entire body gets dark grey to black. At this time ecdysis to the adult occurs. The adult needs about 4-5 days to emerge from the cocoon due to a scarcity of strength and it seems without agility during the first hours of life (Powell, 1938). Lukáš and Stejskal (2004) indicated that in all stages the developmental time was shortest at 27 and 30°C and between these two temperatures no significant statistical difference was registered. Egg development was shortest at 30°C lasting for 1.1 days. Similarly larval development lasted for 5.2 days at 30°C but in contrast the shortest pupal development was 5.2 days at 27°C. The duration of the life is quite different between the two sexes and the mating does not influence it. The males live about 6 days and the female lives an average of thirty-five days. The male doesn't consume food during his life (Cheng et al., 2003, 2004; Powell, 1938).

The way adopted by *C. tarsalis* female to locate hosts involves two main systems. The first is the sight because the wasp has developed eyes, which can help it when some light is present and the second is constituted by chemical trails deriving from hosts (Howard et al., 1998). The latter was firstly reported for *C. waterstoni* (Howard and Flinn, 1990)*,* which is able to locate *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae) following the trails laid by the beetle. Howard et al. (1998) hypothesized that the host's cuticular hydrocarbons, provide the first signal to the wasp, even though *C. tarsalis* needs to perceive movements from the larvae before the attack. Also, *C. tarsalis* uses stimuli deriving from the trails and from the surface of *O. surinamensis* larvae to recognize its host (Collatz and Steidle, 2008). Another suitable way followed by *C. tarsalis* to locate the larvae of the beetle is given by the odours of faeces (Collatz and Steidle, 2008). These odours are host-specific and when the female locates them it can discriminate areas with host activities.

The female of this natural enemy is very active and spends much time searching for a new host (Powel, 1938). A sequence of behaviours for the female involves: searching for hosts, followed by antennal contact, climbing onto the host, biting and stinging it (Howard et al., 1998). These were followed by 15-30 s of immobility until the host's movement ceased. Afterwards the wasp moves the host for a short distance and goes up it. The wasp then moved away, and began grooming and resting (Howard et al., 1998). Occasionally more than one sting is required to subdue the host. Howard et al. (1998) studied the behaviour of *C. tarsalis* female when she was kept in front of a dead larva. In this circumstance, the wasp investigates the host but didn't bite or sting it although host feeding on the prefered dead host was observed. It was hyphotized that the parasitoid perceived dead hosts as a just paralyzed.

C. tarsalis is an useful biological control agent against the sawtoothed grain beetle and the merchant grain beetle, *O. mercator*, being able to parasitize both larvae and pupae (Cheng, 2003; Collatz et al., 2009; Lukáš and Steiskal, 2004; Lukáš, 2005). In a previous book (Gordh and Móczár, 1990) *C. tarsalis* was reported as a parasitoid of different species of genus *Sitophilus* Schoenherr (Coleoptera: Dryophthoridae) as well as *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and again, Howard et al., (1998) indicated this natural enemy as an obligate parasitoid of the sawtoothed grain beetle. In order to put order among the possible pests associated to *C. tarsalis*, Howard et al., (1998) investigated the possible alternative use of the wasp to control *Sitophilus* spp. and *T. castaneum* as reported by Gordh and Móczár (1990). The authors reported *C. tarsalis* only examined both the hosts with antennae but no evidence regarding the host recognition was observed.

The literature offers some examples of combined uses of *C. tarsalis* with other biological control agents (Tab. 1). A first study considered the combination between *C. tarsalis* and the entomopathogenic fungus *Beauveria bassiana* (Balsamo) (Hyphomycetes: Moniliales), but it concluded that the eggs laid by the wasp didn't survive and that the parasitoid showed a level of susceptibily to the fungus similar to that of its host (Lord, 2001). Ždárková et al. (2003) evaluated the compatibility of *Cheyletus eruditus* (Schrank) (Acari: Cheyletidae) and *C. tarsalis* as combined agents against *O. surinamensis*. The study showed how to apply contemporarily the two natural enemies, the control of beetle populations was higher than treatments with each natural enemy alone. Lord (2006) evaluated the interactions between *C. tarsalis* females and the parasitic protozoa *Mattesia oryzaephili* Ormières (Neogregarinorida: Lipotrophidae). According to the author, these biocontrol agents can interact either complementarily or synergistically. In both cases, the capacity to suppress pest beetles was documented. The wasp moving around the stored product environment can easily disseminate the pathogen enhancing its action, although the transmission of the *M. oryzaephili* takes place through the process of oviposition. If on one hand using *M. oryzaephili* the life duration of *C. tarsalis* is reduced, on the other hand the lifespan of the wasp is long enough to produce benefit when the combination parasitoid-neogregarine is adopted. The combination didn't influence the behaviour of this biocontrol agent.

1.3 *Cephalonomia waterstoni*

C. waterstoni is an arrhenotokous ecto-parasitoid, reported by Sheppard (1936) as a parasite of *C. ferrugineus*. The morphological features of this bethylid were reported by Gahan (1931) and Richards (1939). According to the authors, *C. waterstoni* female is black shining, 1.6 mm in length with 12-segmented antennae which are black except for the second and part of the third antennomere. Reichmuth et al. (2007) indicated that *C. waterstoni* presents a thorax strongly compressed dorsoventrally and the median and lateral carinae on the propodeum are not clearly visible. The basal and the median cells of the forewing are absent (Gahan, 1931; Reichmuth et al., 2007). Rilett (1949) indicated aspects regarding the male's morphology; it is shorter than the female, with a less rectangular-shaped head and longer antennae. The scape is brownish-black whereas it is yellowish in the case of female.

After its emergence, the female starts to lay eggs soon following a scheme that can be referred to one of the following cases: a) the female lays a single female egg, b) two eggs which evolve in a male and a female and c) three eggs which develop in two females and one male. The *C. waterstoni* female is able to lay eggs on the pupae of *C. ferrugineus*. The parasitoid lays an average of one to three eggs per day, although a peak of 8 eggs in a 24-hrs period was registered (Finlayson, 1950a; Rilett, 1949). After 30 hours the egg hatches and the new emerged larva, keeping the same position occupied by the egg, begins to feed sucking up the haemolymph of its host, which will be reduced to an empty shell. After the complete consumption of its host, the larva immediately spins a cocoon close to the feeding place (Finlayson, 1950a; Rilett, 1949).

Similarly to other parasitoids, *C. waterstoni* is able to locate hosts recognizing their kairomonal cues left on infested substrates (Howard and Flinn, 1990). Immediately after the female wasp encounters a larva, she starts the host recognition touching it with her antennae. This process is quite fast completed, and the parasitoid attacks the prey suddenly. The female has powerful mandibles, so when she bites a host it is impossible for the host larva to escape (Rilett, 1949). Although the host larva is often larger than the *C. waterstoni* adult and it tries to avoid the paralysis moving continuously, when the parasitoid attacks its host the submission and consequently the paralysis is always obtained. Due to the defense reaction the wasp has in some cases to fight for several minutes, and in some circumstances the parasitoid female has to make many attempts before it is successful in stinging the host. The sting is inserted in a randomly chosen part of the body of the host and the action of the injection is fast causing the paralysis after few seconds. The venomization is so prolonged that the host larva has no opportunity to recover (Rilett, 1949). The parasitism rate will not increase even if more hosts are found (Howard and Flinn, 1990), because the female wasp may attack more hosts than she can oviposit on. In fact *C. waterstoni* female lays only two eggs per day, but it was observed that the parasitoid could paralyse up to 15 larvae per day

(Finlayson, 1950a; Flinn, 1991). In order to explain this behaviour, Flinn and Hagstrum (1995) indicated that when host density is high, female wasps spend more time stinging hosts rather than oviposit on them. Like other Bethylidae after the larva is paralyzed, the parasitoid carries it off to a safe place where one or more eggs will be laid. The venom produces an effect similar to that of other Bethylidae, consisting in continued, uncoordinated and rhythmic movements of the host's legs (Finlayson, 1950a; Flinn, 1991; Rilett, 1949).

In laboratory cultures the sex ratio of *C. waterstoni* was 2:1 (female, male), at 25°C the complete lifespan of females was about three weeks and that of males was much shorter. At 32°C and 75% relative humidity the life cycle is completed within 12 to 13 days (Finlayson, 1950a; Rilett, 1949). In several pubblications, *C. waterstoni* was reported as an external parasitoid of larvae of *Cryptolestes minutus* (Olivier) (Coleoptera: Cucujidae) and *C. ferrugineus* (Finlayson, 1950b; Howard and Flinn, 1990; Rilett, 1949) and the presence of this parasitoid was indicated in specimens collected from stored grain, in which simultaneously the rusty grain beetle was registered as a pest (Flinn, 1991; Sinha et al., 1979). Rilett (1949) reported a rapid population increase of *C. waterstoni* in wheat infested by the rusty grain beetle under laboratory conditions, and at the same time the presence of the parasitoid caused a marked delay in the increase of the *C. ferrugineus* population. Hagstrum (1987) and Reichmuth et al. (2007) reported the ability of *C. waterstoni* in maintaining the rusty grain beetle population below the economic damaging threshold. Further suggestions concerning the suitability of *C. waterstoni* in biological pest control programs were reported by Flinn and Hagstrum (1995). The authors emphasized the ability of this parasitoid to find its host, which usually develops between grains or coarse substrate. One of the most important characteristics of *C. waterstoni* is its capacity to penetrate into the substrate. This behaviour should be taken seriously into account when the use of *C. waterstoni* as biocontrol agent is evaluated. The first author who reported this aspect was Finlayson (1950a), but further evidence, obtained during a field study, was reported by Flinn et al. (1994) who showed the ability of *C. waterstoni* to spread from one bin to another, even though the bins were tightly sealed. Reichmuth et al. (2007)

confirmed this ability indicating as the wasp is capable to enter narrow cracks and crevices when looking for possible hosts. *C. waterstoni* is able to attack different species within the genus *Cryptolestes* but when a choice was provided, the parasitoids preferred *C. ferrugineus* (Finlayson, 1950b; Flinn, 1991). In the same experiment about half to two-thirds of the *C. minutus* and *Cryptolestes turcicus* (Grouvelle) (Coleoptera: Cucujidae) were not attacked or if attacked, were not seriously damaged. Finlayson (1950b) supplied larvae of *O. surinamensis* and *O. mercator* to *C. waterstoni* but no egg laying was registered even though few larvae were stung and paralysed.

Like many parasitoids, when the *C. waterstoni* female is in contact with a host larva, it generally has to decide whether to feed or to lay eggs. This natural enemy feeds upon first, second and third instar larvae whereas egg laying was observed on fourth instars only. The wasp is able to find hosts that are located in any region of the grain mass (Flinn, 1991). The female feeds of her prey performing a hole through the cuticula and then sucking the haemolymph (Howard and Flinn, 1990). The male doesn't feed on the body fluids and seems to be passive when it is confronting with host larvae.

In simulation models of *C. waterstoni*, Flinn and Hagstrum (1995) demonstrated how the use of the appropriate time of release could give better results in controlling *C. ferrugineus*. According to the authors the "optimal timing of parasitoid release" is strictly correlated to the possibility to find first produced fourth-instar beetle larvae by the parasitoid. In other words, the most effective control will be obtained when parasitoids are released in time to suppress the newly formed fourth-instars. Based on field data, Flinn and Hagstrum (1995) estimated that the immigration rate for *C. ferrugineus* is approximately 10 beetles per 27 ton/d. If it is assumed that about 200 *C. ferrugineus* immigrate into an 81-ton (3000 bu) bin during the first week, then a good control should be obtained by releasing 200-400 parasitoids 20 d after the grain was stored.

As demonstrated by Lord (2006) the wasps can serve as mechanical vectors of *M. oryzaephili* enhancing the pathogen's dissemination. *M. oryzaephili* is diffused by the mandibles and the ovipositor during the normal activities of the biocontrol agent. Although the infection with

M. oryzaephili reduced the lifespan of *C. waterstoni* by 21%, the author reported that the survival period of the wasp is sufficient to benefit the biological control efficacy of the wasp-neogregarine combination. As for other bethylid species, also for *C. waterstoni* it has been observed that it can cause irritation to man by stinging (van Emden, 1931; Finlayson, 1950a).

1.4 *Holepyris hawaiiensis*

Among the Bethylidae of stored products pests here considered, *Holepyris hawaiiensis* (Ashmead) (Hymenoptera: Bethylidae) is the only natural enemy able to develop upon Lepidoptera larvae. This parasitoid is also the least studied. Bridwell (1920) reported a brief description of the female as follow: "*Smaller; antennae dusky at tip; punctures of head finer; submedian ridges of propodeum nearer the median, more distinct and shorter; the area lateral to them more finely striate anteriorly and more coarsely posteriorly*".

Bridwell (1920) did some observation on the behaviour of this parasitoid working with the following Lepidoptera: *Ereunetis flavistriata* (Walsingham) (Lepidoptera: Tineidae), *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) and *Corcyra cephalonica* (Stainton) (Lepidoptera: Pyralidae). Similarly to other females of the same genus *H. hawaiinensis* lays a single egg. It is deposited on the dorsum of the ninth abdominal segment (Bridwell, 1920; Pillault, 1976; Shull, 1940). The behaviour of *H. hawaiiensis* agrees with those reported for other Bethylidae indicated as biological control of stored products pests. When the female wasp is in front of a larva, she attacks it immediately, then she measures the host with her antennae and finally she brings the paralyzed prey to a hidden place. The *H. hawaiiensis* female stings the larva at the throat and she is able to feed attacking the leg (Bridwell, 1920). This parasitoid naturally searches for hosts in crevices and hidden places (Pemberton, 1932).

Bridwell (1919) found *H. hawaiiensis* in warehouses located at Honolulu and indicated that Ehrhorn reared this natural enemy from *Ephestia cautella* (Walker) (Lepidoptera: Phycitidae) (Tab. 1). Pemberton (1932) stated that this wasp was reared upon small Lepidoptera occurring in stored foods on dried and broken Algaroba seed pods. Similarly to *C. gallicola*, this natural enemy can cause irritation due to a sting (Pemberton, 1932).

1.5 *Holepyris sylvanidis*

The adult can easily fly, having a 1.5 mm long wing. The antennae count 13 segments. The legs are yellowish brown (Reichmuth et al., 2007). The female has a body length of 2.96 ± 0,27 mm and its egg is 0.51 mm in length. Typically the female glues the egg between the 4th and 5th segments ventrally. An egg is sometimes laid behind the hind pair of legs, *i.e.* 1st abdominal segment dorsally but in some cases it can be laid between the 6th and 7th abdominal segments ventrally. The incubation period ranges from 1.5-1.8 days (Ahmed and Islam, 1988; Reichmuth et al., 2007). Ahmed and Islam (1988) reported data about the length of larva (0.49 ± 0.03 mm), the headcapsule width (0.15 ± 0.01 mm), the male pupal length (1.60 ± 0.15 mm) and the width of his headcapsule (0.53 mm), the female pupa (1.95 ± 0.23 mm) and the width of her headcapsule (0.53 mm). After *H. sylvanidis* larva has completely eaten the body content of its host, it spins a cocoon few centimetres away far from the feeding place. The prepupal period lasts for 4.4 days and the pupal period lasts for 9-10 days. The complete development of this parasitoid takes about 5 weeks at 25°C (Reichmuth et al., 2007).

When a female meets a larva of *Tribolium* spp., she starts the host recognition which is followed by the injection of venom. After the venomization and the subsequently paralysis, the *H. sylvanidis* female brings the host to a potential hiding place where the egg will be laid. Although the host larva is not completely able to move due to the paralysis, during the egg laying and for the next days, it will be still alive. The paralysis involves continuous and uncoordinated movements of legs and head (Ahmed et al., 1997). Male behaviour was not reported until now (Lorenz et al., 2010; Reichmuth et al., 2007). Regarding the way of attack, it was accurately reported by Ahmed et al. (1997): the female firstly grabs the host at the 8th abdominal segment, then she twists her body curving the abdomen over the host larva. The abdominal tip of the parasite remains in touch with the larva for some seconds and in this phase the wasp moves the antennae continuously. *H. sylvanidis* female often bites the host, because it is a synovigenic parasitoid and need to feeds from the host's body fluids in order to obtain nutrients to mature the eggs. As it is common in synovigenic species, *H. sylvanidis* female emerges with only a part of her eggs riped, consequently she needs to nourish especially of protein to develop the rest of the eggs (Ahmed et al., 1997).

Considering that larvae are often hidden under thin layers of the substrate, in aeration ducts, in machineries and in areas that are difficult to clean, employing the wasp as a biocontrol agent, it is possible to get these critical environments (Lorenz et al., 2010; Reichmuth et al., 2007). From an applicative point of view, *H. sylvanidis* female is capable to penetrate different thick layers of fine coarse grist when it searches for hidden larvae. Lorenz et al. (2010) reported that the host finding capacity is hindered by a decrease in particle size. To be more precise, the parasitoid can penetrate easily through 1 and 2 cm fine grist, at 4 cm the wasp is less successuful and it can't penetrate deeper than 8 cm into coarse grist. Considering that the host, the confused flour beetle, often lives hidden under milled grain (Sokoloff, 1974), the capability of *H. sylvanidis* to penetrate the substrate became crucial to obtain good results when this wasp is considered for biological control. Eliopoulos et al. (2002) reported *H. sylvanidis* as the most "dominant" parasitoid collected in storage facilities in Greece, whereas in terms of frequency this parasitoid was the second. In terms of frequency Eliopoulos et al. (2002) showed that the major presence of this parasitoid was on flour although the wasp was also collected in grain and dried fruits. In the same study the author indicated *H. sylvanidis* as able to develop high populations both in grain and flour, reaching 59% and 61% of collected adults respectively. Eliopoulos et al. (2002) stated that if *H. sylvanidis* and *C. tarsalis* are contemporary present in the same substrate, the first is able to suppress the latter. Two explanations were suggested by the authors: 1) *H. sylvanidis* is capable to disperse better and can reach the host easily, b) *H. sylvanidis* is able to develop in a wide host range whereas *C. tarsalis* parasitizes almost exclusively *O. surinamensis* and occasionally *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae).

Regarding the possible hosts of *H. sylvanidis*, the information is scattered (Tab. 1). In effect, Eliopolus et al. (2002) reported *H. sylvanidis* as a parasitoid of a wide range of pests: *C. ferrugineus*, *O. mercator*, *O. surinamensis*, *T. castaneum* and *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae). Lorenz et al. (2010) and Reichmuth et al. (2007) indicated the wasp as biocontrol agent of *T. confusum*. Ahmed et al. (1997) reported that the wasp attacks all the six instars larva of *T. confusum* but host feeding was observed only for larvae from the first to the fourth instars. The authors reported no host feeding on pupae of *T. confusum*. Ahmed et al. (1997) reports as that the highest mortality rate (70%) was for the fourth instar of *T. confusum* whereas the lowest was for the first instar. These findings clearly indicated the preference for the fourth instar when all the larval instars larva were considered. Host feeding did not occur in male parasitoids but it is a typical activity exerted by the female of the wasp (Ahmed and Islam, 1988). This fact can be important if we consider that host feeding in *H. sylvanidis* is an essential factor causing death of smaller hosts and it depends on the age distribution of the host population. Adler et al. (2012) evaluated the efficacy of releases of *H. sylvanidis* in a five floors mill after heat treatments. The heat treatments were conducted in April and during the period between May and November, about 300 unsexed *H. sylvanids* were released every two weeks in batches of 150 individuals in the first and second floor. The presence of pests was also monitored. Approximately 2,350 females were released. The number of confused flour beetles remained low until November but it increases considerably in January especially in the cellar around the base of the grain elevator.

1.6 *Laelius pedatus*

Laelius Ashmead is a genus of small black wasps including 15 described species (Mayhew and Heitmans, 2000). The most known and widely distributed species of this genus is the arrenotokous *L. pedatus*, which has been widely reported as a semi-gregarious ectoparasitoid of Dermestidae larvae (Mayhew, 1997b; Mayhew and Heitmans 2000; Mertins, 1980). Both male and female are dark castaneous to nearly black with forewing hyaline showing a reduced venation and conspicuous setae. The female is 2.51 mm in length and the male is 1.97 mm. The number of antennal segments is 13. Tibia, tarsus and antennae are yellow brown (Azevedo, 2014; Reichmuth et al., 2007).

L. pedatus has been studied by evolutionary biologists, who have focused their attention on its oviposition. The eggs laid by the female are anhydropic (Mayhew and Heitmans, 2000) and when laid they are not firmly attached to the host. The time required between successive releases of eggs is about 30 minutes (Mertins, 1980; Morgan and Cook, 1994). During ovipositions, the female parasitoid moves its abdomen towards the caudal end of the host. One *L. pedatus* female lays approximately 24 clutches and 58 eggs during its lifetime but how the number of eggs is distributed among clutches varies considerably. The female wasp normally lays three eggs per host, although values ranging from one to eight eggs per clutch are reported (Mayhew, 1997b; Mayhew and Heitmans, 2000). When a female lays a solitary egg, the biggest host is chosen to lay female egg, in contrary in smallest hosts, a single male egg is laid (Mayhew, 1997b).

According to literature, clutch size can be directly influenced by the larval weight and/or dimensions (Klein et al., 1991; Mayhew, 1997b; Mertins, 1980). Although the wasp investigates its host with the antennae, the mechanisms employed by *L. pedatus* to determine the host dimension remains unclear. The fact that a female chooses a host depending on its volume is not casual and could be explained with the “conditional sex expression” strategy, which reports that the female can obtain the best benefits from large hosts (Klein et al., 1991). Other variables influencing the number of eggs laid are: the time of exposure to hosts, the age, the nutritional state and the mating status (Klein et al., 1991). Usually the last egg deposited by the wasp in a clutch is male (Mayhew and Heitmans, 2000).

The daily average number of eggs laid by a female wasp is 1.42 when larvae of *Trogoderma angustum* (Solier) (Coleoptera: Dermestidae) were adopted as a host. At low (below 10%) and high (above 90%) humidity and 28°C, the female wasp laid 62 and 25 eggs respectively. The best oviposition activity was registered at temperatures between 25°C and 28°C and at 15°C no oviposition occurred (Reichmuth et al., 2007). Mertins (1980) indicated 4.0 ± 0.6 days as a mean period between oviposition and egg hatching. Immediately after emergence, the first instar larva makes a hole through the host's exoskeleton in order to feed. The larvae grow rapidly completing its development in 3-4 days (Mayhew, 1997b; Mertins, 1980). Mertins (1980) reported three larval instars for *L. pedatus*. At 28°C the total developmental time from eggs to adult was 34.7 days. The lifespan of *L. pedatus* ranges from 27 days for females to 9 days for males (Mertins, 1980), but the lifespan of females can be influenced by temperatures according to the following values: three weeks at 35°C up to 16 weeks at 20°C (Reichmuth et al., 2007).

The female has a full control upon the sex of its offspring (Mertins, 1980). No information is detected regarding mating sequences, but mating opportunities can change according to the develop condition, because females emerging from gregarious clutch can mate immediately after the emergence whereas those emerging from solitary clutch do not have the same opportunity (Mayhew, 1997b).

L. pedatus females show an adaptive behaviour, which has not been reported for the other bethylids attacking stored product pests. It consists of a practice involving the removal of the eggs laid by conspecific females in order to lay an own clutch of eggs upon the same host. This ovicide tactic needs only little time to remove eggs but advantages in competition resources for the offspring can be high (Mayhew and Heitmans, 2000). Although the literature offers examples of ovicide strategy and this parasitoid shows a high handling time, no brood guarding was reported (Mayhew, 1997a). Throughout the developmental period, the offspring of *L. pedatus* could undergo attacks by different natural enemies, which cause it's died reducing the reproductive efficacy. The main factors producing mortality are: predatory mites which kill eggs, sometimes eggs become red indicating a possible bacterial infection and a hyperparasitoids *Melittobia acasta* (Walker) (Hymenoptera: Eulophidae) which attacks larvae and pupae (Mayhew and Heitmans, 2000).

L. pedatus female moves very quickly in search for hosts and when it is in front of a host, the recognition starts immediately. If the host is accepted, the female jumps upon the prey but the host larva reacts erecting dense caudal tufts of hastisetae (Mertins, 1980). This defensive mechanism sometimes allows the host to escape (Klein et al., 1991) but the attempts to be free from the parasitoid often fail, because the wasp clings to the host with all six tarsi and bites it (Mertins, 1980). Afterwards, the wasp curls the abdomen and stings the host usually at one of the coxal articulations, most often the prothoracic. The sting is very efficient producing an immediate paralysis, but it is incomplete showing the host typical contractions. Even though one sting is sufficient to make the host unable to recover, in some circumstances the larva is stung again. Similarly to other Bethylidae, after the paralysis, *L. pedatus* female bring the host to a safe place where the oviposition takes place (Klein and Beckage, 1990; Reichmuth et al., 2007). Before laying eggs, the parasitoid depilates part of the host body using its mandibles. After the complete depilation, of the posterior part of the ventral mesothorax across the fifth or sixth abdominal sternum, the host is ready for oviposition (Mertins, 1980). Although Mertins (1980) reported that no host-feeding was observed, Mayhew and Heitmans (2000) stated that *L. pedatus* adults can feed of host's haemolymph, from which they can obtain nutrients and water. Usually the female bites the host's legs or antennae.

The female wasp acts opportunistically and when a larva is encountered, it will always be stung and paralyzed. According to the literature, this natural enemy can be adopted as a control agent of stored product pests (Klein et al., 1991; Klein and Beckage, 1990; Yuntai and Burkholder, 1990). *L. pedatus* is a negatively phototactic wasp, this fact enhances its capacity to penetrate into wheat and successfully parasitize host larvae of *Trogoderma granarium* Everts (Coleoptera: Dermestidae) at a depth of 90 cm (Mertins, 1980). At a parasitoid/host ratio of 1/25, *L. pedatus* reduced populations of *T. granarium* by 75-80% within 6 to 8 weeks. Yuntai and Burkholder (1990) stated that *L. pedatus* can parasitize and kill larvae of *Trogoderma variabile* Ballion (Coleoptera: Dermestidae). It involves different performances upon different hosts. For example, Klein et al. (1991) found that *L. pedatus* laid more eggs on *T. variabile* compared to those reported by Mertins (1980) on *Anthrenus verbasci* (L.) (Coleoptera: Dermestidae). Al-kirshi (1998) reported another example; *i.e.* one *L. pedatus* female paralyses 74 larvae of *A. verbasci* and only 44 larvae of *T. granarium*. On average, only one third of the paralysed larvae of *A. verbasci* are used for oviposition.

According to different authors, *L. pedatus* is a parasitoid of the following Dermestidae: *Anthrenus flavipes* (LeConte), *A. sarnicus* Mroczkowski, *A. verbasci, Trogoderma glabrum* Herbst, *T. angustum*, *T. granarium*, *T. variabile* (Klein and Beckage, 1990; Mayhew and Heitmans, 2000; Mertins, 1980; Reichmuth et al., 2007). The wasp was reared in laboratory conditions using *A. flavipes* and *T. variabile* as hosts (Klein and Beckage, 1990; Mayhew, 1997a) (Tab. 1).

1.7 ***Plastanoxus westwoodi***

P. westwoodi is a parasitoid associated with the flat grain beetle, *Cryptolestes pusillus* (Schönherr) (Coleoptera: Cucujidae) (Rahman and Islam, 2006) and *C. turcicus*. This biocontrol agent is an external parasitoid able to parasitize fourth instar larvae and pupae of this beetle, although it is also capable to paralyze and feed on first through third instars (Ahmed and Khatun, 1996a; Rahman and Islam, 2006). The adult female is 1.35 ± 0.11 mm in length and 0.23 ± 0.02 mm in width, with a slender body shiny black. The male is 1.06 ± 0.08 mm in length and 0.18 ± 0.01 mm in width.

At the beginning of the copulation the female tries to drop the male from the abdominal region shaking her body rapidly (Ahmed and Khatun, 1996a). This behaviour was not reported for other bethylids of stored product pests herein considered.

Within 24 hours after mating, *P. westwoodi* female immediately searches for host upon which to lay eggs (Ahmed and Khatun, 1996b). The eggs are laid individually on the following parts of the abdominal segments ventrolaterally: between 2nd and 3rd segments or between the 3rd and 4th segments. Rarely, the egg is laid on the 1st thoracic segment dorsally. When the egg is laid on the pupa, the parasitoid prefers the 3rd and 4th abdominal segments ventrolaterally. The egg is 0.30 ±

0.03 mm in length, oval, whitish translucent and gradually it becomes pale yellowish at the middle (Ahmed and Khatun, 1996b). The average incubation period is 1.7 ± 0.13 days. The newly hatched larva inserts its mouthparts in the cuticle of the *C. pusillus* larva, devouring the entire host body content in 1.5-2.0 days. Although *P. westwoodi* prefers to oviposit on fourth instar larvae, this natural enemy can complete its cycle on all instar larvae of *C. pusillus* except the 1st instar, probably because this instar is too tiny to receive eggs (Ahmed and Khatun, 1996a). Developmental time from egg to adult takes 12-15 days at 27 ± 1°C and 70 ± 5 % RH (Rahman et al., 2008).

During their studies on the biology of *P. westwoodi,* Ahmed and Khatun (1996b) reported three different larval instars and their relative morphological differences. The first instar larva is creamy-white, the second is pale yellow and the third is yellowish. Regarding the dimension, the first instar is 0.46 ± 0.04 mm in length and 0.17 ± 0.01 mm head capsule, the second instar is 0.96 ± 0.09 mm in lenght and 0.19 ± 0.01 mm head capsule width, the third instar is 1.08 ± 0.03 mm in length and 0.20 ± 0.02 mm head capsule. The larva is ovoidal during the first and second instars and it becomes elongated during the third. The duration of the three stages shows some differences at 30°C and 70% RH, in which the first instar lasts for 1 day, the second lasts for 1.5 and the third lasts for 4.15 days. The prepupa measures 0.77 ± 0.08 mm in length and 0.32 ± 0.01 mm in width, it is pale yellowish and its body is elongated. The prepupa remains more or less motionless and fixes itself on a place where it spins a cocoon. The pupa is 1.02 ± 0.02 mm in length, 0.34 ± 0.02 mm in width, creamy-white, but soon the eyes and some of the abdominal portion get blackish.

Distinct levels of temperatures involve different biological performance on *P. westwoodi.* The fastest developmental time was registered at 35°C but at 25°C the wasp is able to maximize its progeny production and this temperature optimizes the longevity of females (Rahman and Islam, 2006). The entire developmental period of *P. westwoodi* could be influenced by the larval instar on wich it feeds. It was supposed (Ahmed and Khatun, 1996a) that the wasp obtains proper nourishment from fourth instar larvae and due to its high level of nutrients, this instar reduces the developmental period of *P. westwoodi* if compared to pupa and prepupa. Ahmed and Khatun

(1996b) reported the super-parasitism for *P. westwoodi*, also indicating that two larvae developed on the host and both emerged as adult.

P. westwoodi female is a restless wasp, which always searches for a suitable host (Ahmed and Khatun, 1996b). When one host is encountered, the female immediately paralyzes it by attacking its thoracic and abdominal region with the ovipositor. Rahman et al., (2008) reports as *P. westwoodi* is an efficient and successful biological control agent in maintaining *C. pusillus* populations under damaging levels (Tab. 1). During laboratory studies, the population of the the flat grain beetle suddenly decreased to a considerable level by *P. westwoodi*, but unfortunately, until now, no information is available on the suppressive effect of the wasp upon population of *C. pusillus* under natural conditions (Rahman et al., 2008). Considering its ability to parasitize all the stages of *C. pusillus*, *P. westwoodi* may play an important role in the biological control of this beetle.

1.8 Discussion

The bethylids are without doubt less studied as biological control agents than other parasitoids. The features herein reviewed offer elements that make Bethylidae suitable as biological tools against some of the most important pests of stored products. They can play a primary role in finding novel strategies to control pests, especially in case of combined uses with natural enemies belonging to other families. Here it has been reviewed seven wasps as candidate as biocontrol agents of stored products, but considering that the family consists of 2,200 species (Hawkins and Gordh, 1986), it is not possible to exclude that in the future the list of possible bethylids species will become larger than those which are herein reported. From this revision emerges that a lot of papers have been mainly focused on the biological aspects and only in few circumstances application for biological control were treated. The majority of authors linked each Bethylidae to several possible hosts, but we didn't find sufficient empirical evidence to support these relationship. In our opinion there is an urgent need to understand definitively, what are the real hosts against which, the biocontrol agents here reported can be effectively employed.

Although there are several unclear aspects, on the other side from the analysis of the *status* of the natural enemies herein discussed some important elements emerged, which should be seriously taken into account when these bethylid species are evaluated as biocontrol agents. For example *C. tarsalis* is reported as the principal natural enemy of *O. surinamensis* and *O. mercator* (Powell, 1938). It has shown benefits when used in combination with other biological organisms (Lord, 2006; Ždárková et al., 2003*). C. gallicola* outside and inside buildings is a wasp resistant to adverse climatic conditions, it is not attracted by light traps and it is able to penetrate into cracks and crevices when searching for hosts (Kearns, 1934a). For this wasp some doubt remain in relation to its capacity to cause dermatitis to humans. *H. sylvanidis* is among the less studied Bethylidae of stored products pests even though it is a promising candidate for the control of *T. confusum*. Its capacity to penetrate into different substrates in order to find hosts was demonstrated by Lorenz et al. (2010). *Holepyris hawaiiensis* is the only bethylid reported as a biological control agent against Lepidoptera infesting stored products. Similar to *C. gallicola*, this natural enemy can cause irritation to human due to its sting (Pemberton, 1932). *L. pedatus* is a wasp studied from an evolutionary point of view and, although the knowledge of its applicative characteristic is lacking, it has shown interesting features like the capacity to penetrate at a depth of 90 cm of a grain mass and successfully parasitized host larvae of *T. granarium*. *P. westowoodi* can parasitize all the stages of *C. pusillus,* and it is considered a very high host specific parasitoid of this beetle but no information is available on the suppressive effect of the wasp upon population of *C. pusillus* under natural conditions (Rahman et al., 2008).

From our study it emerges that each bethylid could be employed against a group of pests. It involves that the Bethylidae here reported can be used in several cases of infestation, covering in this way an interesting number of possible cases. Unfortunately, with regard to the methods of use we have encountered a lack of knowledge, except for *C. waterstoni* and in one case for *H. sylvanidis*. In the first case, effort has been done by Flinn et al. (1994) and Flinn and Hagstrum

(1995), who had evaluated aspects like the optimal timing of release, and the response of this natural enemy to different host densities and different temperatures. For *H. sylvanidis,* Adler et al. (2012) evaluated the efficacy of releases in a five floors mill after heat treatments.

The study of the literature regarding the Bethylidae of stored product pests has shown only in rare circumstances aspects like rearing or collecting methods. At this moment, due to the lack of knowledge present, the information collected are not able to furnish elements to make relevant the use of the most part of these biocontrol agents, especially if we compared them with other Hymenoptera parasitoids such as Trichogramma spp., Braconidae and Pteromalidae. For these reasons, further studies are absolutely needed to solve most of the questions herein indicated.

Table 1
References and hosts of Bethylidae of stored products pests. Experimentally confirmed hosts.

Biocontrol agents	Host (s)	Reference
C. gallicola	*Araecerus fasciculatus* (Degeer) (Coleoptera: Anthribidae) *Gibbium psylloides* (De Czepinski) (Coleoptera: Ptinidae) *Lasioderma serricorne* (F.) (Coleoptera: Anobidae) *Niptus hololeucus* Faldermann (Coleoptera: Ptinidae) *Ptinus fur* (Linneaus) (Coleoptera: Ptinidae) *Ptinus tectus* Boieldieu (Coleoptera: Ptinidae) *Stegobium paniceum* (L.) (Coleoptera: Anobidae)	Lim et al. (2007), Itoh (1980).
C. tarsalis	*Oryzaephilus mercator* (Fauvel) (Coleoptera: Silvanidae) *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae)	Cheng et al. (2003), Collatz and Steidle (2008), Lord (2001, 2006), Ždárková et al. (2003).
C. waterstoni	*Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae) *Cryptolestes minutus* (Olivier) (Coleoptera: Cucujidae)	Flinn (1991), Flinn and Hagstrum (1995), Reichmuth et al. (2007).
H.hawaiiensis	*Corcyra cephalonica* (Stainton) (Lepidoptera: Pyralidae) *Ephestia cautella* (Walker) (Lepidoptera: Phycitidae) *Ereunetis flavistriata* Walsingham (Lepidoptera: Tineidae) *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae)	Bridwell (1919, 1920).
H. sylvanidis	*Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae) *Oryzaephilus mercator* (Fauvel) (Coleoptera: Silvanidae) *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) *Tribolium confusum* J. du Val (Coleoptera: Tenebrionidae)	Adler et al. (2012), Eliopoulos et al. (2002), Lorenz et al. (2010), Reichmuth et al. (2007).
L. pedatus	*Anthrenus flavipes* (LeConte) (Coleoptera: Dermestidae) *Anthrenus sarnicus* Mroczkowski (Coleoptera: Dermestidae) *Anthrenus verbasci* (L.) (Coleoptera: Dermestidae) *Trogoderma angustum* (Solier) (Coleoptera: Dermestidae) *Trogoderma glabrum* (Herbst) (Coleoptera: Dermestidae) *Trogoderma granarium* Everts (Coleoptera: Dermestidae)	Klein and Beckage (1990), Klein et al. (1991), Mayhew and Heitmans (2000), Reichmuth et al. (2007).
P. westwoodi	*Cryptolestes pusillus* (Schönherr) (Coleoptera: Cucujidae) *Cryptolestes turcicus* (Grouvelle) (Coleoptera: Cucujidae)	Rahman et al. (2008). Schöller (personal observation)

2 The biology of *Holepyris sylvanidis*

2.1 General aspects

H. sylvanidis has been reported as a parasitoid of the confused flour beetle *T. confusum* (Ahmed and Islam, 1988; Hagstrum et al., 2012). *Tribolium confusum* is a cosmopolitan pest which lives in almost any kind of flour, cracked grain, breakfast cereals (Park, 1934). As reported by Hagstrum et al. (2012) this pest occurs in flour mills, feed mills, warehouses, retail grocery stores, boxcars, semolina mills and bakeries. Sutton et al. (2011) indicated *T. confusum* as a pest of stored grains, cereal products, fruit and nuts. Also, the pest was reported in empty cargo containers, farm grain bins, farm storages, grain elevators, peanut shelling plants, residences and pet stores (Hagstrum and Subramanyam, 2009). In flour mills, the direct contamination, could have heavy economic consequence including costs associated with treatment and monitoring, rejection and return of contaminated products and loss of consumers trust (Campbell and Hagstrum, 2004). Previous studies (Ahmed et al., 1997; Ahmed and Islam, 1988) have only partially clarified the biology of *H. sylvanidis,* thus the main biological features of this natural enemy remain strongly unknown until now. It is probably due to the difficulties to collect and to rear this wasp under laboratory conditions. However, so far no viable rearing protocols have been developed for this natural enemy. In order to contribute toward the development of recommendations for application of Bethylidae in biological pest control programs, observations on the biology of *H. sylvanidis* are here reported together with a protocol to rear *H. sylvanidis* under laboratory conditions. A wild strain of *H. sylvanidis* collected at Enna in a grain centre warehouse was investigated. Similarly to Ahmed et al. (1997) the wasp was found on wheat infested by *T. confusum*. According to Trematerra et al. (2007), *T. confusum* is dominant in areas with Mediterranean conditions. In this study, the following aspects are reported: (I) the life history for male and female, (II) mating with emphasis to duration and courtship times, (III) preoviposition period and clutch size during the first 24 hours of life, (IV) fecundity, (V) longevity of males and females under different nutritional states, (VI) a description of the attack.

2.2 Materials and methods

2.2.1 Parasitoid rearings

T. confusum was reared in a 12 cm diameter Petri-dish containing 20 g of flour (*Triticum aestivum*) and 5% of brewers' yeast as substrates (Athanassiou and Kavallieratos, 2014). *T. confusum* adults were sieved out using a 0.6 mm sieve one time a week and kept on fresh flour and brewers' yeast, as reported above, to lay new eggs. Sieving the content, the flour containing the eggs was collected and afterwards it was maintained at 27 ± 1°C and 65 ± 5% relative humidity (RH). After about 23 days of incubation, fourth instars larvae were obtained. In order to rear the parasitoid, two females and one male of *H. sylvanidis* were kept in a 12 cm diameter Petri-dish with fourth instars larvae of the confused flour beetle (Ahmed et al., 1997). Ten fresh fourth instar larvae were added to each Petri dish twice a week. The parasitized larvae of the beetle were collected three times a week and kept at 27 ± 1°C and 65 ± 5% RH. In order to favourite the natural behaviour of Bethylidae, which hide the paralyzed host larva in a safe place, where the oviposition will take place (Griffiths and Godfray, 1988), the undulated part of a cardboard was added to the Petri dish. As reported by Ahmed et al. (1997), *H. sylvanidis* feeds on its host in order to enhance egg production and subsequently oviposition. An energetic and high protein diet has been provided to *H. sylvanidis* aiming at a reduction of host feeding and increased oviposition. If on one hand the importance of sugar in enhancing egg production has been discussed in some manuscripts, on the other hand the literature offers some examples of the importance of amino acids in egg production (Gilbert, 1972; Murphy et al., 1983). The diet provided to *H. sylvanidis* was composed of: water (1 ml), honey (0.1 g), pollen (7 pieces) and royal jelly (0.05 g) and has been provided putting a drop of the solution in a 1 x 1 cm filter paper.

2.2.2 Life history

In order to evaluate the duration of the life stages, eggs laid by *H. sylvanidis* upon fourth instar larvae were collected daily, kept individually in a Petri dish (4 cm diameter) and maintained at 27 ± 1°C and 65 ± 5% RH. Each egg and the successive stages were observed daily until a new emerged adult was obtained. After the emergence, each adult was sexed. During the observations the following aspects were registered for both sexes: (I) days from egg to larva, (II) days from larva to pupa, (III) days from pupa to adult, (IV) days between egg and penetration of the larva into the body of the host, (V) days between egg and the complete consumption of the host larva, (VI) days between the first emission of silk and pupal stage. The larval stage began when the head of the larva emerged from the chorion as reported by Powel (1938). Finlayson (1950a) evaluated the pupal stage opening the cocoon. In the case of *H. sylvanidis*, considering that the pupa is very delicate and it could die easily during manipulation, it was preferred to avoid opening the cocoon by cutting it during the experiments.

2.2.3 Duration of mating

Mating was evaluated in a room in which the temperature was maintained at 27 ± 1°C and 65 ± 5% RH. As reported by Ahmed and Islam (1988) the mating occurs soon after the emergence and Ahmed et al. (1997) stated that when the mating occurs, the male suddenly jumps over the female and pushes his copulatory organ. This experiment was conducted in a Petri dish (4 cm x 1.2 cm) as arena. A new Petri dish was used for each observation in order to eliminate stimuli left by the previous examined wasps. Unmated, one day old females and males were evaluated. In order to be sure that the adults were unmated, cocoons were isolated from the rearings 4-5 days before adult emergence. One female was introduced into the cage and subsequently one male. Time from the introduction of wasps and the beginning of copulation (precopulatory time, PT), the duration of copulation (copulatory time, CT) and the total time duration (TT) were registered. The count of CT started when the male mounted the female and finished when the male dismounted from the female.

2.2.4 Pre-oviposition period

The pre-oviposition period was investigated holding cocoons individually until newly emerged adults were obtained. Two experiments were carried out. The first experiment was conducted keeping a new emerged female (< 1 day old) and a new emerged male (< 1 day old) in a Petri dish (12 cm) containing 5 fourth instar larvae of *T. confusum*. The second experiment was conducted maintaining in the Petri dish only one unmated *H. sylvanidis* female (< 1 day old) with 5 fourth instar larvae. The number of larvae provided was chosen according to Finlayson (1950a). The author indicated that new fresh larvae were added when those already present were paralysed or dead. In the case of *H. sylvanidis* paralysis is an important step before the oviposition, so new fresh larvae were provided only when the old were used as a food source and offered no opportunity to lay eggs. The larval instars were chosen according to Ahmed et al. (1997) who indicated the fourth instar as the favourite for oviposition. A piece of undulated cardboard (2 x 2 cm) was inserted in the Petri dish. The pre-oviposition period was calculated computing the days between the emergence of the female and the first egg laid (Finlayson, 1950a).

During the pre-oviposition experiments, the hypothesis has also been investigated if the mating might affect the first 24 hours of oviposition. To investigate this, the number of eggs laid during the first 24 hours of oviposition by mated and unmated females were registered, respectively. Recording of the eggs released in this time period started when the first egg laid was observed.

2.2.5 Fecundity

In order to evaluate how many eggs are laid by mated and unmated females during their entire lifespan an experiment was conducted. A 12 cm Petri dish containing a piece of undulated cardboard, was provided to each mated female. Each repetition included a female and a male. The same procedure was used for unmated females but in this case no males were included in the experiment. Weekly 15 fourth-instar larvae (Ahmed et al., 1997) were supplied to each female. The 15 fourth instar larvae were distributed as follow: on Monday 5 larvae were added to each Petri dish and on Friday a new petri dish containing 10 fresh larvae was offered to each female. The experiment was conducted at 27 ± 1°C and 65 ± 5% RH. Eggs laid by the parasitoid on the hosts were collected two times a week on Monday and Friday and registered in a data sheet.

2.2.6 Longevity of females

The longevity of starved females and females nourished with fourth instar larvae was tested at 27 ± 1°C and 65 ± 5% RH. Mated females (0-1 day old) were then transferred to plastic vials (2.6 cm x 0.6 cm) and two times a week 5 fresh larvae were provided. The cap was cut making a circular hole (0.75 cm diameter) and a piece of thin woven was used in order to facilitate airflow. In his experiment Finlayson (1950a) discarded some tubes because during the examinations some daughter females emerged. In order to avoid this, when new larvae were supplied the old ones were eliminated. Starved females were also kept in a plastic vial (2.6 cm x 0.6 cm) and in the same environmental condition but without a food source.

2.2.7 Longevity of males

The longevity of males was studied keeping cocoons individually in Eppendorf vials (1.5 ml) and by collecting daily the newly emerged males. They were kept in a plastic vial (2.6 cm x 0.6 cm) at 27 ± 1°C and 65 ± 5% RH. The cap was cut making a circular hole (0.75 cm diameter) and a piece of thin woven was used in order to facilitate airflow. Three different experiments were set up: (I) starved males, (II) males nourished by a sucrose solution, (III) males nourished by a sucrose and pollen solution. The sucrose solution was obtained mixing water (2.5 ml) and sucrose (0.5 g) for 1 minute, the sucrose pollen solution was obtained adding to a sucrose solution 4 pieces of pollen, which were dissolved in the solution. In all the cases in which nourishment was provided, a drop of food solution was provided to each male from the top of the woven twice a week. Each vial was checked every day after the beginning of the experiment until the death of each wasp.

2.3 Statistical analysis

Data were analysed using AnalystSoft Inc., StatPlus: mac – statistical analysis program for Mac OS. Version v5. All the means reported in this study are followed by standard error and for all the tests here reported α = 0.05 has been considered as significance level. The following comparisons, between males and females or mated and unmated females, were explored studying the normality with the Shapiro-Wilk test and comparing the means according to the non-parametric Whitney-Mann-U test: duration of the different life stages, pre-oviposition period and fecundity. The frequency in which the oviposition has been registered for mated and unmated females was analysed computing the χ^2. The regression analysis was performed in order to study the oviposition over time for mated and unmated females. The average longevity of nourished and starved females was explored assessing the normality according to Shapiro-Wilk test and comparing the means with the parametric two-tailed T-test. The average longevity of nourished and starved males was compared, after the study of the normality according to Shapiro-Wilk test, performing out the Kruskall-Wallis test, followed by all pairwise multiple comparisons with the non-parametric Whitney-Mann-U test.

2.4 Results

2.4.1 Life History

The egg is 0.32 ± 0.003 mm ($n = 50$) in length and 0.13 ± 0.001 mm ($n = 50$) in width. This stage lasts for 2.37 ± 0.11 days ($n = 35$) in male and 2.31 ± 0.09 days ($n = 35$) in female and there was no significant difference in duration between the sexes ($P > 0.05$) (Tab.1). Larval stage lasts for 8.8 ± 0.17 days ($n = 35$) in male and 8.86 ± 0.16 days ($n = 35$) in female and there was no significant difference in duration between the sexes, too ($P > 0.05$) (Tab. 1). The pupal stage lasts for 11.94 ± 0.15 days in male ($n = 35$) and 13.26 ± 0.23 days ($n = 35$) in female and when the means were compared a strong significant difference was found ($P < 0.0001$) (Tab. 1). The time computed from egg to larval penetration is 5.67 ± 0.12 days for both male ($n = 30$) and female ($n = 30$). After the larva finished to feed, only an empty shell of the host remains, which shows a rounded hole 0.38 ± 0.01 of diameter ($n = 30$). The time computed from the oviposition to the complete consumption of the host larva is 6.9 ± 0.17 days in male (*n* = 30) and 6.4 ± 0.15 days in female (*n* = 30). Subsequently the larva spins a white cocoon and after 7.3 ± 0.18 days (*n* = 30) in male and 7.0 ± 0.21 days in female (*n* = 30) pupation takes place.

Table 1 Development time (days ± SE) of *Holepyris sylvanidis* egg, larva and pupa under laboratory conditions (27 ± 1°C; 65 ± 5% RH).

Stage	Male	Female
Egg	2.37 ± 0.11a	2.31 ± 0.09a
Larva	8.80 ± 0.17a	8.86 ± 0.16a
Pupa	11.94 ± 0.15a	13.26 ± 0.23b
Total	23.11 ± 0.21a	24.43 ± 0.28b

Means within rows followed by different letters are statistically different (P < 0.05) according to Whitney-Mann-U test.

2.4.2 Duration of mating

When the wasps were kept in the Petri dish, after a mean period of 208.6 ± 30.2 s (*n* = 30) the copulation began (PT) (Fig. 1). The mean copulatory time was (CT) 119. 5 ± 8.2 s (*n* = 30), the mean total time duration (TT) was 328.1 ± 30 s (*n* = 30).

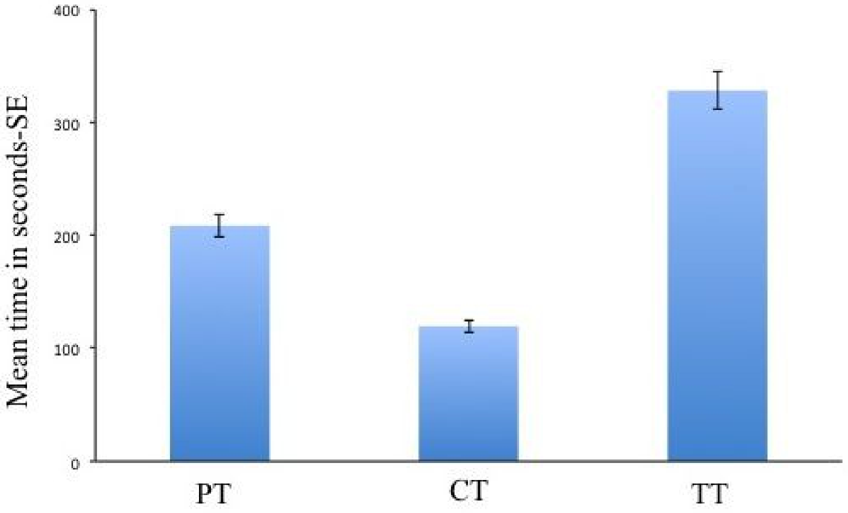

Fig. 1 Precopulatory period (PT), copulatory period (CT) and total duration of the mating (TT) of *H. sylvanidis*. Data are reported as mean in seconds and standard errors of the mean.

2.4.3 Pre-oviposition period

There was a significant statistical difference ($P < 0.05$) between the means of the mated (3.68 ± 0.28 days) ($n = 50$) and the unmated (5.36 ± 0.51 days) ($n = 50$) females (Fig. 2). When the average number of eggs laid during the first 24 hours of life were compared, no significant difference ($P > 0.05$) between mated (1.4 ± 0.11 egg) ($n = 35$) and unmated females (1.3 ± 0.07 egg) ($n = 35$) was registered.

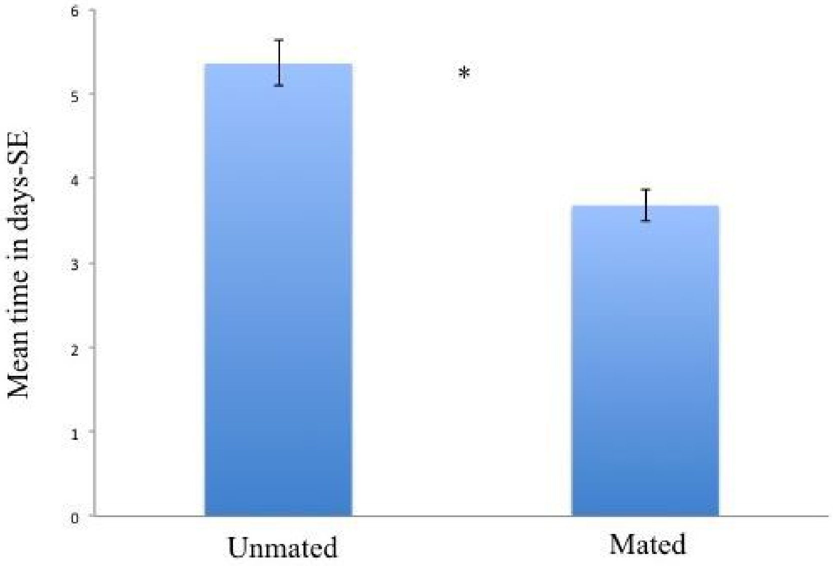

Fig. 2 Pre-oviposition period for unmated and mated *H. sylvanidis* females. The means are reported in days ± standard error and show a significant statistical difference ($P < 0.05$) according to the non-parametric Whitney-Mann-U test. * = significant difference.

2.4.4 Fecundity

Mated females laid a mean of 15.11 ± 1.1 eggs (*n* = 34) and unmated females laid 17.7 ± 1.1 eggs (*n* = 34) during the entire lifespan. The difference between the average number of eggs laid by mated and unmated females was not significant ($P > 0.05$) (Fig. 3). In terms of frequency unmated females laid eggs more times than the mated female (Tab. 2) and this frequency is influenced by the mating status χ^2 ($P < 0.05$). There was a significant negative correlation between eggs laid and time for mated (Fig. 5) ($P < 0.0005$, $R^2 = 0.153$, $y = -0.0272x + 0.52$) and unmated females (Fig. 6) ($P < 0.0005$, $R^2 = 0.092$, $y = -0.0146x + 0.418$).

Table 2 Contingency table. Incidence of the mating on the frequency of oviposition during the life time.

	Mated	Unmated	Total
Oviposition	254	316	570
No oviposition	99	186	285
Total	353	502	

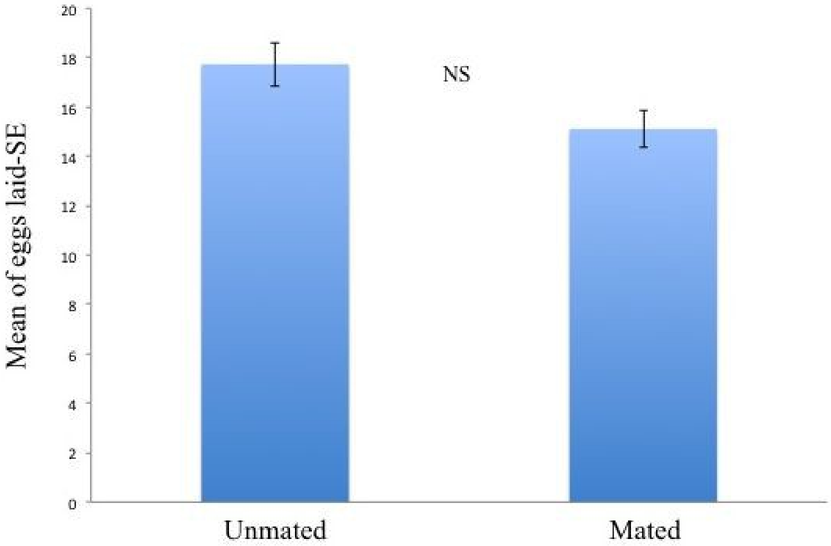

Fig. 3 Eggs laid by unmated and mated females during the lifetime. The means are reported as number of eggs laid ± standard error and don't show a significant statistical difference ($P > 0.05$) according to the non-parametric Whitney-Mann-U test. NS = no significant difference.

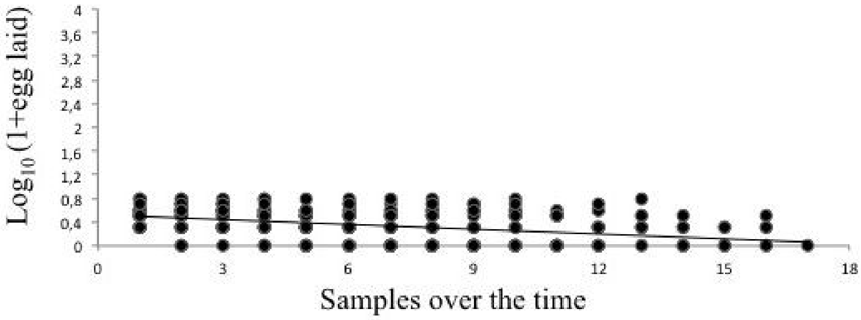

Fig. 4 Effect of time on the number of eggs laid by mated females. The number of eggs laid by each female was recorded two times a week following a Monday-Friday scheme for the entire lifetime. The regression line shows a significant decline over the time ($P < 0.0005$, $R^2 = 0.153$).

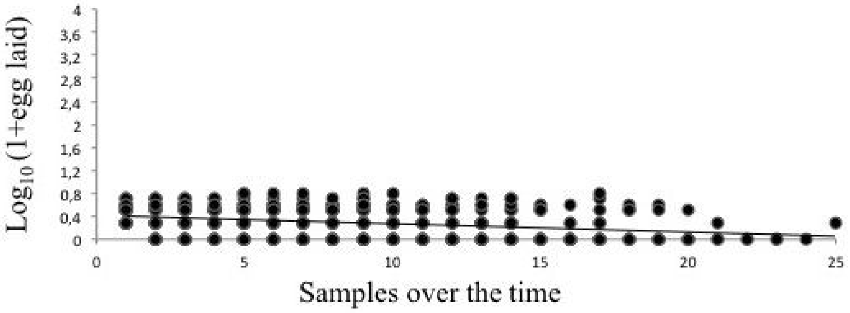

Fig. 5 Effect of time on the number of eggs laid by unmated females. The number of eggs laid by each females was recorded two times a week following a Monday-Friday scheme for the entire duration of the life. The regression line shows a significant decline over the time ($P < 0.0005$, $R^2 = 0.092$).

2.4.5 Longevity of females and males

The females nourished with fourth-instar larvae lived significantly longer (48.6 ± 2.7 days) ($n = 45$) ($P < 0.0001$) than starved females (8.1 ± 0.5 days) ($n = 45$) (Fig. 6). The starved males lived on average for 7 ± 0.2 days ($n = 45$), the males nourished with the sucrose solution lived on average for 18.18 ± 1.64 days ($n = 45$) and the males nourished with the sucrose solution and pollen lived on average for 35.48 ± 4.97 days ($n = 45$). There was a significant difference between the mean longevity of males nourished with the sucrose solution and the mean longevity of starved males ($P < 0.005$), the mean longevity of males nourished with the sucrose solution and pollen was significantly longer than the mean longevity of starved males ($P < 0.0005$). There was no significant difference between males nourished with the sucrose solution and males nourished with the sucrose solution and pollen ($P > 0.05$) (Fig. 7).

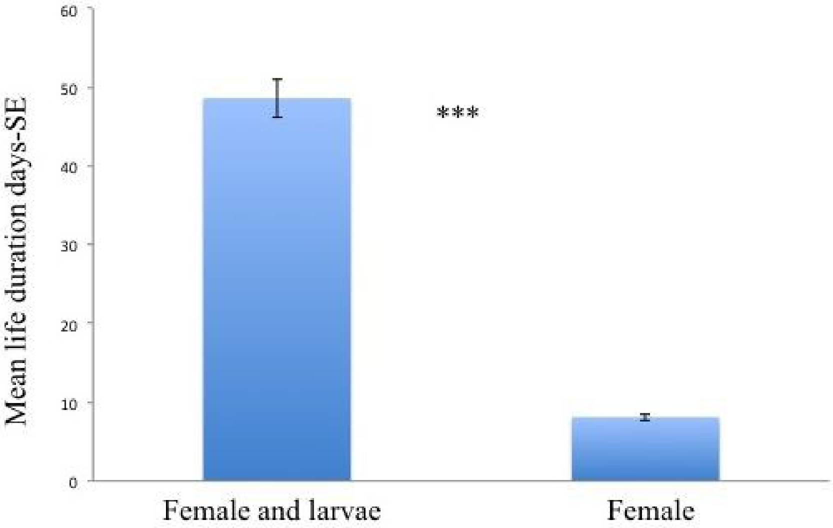

Fig. 6 Longevity of starved females and females nourished with fourth-instar larvae. The means are reported in days ± standard error and show a strong significant statistical difference according to two-tailed T-test, $P < 0.0001$. *** = strong significant difference.

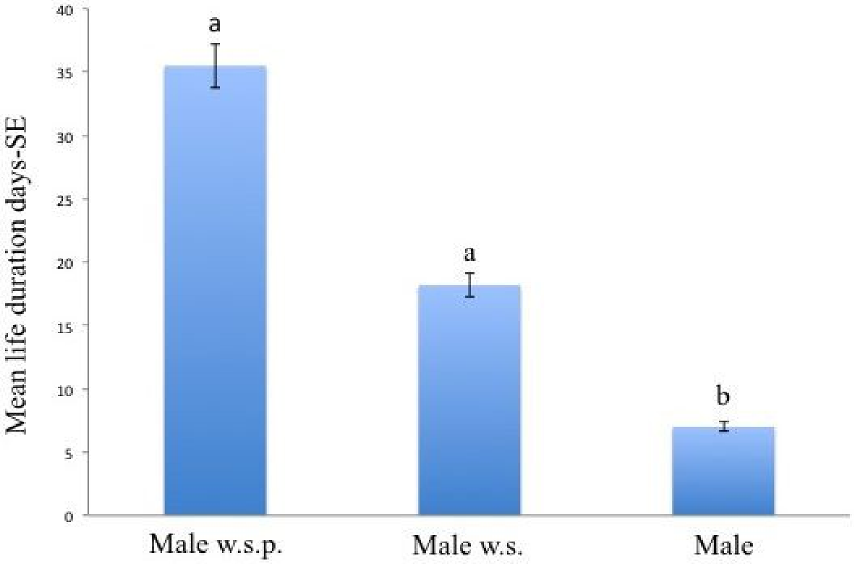

Fig. 7 Longevity of males nourished with sucrose and pollen solution (w.s.p.), males nourished with sucrose solution (w.s.), starved males. The means are reported in days ± standard error. Data in each bar followed by the same letter are not significantly different according to non- parametric Kruskal–Wallis test followed by all pairwise multiple comparisons with Whitney-Mann-U test ($P < 0.05$).

2.5 Discussion

2.5.1 Life History

H. sylvanidis is an idiobiont semiectoparasitoid, which lays a solitary egg on the venter of its host. The egg is elongated, slightly curved and white, its poles are rounded and it is normally placed longitudinally between the fourth-fifth segments on the host's venter but sometimes the egg is laid in other positions: at the fifth segment longitudinally or as reported by Ahmed and Islam (1988) between the sixth and seventh segments longitudinally. Only in rare cases eggs were laid transversally or at the dorsal side.

In some circumstance it has been observed two eggs per host, so we confirm the superparasitism stated by Ahmed et al. (1997). During the present study, this phenomenon was further investigated, keeping a single mated female with 5 larvae provided twice a week. According to the definition of van Dijken and Waage (1987), self-superparasitism is reported here the for *H. sylvanidis*. The literature offers examples of Bethylidae which can lay more than one egg per host

(Finlayson, 1950a) or the sex allocation can vary when a clutch of eggs is laid (Mayhew and Godfray, 1997). In the case of *H. sylvanidis*, at this moment, it is only possible to formulate some hypothesis to explain both the superparasitism and the self superparasitism, *e.g.* the female tries to equilibrate the sex allocation among off-spring, or she is not able to distinguish between a parasitized and unparasitized host due to a lack of intensity of the chemical marker laid with the first egg (van Dijken and Waage, 1987). The self-superparasitism is of interested considering that this phenomenon involves siblicide and parent-offspring conflict (Mackauer and Chau, 2001).

The larva of *H. sylvanidis* has white poles and it is entirely white at the beginning of this stage becoming brownish at the centre after few days, it is legless and setae are absent. This stage begins when the head appears at the superior extremity of the egg and the parasite larva occupied, until it doesn't penetrate, the same position on the host as in its egg stage. The head is narrower than the abdomen, which is strongly pointed at the end parts. Characteristic larval traits are discernible after some days of the eclosion, *e.g.* white grains (feces) are present at the centre of the body and the sucking movements of the digestive tract are detectable through the transparent cuticle. The white grains move initially through the digestive tract but afterwards they become fixed.

After eclosion, the larva inserts the mouthparts into the host body to feed. Removing the egg from the host larva, it seems that *H. sylvanidis* female doesn't practice holes or incisions to favourite the feeding of her off-spring. During the larval stage the growth is rapid and when the larva becomes about two or three times bigger than the initial dimension, it penetrates with the anterior part into the host and sucks up the body fluid content leaving half of its body inside the host and the other half outside. During the study it was not possible to evaluate the number of larval stages because no exuviae were observed. A similar aspect has been reported for other Bethylidae like *C. waterstoni* (Finlayson, 1950a) and *L. pedatus* (Mertins, 1980).

After the larva has finished to feed from its host, it leaves the empty shell and starts to produce the silk net in order to pupate. In some cases a proper cocoon was not formed because it doesn't enveloped completely the larva and it was only reduced to a tangled mass of threads.

However, pupae that were not enclosed in the cocoon successfully reach adulthood. During the study of life stage a typical meconium was observed during the metamorphosis. This phenomenon was similarly observed by Rilett (1949) for *C. waterstoni*. The pupa has structures whose make it similar to the adult, so all the appendages and the eyes can be fully distinguished. In this phase the antennae and the legs are closely attached to the body. This preimaginal stage can respond to external stimuli moving the abdomen. The pupa is initially white but becomes dark during the days. How it changes colour is of interest, the large compound eyes are brownish by the end of the first day of pupation and by the second day they are dark, the thorax first becomes dark followed by the abdomen and the head. The last part, which change colour is the intersegmental membrane of the abdomen, becoming completely dark after one day the new emerged adult was seen. As we expected the differentiation between the two sexes occurs during the pupal stage. The strong significant difference here reported is an indicator of the more biological complexity of the female and it reflects how big is the variance in the behaviour of the two sexes. Males are smaller then female and they have only shown simple life patters, *e.g.* they don't attack larvae or don't bring them in places where oviposition takes place. In contrast, females have displayed a lot of complex patterns, which were not studied until now and for this reason they need further analyses.

2.5.2 Duration of mating

The courtship starts when the male verifies the opportunity to mate approaching the female and touching her with its antennae. Escaping females indicate that they are not ready to mate. A similar behaviour was reported by Cheng et al. (2003) for *C. tarsalis*. On the other hand, when the female is receptive, the wasps vibrate their antennae respectively, then the female turns the body of 180 degrees and as reported by Ahmed et al. (1997), the male suddenly jumps over the female and the copulation occurs. At this stage the male is fixed to the female. Bodies made a 90° angle during this phase and the antennae of both sexes are stationary. In some circumstances, especially when the male doesn't start the courtship, the female touches with the antennae the male's abdomen from behind, probably aiming at to stimulate the beginning of a courtship.

It is possible to confirm the observation of Ahmed et al. (1997) who indicated the female as stationary during the copulation, and on the other hand it is possible to report how in some circumstances the female runs around the cage when the bodies assume the copulatory position. The male shows rhythmic movements of his body during the copulation when the female is stationary. These movements were not detectable when the female ran around the cage. Our observations were conducted in a 34.5 cm^3 Petri dish, inside which it is possible to assume the mean time required for mating (courtship and copulation) as TT, when one female and one male are inside the cage. This aspect is of importance because larger Petri dishes or the tube in which the wasps were kept, more time is required until mating.

2.5.3 Pre-oviposition period

H. sylvanidis reproduces according to haplodiploidy (arrhenotoky) sex determination, in which the unfertilized egg develops into a male and the fertilized egg into a female. Haplodiploidy involves a control over the sex ratio, it means that a female can control the access of sperm to egg by itself in order to establish the best sex allocation. This type of reproduction is of interest for behavioural ecologists, who have conducted some studies on Bethylidae revealing factors influencing their strategies of reproduction (Hardy et al., 2000; Khidr et al., 2012).

How the sex is allocated among offspring can change according to environmental conditions and other influences have also been attributed to population structure, resource quality, seasonality and patterns of inheritance (Werren, 1987). The data here presented show that the pre-oviposition period of the mated females is significantly shorter than that of unmated females. This fact is of interest, because unmated female shows a sort of reluctance to lay unfertilized eggs. Similar evidence was reported for *L. pedatus* by Mayhew and Heitmans (2000) and the study of Yamasaki (1982) on *C. gallicola* agree with the longer pre-oviposition period of unmated *H. sylvanidis* females. The longer pre-oviposition time of unmated female can be explained according to Guertin et al. (1996) who stated that a female wasp may face a trade-off between seeking hosts before mating and producing only sons, or searching for mating with the possibility of producing both sons and daughters afterwards.

H. sylvanidis is a synovigenic species (Ahmed et al., 1997) and as reported by Lauzière et al. (1999) host feeding is a relevant characteristic of synovigenic parasitoids. Host feeding primarily serves to supply nutrients for egg maturation. This fact could influence the pre-oviposition period but also could play a sort of influence on the time required for successive egg laying. For example, *Cephalonomia stephanoderis* Betrem (Hymenoptera: Bethylidae) (Lauzière et al., 1999) can extend its pre-oviposition period behaving as predators to survive and secure nutrients for vitellogenin-rich oocyte formation. In the literature, evidences are reported about the relationship between pre-oviposition period and environmental conditions. For example, in the case of *C. waterstoni* the mean pre-oviposition period was 1.7 days at 30°C and 80% RH, it was 5 days at 25°C and 80% RH (Finlayson, 1950a) whereas for *C. stephanoderis* the mean pre-oviposition time decreased when the number of hosts available increased (Lauzière et al., 1999).

Considering the lack of knowledge regarding the correlation between environmental conditions and food sources on the pre-oviposition period of *H. sylvanidis*, further investigations are required. In the literature of the most important Bethylidae candidate as biocontrol agents, there is no information available about the mean number of host larvae used as a food source before to lay the first egg.

The data here presented show that the mating status doesn't influence the oviposition strategy during the first 24 hours of life. This result can be explained according to a physiological status of *H. sylvanidis* female, which is able to mature during the first 24 hours of life only 1.4 ± 0.11 eggs when she is mated and 1.3 ± 0.07 when she is unmated.

2.5.4 Fecundity

Our results have shown a slightly smaller quantity of eggs released by mated female when compared with unmated female. This result is in contrast with the study of Cheng et al. (2003) in which an higher average of eggs laid for mated females when compared with unmated females has been reported, but the literature (Hardy et al., 1998) offers one example in which mated females of *Colpoclypeus florus* (Walker) (Hymenoptera: Eulophidae) laid slightly smaller clutches than virgin females. Under the experimental conditions here reported, the average number of eggs laid by both mated and unmated female of *H. sylvanidis* is less than those reported for other Bethylidae considered as a biocontrol agent of stored products pests, *i.e. C. tarsalis* 85 eggs (Powell, 1938) and *C. waterstoni* 102 eggs (Finlayson, 1950a). Until now nothing is known regarding the quantitative aspects of the oviposition of the solitary ectoparasitoid *H. sylvanidis*, but Infante (2000) during his studies on the solitary ectoparasitoid *P. nasuta*, reported different oviposition rates at different temperatures. This natural enemy of *H. hampei* lays 322 eggs at 27°C and only 33 at 18°C.

During the experiments here reported only fresh fourth-instar larvae were provided to *H. sylvanidis* females, being this instar the favourite to lay egg (Ahmed et al., 1997). *H. sylvanidis* is a synovigenic parasitoid (Ahmed et al., 1997), it involves that the female has to feed to mature their own eggs. Female of synovigenic species emerge with only a fraction of mature egg (Lauzière et al., 2001) and can be subdivided in two groups: autogenous which mature some eggs without first feeding and anautogenous which must feed during a variable pre-oviposition period to stimulate oogenesis. Females feed on haemolymph and other tissues of their hosts to get the required level of nutrients to mature eggs (Lauzière et al., 2001). *H. sylvanidis* is also a nonconcurrent host feeding parasitoid being able to use the larva either for oviposition or as a food source. To explain the low oviposition rate obtained during the current study, it is possible to hypothesize an influence by the fact that only large larvae have been offered to both mated and unmated females. In this environment the favourite food source; *e.g.* small larvae, were lacking. Other elements could have played a primary role upon the quantitative aspects of the oviposition. For example, Godfray (1994) reported that egg loads can vary according to two main factors; parasitoid's capacity to replenish egg and its carrying capacity. Furthermore, nothing is still known regarding the influence of temperatures, humidity and number of larvae presents in the environment on the oviposition rate of *H. sylvanidis*. In terms of frequency of oviposition unmated females were more active than mated females. It could be due the fact that *H. sylvanidis* mated females have to allocate the sex among their offspring. To do so, mated females have to evaluate continuously the quality of the larvae present in the environment. In involves costs in term of time required to find the best larva upon which to lay a fertilized egg. During the experiments here reported the larvae were renewed two times a week, it probably played an important role in the frequency of oviposition. Instead, unmated females have only to lay the eggs when it is ripe and the reproductive expectancy for them is of secondary importance.

During our observations, an egg limitation has been noticed for *H. sylvanidis* female, because she ran out of eggs before all possible oviposition sites were used (Deas and Hunter, 2014). This involves that like other egg limited parasitoids, with low fecundity, *H. sylvanidis* has to be selective when she has to determine if the egg will be fertilized or not. In several cases the females ceased to lay egg from 1 to 2 weeks before they die. This aspect seemed more pronounced for unmated females for which it has been observed that some females didn't lay eggs for 4 weeks before dying. The data here indicated a significant decline of egg laying over time. It is possible to suppose that over time, the resources necessary to develop eggs in the body of females are reduced.

2.5.6 Longevity of females and males

Providing parasitoids with a suitable food source is a promising method to maximize survival, pest suppression and the establishment of beneficial insects in an environment (Hausmann et al., 2005). Longevity can play a primary role on the impact of parasitoids on the pest population because it is has been observed that well-fed parasitoids are more active and more focused in seeking their host. On the contrary starved females could be less active and lay fewer eggs like in the case of *Rhyssa persuasoria* (L.) (Hymenoptera: Ichneumonidae) (Hocking, 1966; Wäckers, 2002). The energy cost that a female has to meet during her lifetime is generally related to egg production and host-finding (Hoogendoorn et al., 2002). When a female parasitoid is in front of a larva, she has to establish whether to use the host for current reproduction (oviposition) or for future reproduction (host-feeding). This fact mostly depends on the physiological state, which consequently involves different behaviours in the environment (Chan and Godfray, 1993; Pereira and Zucoloto, 2006; Rivero and Casas, 1999). The food elements needed by an insect can vary according to their life stage, growth, development and reproduction.

The goal of this study was to clarify which macronutrient is involved in the longevity of *H. sylvanidis*. This natural enemy is synovigenic, it involves that the female must get the nourishment to mature eggs. According to Ahmed et al. (1997), *H. sylvanidis* females can attack and host-fed on all larval stages except to fifth and sixth. In the current study only fourth instar larvae were provided as a food source. This instar has been reported (Ahmed et al., 1997) as the favourite for the egg-laying but the data here reported indicate the suitability of the fourth instar as a food source. During the current observational study, it has been observed that the parasitoid feeds primarily from the host haemolymph but it is also able to eat from tissues of the host larvae. According to Famadiro and Heimpel (2001), it is possible to hypothesize that the sugar source that could be used by a female is the trehalose because it is the most dominant sugar in insect haemolymph. The biochemical advantage deriving from the trehalose is that this molecule can be stored in relatively high concentration in body fluids (Becker et al., 1996). The literature offers several examples regarding the longevity of Bethylidae, *e.g.* *C. gallicola* females reared on fresh hosts survived on average for 94 days and when maintained without host supply they survived on average for 10-20 days (Yamasaki, 1982). Van Emden (1931) found that *C. gallicola* females lived on average for 58 days at 21.9°C, 64 days at 19.1°C and 75 days at 18.7°C when fed on hosts. *C. waterstoni* females lived on average for 37.5 days at 25°C and 80% RH and 30 days at 30°C and 80 RH in presence of larvae. In *C. tarsalis* both fertilized and unfertilized females lived on average for 35 days and the males lived for 6 days (Powel, 1938). Mated females of *C. hyalinipennis* lived on average 57 days (Pérez and Hardy, 1999).

To replenish the energy invested in their activities, parasitoids need to feed. Natural enemies larvae feed on the host larvae but adults are not always able to feed from host sources. *H. sylvanidis* females can feed from almost all the larval instars when these are present, but the male is not able to do this showing a strong indifference toward host larvae. It is due to the fact that the male is not able to attack the larvae, instead it is strongly attracted by non host food source. In this context, it is possible to expect that under natural conditions the male feeds from nectars and pollen. Male longevity in parasitoid wasps can be studied using essentially the same approach as female longevity (Hoogendoorn, 2002). When *H. sylvanidis* males have been fed by the diets (sucrose solution, sucrose solution and pollen) their lifetime was significantly higher than starved males. The sucrose pollen solution provided to males showed a statistical significant effect when compared with starved males but not when compared to males fed with the sugar solution. These results agreed with those of Kaspi and Yuval (2000) who reported no significant difference in male survival between sugar-fed and protein-fed males in *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). Consequently, it is possible to hypothesize that proteins are involved, during the adult stage of this natural enemy, in other processes. Kaspi and Yuval (2000) stated that when *C. capitata* males have been fed by protein, under laboratory conditions, they emit pheromone sooner and copulate more frequently. The energy cost of males is related to the trade-off between sperm production and time for finding mates. The costs to produce sperm are poorly known and those involved in finding females can vary according the mating system. In outbreeding systems, male disperse to find a female and in inbreeding systems male emerges early than female and generally wait at the site of emergence (Hoogendoorn, 2002). Until now nothing is know about sperm depletion and the effects of multiple mating on *H. sylvanidis* females during their life. If in the future sperm depletion over the time and the effects of multiple mating will be confirmed, getting a long longevity of males could make a contribution to a more effective and reliable performance of this parasitoid in biological pest control programs.

The main energy source for adult parasitoids is sugar (Famadiro and Heimpel, 2001). Sigsgaard et al. (2013) hypothesized that insects use sugars to aid the digestion of pollen. Famadiro and Heimpel (2001) have shown that the lifespan of adults can be increased of 10-fold by sugar feeding. Insects after the emergence need to acquire resources for survival and reproduction. The energy deriving from sugar is available in four forms: (I) as monosaccharides fructose and glucose ready for immediate absorption in the gut; (II) as disaccharide trehalose circulating in the hemolymph; (III) as polysaccharide glycogen stored in the fat body and sometimes in the flight muscle, and (IV) as lipid (triglycerides) in the fat body (Fadamiro et al., 2005).

How *H. sylvanidis* converts the nutrients into longevity remains unanswered. This aspect is of importance because knowing how parasitoids transform molecules could involve a more rationale food source composition. Managing this aspect in the best way can play a relevant role toward a more effective and reliable performance of natural enemies in biological control programs (Hausmann, 2005). If in the future the multiple mating for *H. sylvanidis* females will be confirmed, to get a more high longevity in males could be an interesting tool to manipulate the host-parasitoid dynamics in biological control programs.

2.5.7 Description of the attack

When a female is kept in a Petri dish with larvae she searches for a suitable larva upon which to lay an egg and when she finds one, immediately attacks it. Although the entire body of the host larva is explored, the female pays much attention to the segments close to the legs. During the host recognition, the wasp moves the mouth close to the host in order to touch it with the labial and maxillary palps. If the host recognition provides the right signals, the wasp immediately attacks the larva and once the assault is started, the prey can’t escape. The way in which the attack is conducted shows some differences if compared to those of other Bethylidae candidate as biological control agents of stored commodities. Initially the female grasps the larva with her legs, then she bends her abdomen around the larva and rotates the bodies of 360 degree for three or four times. The struggle is very fast and it is very difficult to perceive when the venomitation takes place. According to Klein and Beckage (1990) ectoparasitoids venoms are of two types: paralyzing agents that induce an immediate and long-lasting paralysis, and venoms that elicit developmental arrest but permit the host to continue feeding temporarily. *H. sylvanidis* venom belong to the first type, although in rare circumstances the paralyzing effect was not permanent and the parasitized larva, with the egg adhering to its venter, became active and crawled in the cage. Similarly to other member of the family, *H. sylvanidis* grasps the larva with her mandibles in order to bring it in a safe place (Ahmed et al., 1997). The female continues to touch the host for some seconds after the injection using the antennae. Presumably, she looks for more confirmation about the good effect of the venom. The host larva becomes almost motionless after the injection. It shows rhythmic movements of the legs and remains curved with the venter on the top. So, it is ready to receive the egg. Sometimes the female attacks the legs of the host larva (Ahmed et al., 1997), biting the intersegmental membrane, probably in order to avoid its escape. When the wasp was put in a Petri dish with all instars larvae, it prefers to parasitize the fourth and the fifth instar larva, no eggs have been found on the first, second, third instars and on pupae.

3 Olfactory responses of *Holepyris sylvanidis*

3.1 General aspects

The parasitic wasp *H. sylvanidis* is a natural enemy of *T. confusum* and it is also able to attack and oviposit on larvae of *T. castaneum*. The knowledge regarding the biology of *H. sylvanidis,* its presence on infested substrates and the possible applications as natural enemy against *T. confusum* in different thick layer of substrates derives from some studies (Ahmed and Islam, 1988; Ahmed et al., 1997; Eliopoulos et al., 2002; Lorenz et al., 2010;) in which, until now, the aspects related to host preference were not empirically evaluated.

The studies conducted until now have only partially clarified the innate behaviour of this parasitoid. Considering that chemical cues are important for parasitoids because they permit to locate the host larvae, to attack and paralyze them (Collatz et al., 2009; Steidle and Schöller, 1997), studying the innate searching behaviour of *H. sylvanidis,* when it is in front of the chemical stimuli released by substrates and hosts, may provide further scientific knowledge useful to understand its biology and the future applicative perspectives. Furthermore, because both *T. castaneum* and *T. confusum* were reported as world-wide distributed pests (García et al., 2005; Park, 1934; Suzuki and Sugawara, 1978), solving the question of the host preference can also be of interest in several cases such as when a biological pest control program has to be performed or when this natural enemy has to be reared. To clarify if a response to chemicals does exit and what is the favourite host of inexperienced *H. sylvanidis* female, its response to different stimuli was evaluated in a four chambers olfactometer. According to Vet and Dicke (1992) chemical information can originate from the herbivore, from its food, from organism associated with herbivore presence, or from interaction between these sources. In order to explore these aspects for *H. sylvanidis*, stimuli deriving from host complexes were primarily investigated and afterwards evaluations on stimuli deriving from substrates, larvae, pupae and adults were conducted with particular attention to the synovigenic status (Ahmed et al., 1997). In this sense, parasitoids can be classified as proovigenic and synovigenic. Females of proovigenic species complete oogenesis prior to emerge and lay eggs soon after their emergence, thus feeding serves to ensure sufficient longevity to oviposit all the eggs. On the contrary, females of synovigenic species emerge without a full complement of mature eggs and they feed to obtain proteins for oogenesis and embryogenesis (Riviero and West, 2005). Synovigenic parasitoids are furthermore classified in: autogenous and anautogenous. The species belong to the first group can mature some eggs without first feeding being able to use some fats reserves for the initial egg production. In this case feeding is helpful to maximize the egg production during the lifetime. The parasitoids belonging to the anautogenous group have to feed to mature eggs (Ueno and Ueno, 2007; Rosenheim and Rosen, 1992; Thompson, 1999). To explain this physiological state, Jervis and Kidd (1986) hypothesized that among anautogenous natural enemies insufficient metabolic reserves are carried over from the larval stage, it involves that feeding behaviour is necessary to compensate for this deficiency. In detail, we studied: (1) if *H. sylvanidis* uses volatiles to locate the presence of its host on infested substrates; (2) the origin of potential volatiles from the components of the host complex; (3) the fecundity on fourth instar larvae of both *T. castaneum* and *T. confusum*.

3.2 Materials and methods

3.2.1 Insects rearing

T. castaneum adults were kept in 9 cm diameter Petri-dish on 20 g of a commercial flour containing: durum wheat flour, rye flour, yeast and malt as substrates. The adults were sieved out using a 0.6 mm sieve three times a week and kept on the commercial flour as reported above, to lay new eggs. *T. confusum* was reared in a 9 cm diameter Petri-dish containing 20 g of flour (*Triticum aestivum*) and 5% of brewers' yeast as substrates (Athanassiou and Kavallieratos, 2014). The adults were sieved out using a 0.6 mm sieve one time a week and kept on fresh flour and brewers' yeast, as reported above, to lay new eggs. Both the flours containing the eggs were kept at 28 ± 1°C and 65 ± 5% relative humidity (RH). After about 23 days of incubation, fourth instars larvae of both the beetle species were obtained.

In order to rear the parasitoids, two females and a male of *H. sylvanidis* were kept in a 9 cm diameter Petri dish with fourth instars larvae of the confused flour beetle (Ahmed et al., 1997).

Ten fresh fourth instar larvae were added to each Petri dish twice a week. The parasitized larvae of the beetle were collected three times a week and kept at 28 ± 1°C and 65 ± 5% RH. In order to favourite the natural behaviour of Bethylidae, which hide the paralyzed host larva in a safe place (Griffiths and Godfray, 1988), where the oviposition will take place, the undulated part of a cardboard was added to the Petri dish. As reported by Ahmed et al. (1997), *H. sylvanidis* feeds of its host in order to enhance egg production and subsequently oviposition. An energetic and high protein diet was provided to *H. sylvanidis* aiming at to obtain less host feeding and more oviposition. The diet contained: water (1 ml), honey (0.1 g), pollen (7 pieces) and royal jelly (0.05 g) and was given putting a drop of the solution in a 1 x 1 cm filter paper.

3.3 Experiments

3.3.1 Bioassay for attractive stimuli

Experiments were performed with 1-3 days old, inexperienced, starved and mated females, which were kept singularly in an Eppendorf vials until the beginning of the observation. According to Vet et al. (1990) inexperienced *H. sylvanidis* females are those adults which had no experience with hosts beyond that occurring during the development. To mate females, a male (1-day old) was introduced into one Eppendorf containing a female and left in the vial for 24 hours. Females were randomly assigned to experiments and after each test they were returned to stock cultures.

Each set of experiment was replicated 30 times and for each replicate a new emerged female was employed. The experiments were conducted in a four-chamber olfactometer (Fig. 1) (Steidle and Schöller, 1997). It consists of an opaque Plexiglas® cylinder (height 4 cm, diameter 19 cm) divided by vertical plates into four chambers. On the top of the cylinder, a walking arena (height 1 cm, diameter 19 cm) consisting of plastic gauze (mesh 0.2 mm) with a rim of heat resistant Plexiglas® (0.9 cm high) was covered with a glass plate. For each test a Petri dish (5.5 x 1.5) containing the odour sample was placed beneath the arena in one chamber and the opposite chamber either contained an empty Petri dish or a Petri dish with another odour sample. The other two chambers remained empty as transition zones. The experiments were conducted in a dark room except for the olfactometer, which was illuminated from above. At the beginning of each experiment, a wasp was placed at the centre of the walking arena and for a period of 600 s its behaviour (walking, resting) and the position of the wasp were registered using the computer software "The Observer 5.0" (Noldus, Wageningen, The Netherlands). The olfactometer was rotated after each observation and no airflow was generated during all the experiments. The time that each wasp spent walking upon the odours or control field when provided in the experiment, was compared, after the study of the normality performed with the Kolmogorov-Smirnov test, by the Wilcoxon-matched pairs test by using the software package StatPlus: mac – statistical analysis program for Mac OS. Version v5. The experiments evaluated the parasitoid's attraction toward the following odour sources: *T. castaneum* host complex (CAH) which consists of 0,5 g whole grains, 0,5 g broken grains, 1-2 instar larvae (*n* = 2), fourth instar larvae (*n* = 2), pupae (*n* = 2) and adults (*n* = 2); *T. confusum* host complex (COH) which consists of 0,5 g whole grains, 0,5 g broken grains, 1-2 instar larvae (*n* = 2), fourth instar larvae (*n* = 2), pupae (*n* = 2) and adults (*n* = 2); whole grains (WHG) which consists of 1 g of whole grains (*Triticum durum*); flour (FLR) which consists of 1 g of flour (*Triticum aestivum*); *T. castaneum* 1-2 instar larvae (CA1-2) (*n* = 10); *T. castaneum* 4 instar larvae (CA4) (*n* = 10); *T. confusum* 1-2 instar larvae (CO1-2) (*n* = 10); *T. confusum* 4 instar larvae (CO4) (*n* = 10); *T. castaneum* pupae (CAP) (*n* = 5); *T. confusum* pupae (COP) (*n* = 5); *T. castaneum* adults (CAA) (*n* = 5); *T. confusum* adults (COA) (*n* = 5).

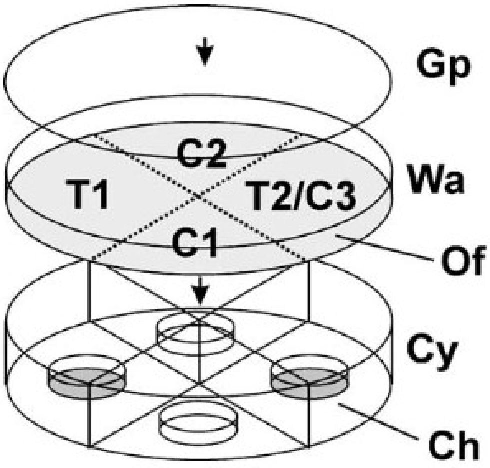

Fig. 1 Olfactometer for testing the response of wasps towards different odour sources. Height 5 cm, diameter 19 cm. Abbreviations: Gp-glass plate; Wa-walking arena, Of-odor fields; Cy-cylinder; Ch-chambers, T1 test field above odour sample, C1 and C2; transition zones above empty Petri dishes; T2/C3: field opposite of T1 with odour sample in Petri-dish or with empty dish.

3.3.2 Bioassay for the fecundity

In order to evaluate the fecundity of *H. sylvandis* on both *T. castaneum* and *T. confusum* fourth instar larvae two sets of experiments were performed. The first set consisted of *T. castaneum* and the second of *T. confusum*. For each set, which consisted of 33 replicates, a female and a male *H. sylvanidis* were kept in a 12 cm Petri dish containing a piece of undulated cardboard and the larvae. Weekly 15 fourth-instar larvae (Ahmed et al., 1997) of the tested beetle were supplied to each female. The 15 fourth instar larvae were so distributed: on Monday 5 larvae were added to each Petri dish and on Friday a new Petri dish containing 10 fresh larvae was offered to each female. The experiments were conducted at 28 ± 1°C and 65 ± 5% RH. The eggs laid by the parasitoids on the host larvae were collected on Monday and Friday and registered in a data sheet. To compare the fecundity upon the two species of beetles, after the study of the normality performed with the Kolmogorov-Smirnov test, a two tailed T-test was calculated.

3.4 Results

Wasps were arrested by the host complexes (Fig. 2), which consisted of grains (whole and broken), larvae (1-2 instar and 4 instar) pupae and adults of the beetles studied. When the host complex of *T. castaneum* was directly compared to that of *T. confusum*, the first was significantly more attractive than the second. To study the exact origin of the odours, substrates-derived and host-derived components of the host complexes were tested individually. The uninfested substrates had no arresting effect on *H. sylvanidis* (Fig. 3). Thus, the chemicals emitted by substrates in the host complex were not attractive. Wasps responded to stimuli deriving from larvae showing a preference for 1-2 instars (Fig. 4), but no preference was showed when fourth instar larvae were compared. The pupae and adults were also attractive (Fig. 5) except when *T. confusum* adults were tested against empty. The wasps were significantly more attracted by odours emitted by *T. castaneum* when this beetle was directly compared to *T. confusum* (Fig. 4a-c; Fig. 5a-d), except when the 4 instars larvae of the two pests were directly compared (Fig. 4d). When the fecundity on the two beetles was compared, the mean number of eggs laid by *H. sylvanidis* female was higher on *T. castaneum* than on *T. confusum* (Fig. 6).

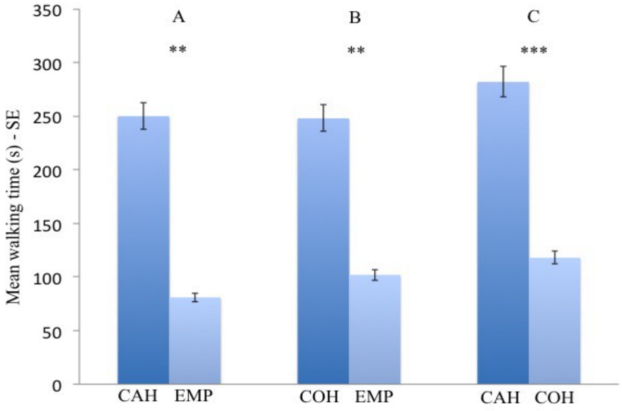

Fig. 2 Abbreviations: CAH-*T. castaneum* host complex; COH-*T. confusum* host complex; EMP-Empty. *** $P < 0.0005$; ** $P < 0.005$ (Wilcoxon-matched pairs test).

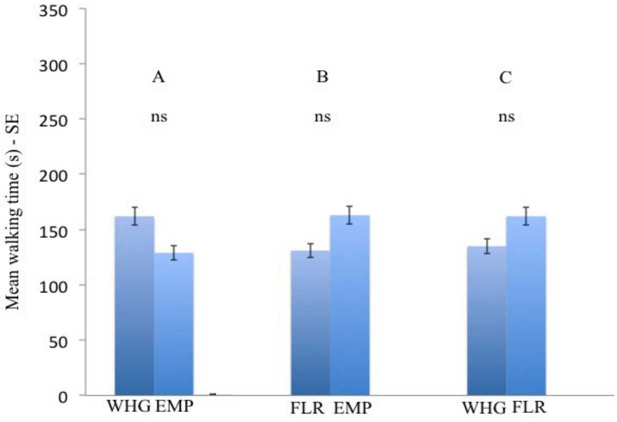

Fig. 3 Abbreviations: WHG-whole grains; FLR-flour; EMP-empty. ns not significant (Wilcoxon-matched pairs test).

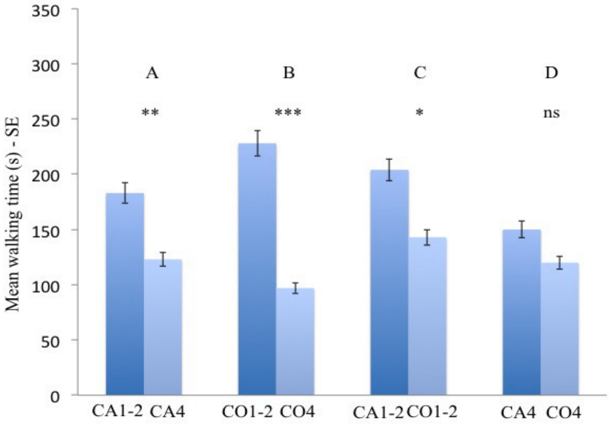

Fig. 4 Abbreviations: CA1-2-*T. castaneum* 1-2 instar larvae; CA4- *T. castaneum* 4 instar larvae; CO1-2-*T. confusum* 1-2 instar larvae; CO4-*T. confusum* 4 instar larvae. *** $P < 0.0005$; ** $P < 0.005$ (Wilcoxon-matched pairs test).

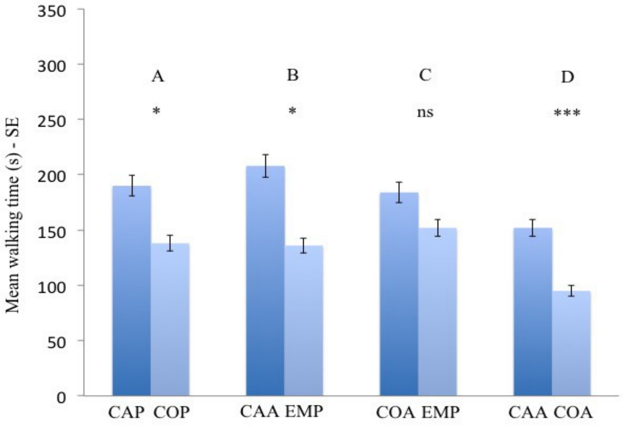

Fig. 5 Abbreviations: CAP-*T castaneum* pupae; COP-*T. confusum* pupae; CAA-*T. castaneum* adults; EMP-empty; COA-*T. confusum* adults. *** P < 0.0005; * P < 0.05; ns not significant (Wilcoxon-matched pairs test).

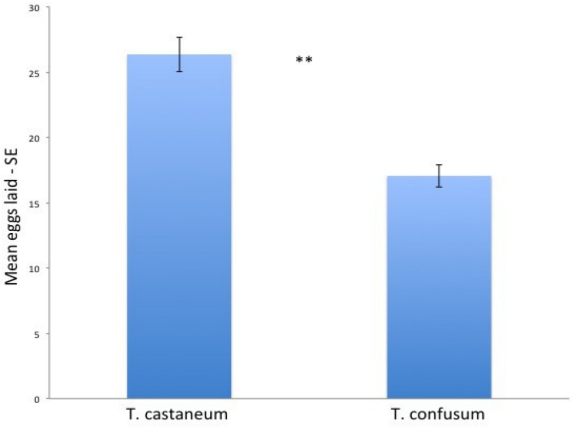

Fig. 6 Fecundity (mean number of eggs laid ± s.e.) by *H. sylvanidis* female on larvae of *T. castaneum* and *T. confusum.* ** P < 0.005 (two tailed T-test).

3.5 Discussion

The data here reported indicates that both the host complexes release attractive stimuli for *H. sylvanidis* females. When the two host complexes were directly compared that of *T. castaneum* resulted the most attractive. After this result was obtained, the subsequent question was to further investigate from which elements of the host complex these stimuli derived. Vinson (1991) reported that in the initial phases of foraging, parasitoids respond to host associated cues rather than to cues actually produced by host itself. Thus, the food sources reported as favourite for both the beetles were tested (Athanassiou et al., 2005; Arbogast, 1991), and these didn't show any significant statistical attraction, indicating that no information derives from uninfested substrates. To explain this result it is possible to assume that cues deriving from the substrates have low reliability and therefore provide only a low innate response potential (Steidle and van Loon, 2008), or that volatile blends released by these substrates, although generally comprise several dozens of volatile compounds, are emitted in small amounts involving that the chemicals emitted are not detectable by *H. sylvanidis* (Schoonhoven et al., 1998). Considering this result, it is possible to expect that stimuli deriving from the food sources of the hosts become reliable and detectable for *H. sylvanidis* only after substrates are damaged by *Tribolium* ssp. The damaging action of pests involves the release of large quantities of herbivore-induced plant volatiles (HIPVs). These are new formed molecules which can be recognized by parasitoids. Furthermore, olfactory experiments showed how natural enemies are able to discriminate by olfactory cues between infested and healthy plants (Dicke, 1999; Steidle and Schöller, 1997; Turlings and Tumlinson, 1990). The experiments conducted with larvae have clearly demonstrated that 1-2 instars are the most attractive and between the two species, the 1-2 instar larvae of *T. castaneum* were significantly more attractive that 1-2 instars of *T. confusum*. Subsequently, the fourth instar larvae of both the species were tested and in this case no significant statistical difference was obtained. Possible explanations for this result can be formulate stating either that the fourth instar larvae of both species don't release odours which are attractive for the parasitoid, or both the fourth instars larvae are equally attractive. The pupal stage revealed again that *T. castaneum* was significantly more attractive than *T. confusum*. When the adults of both species were compared against empty only stimuli deriving *T. castaneum* arrested *H. sylvanidis* female in the olfactometer. When *T. castaneum* adults were directly compared to *T. confusum* adults, the first were significantly more attractive than the second. Although adults do not represent the proper stage for parasitoids, the attractiveness of *T. castaneum* adults observed in the olfactometer can be explained according to Vet and Dicke (1992), who stated that in some circumstances to solve the reliability-detectability problem, stimuli deriving from other stages can be used by the parasitoids to locate the proper host stage (infochemical detour).

H. sylvanidis is a synovigenic species (Ahmed et al., 1997), thus females always showed a strongly preferences toward 1-2 instar larvae. Considering that wasp females were kept without food sources before the tests, the strongly attractiveness of 1-2 instar larvae indicates that these stages provide the reliable stimuli needed to *H. sylvanidis* for feeding and subsequently for eggs maturation.

As the results here reported indicate, the most reliable and detectable chemical stimuli derive from hosts and not from uninfested substrates. Thereby the use of one or more chemicals deriving from hosts plays an important role in the complex of the biological decisions make by of *H. sylvanidis*. In the current study it has been demonstrated that: (I) *T. castaneum* represents the favourite host both from an olfactory and an oviposition point of view; (II) that the innate response of new emerged and starved females is toward 1-2 instars larvae being *H. sylvanidis* female a synovigenic anautogenous natural enemy; (III) infochemical detour signals can be used by this parasitoid to obtain infochemicals. To our knowledge a similar scenario for the location of active hosts has not been reported before. Future studies aim to identify these remarkable chemicals.

4 Olfactory responses of *Cephalonomia waterstoni*

4.1 General aspects

The parasitic wasp *C. waterstoni* is an external, arrenotokous, idiobiont and larval ectoparasitoid described for the first time by Gahan (1931). This natural enemy was indicated as parasitoid of *C. minutus* and *C. ferrugineus* and its presence was reported on specimens collected from stored grain, in which simultaneously the rusty grain beetle was registered as a pest (Finlayson, 1950a; Flinn, 1991; Howard and Flinn, 1990; Rilett, 1949; Sinha et al., 1979). Some studies have investigated the host preference of this wasp reporting that among the different species of the genus *Cryptolestes*, *C. ferrugineus* resulted the most suitable. A valid explanation of this preference is that the rusty grain beetle represents the natural host, which provides the appropriate stimuli to *C. waterstoni* (Finlayson, 1950b; Flinn, 1991). As reported in the literature *C. ferrugineus* is a primary pest and its biological cycle is facilitated by the activities of other pests like *S. granarius* and *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae). The larvae of *C. ferrugineus* are very active among damaged seeds, where they feed mostly on the germ. Furthermore, larvae often remain under the seed coat and pupate there (Minkevich et al., 2002). The presence of *C. ferrugineus* has been extensively reported in different parts of mills. Arthur et al. (2006) reported that in grains remaining inside empty bins, *Cryptolestes* spp. comprised 45% of the insects collected, whereas *Sitophilus* spp. represented 32.4%, and *R. dominica* 9%. Reed et al. (2003) stated that in the discharge spouts, *Cryptolestes* spp. comprised 65% or more of the insects collected in four of the five sampling periods (6 months each). Smith (1985) recorded *C. ferrugineus* adults in 38% of railcars loaded in 1977 and in 53% of the railcars loaded in 1978. In this context, the capacity of *C. waterstoni* to detect and suppress populations of *C. ferrugineus* could be of importance and this natural enemy has to be seriously taken into account as a suitable biocontrol agent. Some authors have indicated the main features of *C. waterstoni* as biocontrol tool against *C. ferrugineus*. For example, Rilett (1949) reported that after a *C. waterstoni* female has attacked a host, it has no opportunity to recover. Lord (2006) demonstrated how this wasp serves as mechanical vectors of *M. oryzaephili* enhancing the pathogen’s dissemination. Howard and Flinn

(1990) underlined the aggressiveness of *C. waterstoni* indicating that the female may attack more hosts than she can effectively use for oviposition. Hagstrum (1987) and Reichmuth et al. (2007) reported the ability of *C. waterstoni* in maintaining the rusty grain beetle population below the economic damaging threshold.

During a biological treatment based on natural enemies, the biggest challenge of wasps is to find hosts, which are very tiny and generally live hidden and scattered in large and complex environments (Collatz and Steidle, 2008). Female parasitoids need to find hosts to match all the biological requests in terms of energy and reproduction. To do so, parasitoids seek hosts on a variety of substrates, moving on and between them (van Alphen and Vet, 1986). When a host is found, the natural enemy has to decide how to use it; *e.g.* maximizing the total genetic contribution toward the next generations or feeding on it in order to meet the best ratio between energy available to find new hosts and number of eggs that will be laid. In a complex environment inside which several trophic levels are present, parasitoids can get stimuli deriving from different living systems (*e.g.* substrates and pests) and in this context they are innately able to discriminate among them showing high degrees of host specificity. There are also different factors influencing the parasitoid's decision such as: the physiological state, the mating status, the age and also the experience acquired during the lifetime (van Alphen and Vet, 1986). The stimuli deriving from hosts which elicit behaviour in parasitoids are restricted to a single host species if the parasitoid is monophagous, to a few related species if oligophagous, or to a more or less wide range of species when the natural enemy is polyphagous (Finlayson, 1950b).

Although some efforts have been done in order to amplify the potential use of this natural enemy in biological control programs (Flinn and Hagstrum, 1995; Lord, 2006), important gaps of knowledge still exist, especially regarding the aspects related to habitat location. Considering the growing interested on biological control programs, the relevance of *C. ferrugineus* as pest and the possible future perspective of this promising Bethilidae wasp, more knowledge on the olfactometric aspects can contribute to improve its practical application as biological control agent. In order to link olfactometric to applicative aspects, we evaluated the behavioural responses of *C. waterstoni* to the volatiles emitted by substrates and three living stages of its host.

4.2 Material and methods

Both the species tested in this study were collected in a Sicilian flourmill near Syracuse. The insect cultures were kept at 28°C in a glass jar with durum wheat kernels damaged by *S. granarius*. The experiments evaluated the parasitoid's attraction toward the following odour sources: healthy grains (HGR), which consist of undamaged and uninfested wheat harvested in Germany in 2013. Infested grains by *S. granarius* (IGR), which were obtained from the stock rears of the Hohenheim university. Dust from mass rearing (DST), which was collected from a culture kept at 30°C and 60 % relative humidity (RH). This culture included durum wheat as substrate and *R. dominica*, *O. surinamensis* and *C. ferrugineus* as pests. The insects and the substrate were kept in a 100 l bin for about 6 months. Dust from Hohenheim's university laboratory (DSH), which was obtained sieving infested durum wheat after an exposition to adults of *S. granarius*. Diet without *C. ferrugineus* (DIT), which consisted of big oats, small oats and wheat in proportion 1:1:2. The diet also contains: one teaspoon of yeast and one of water. Diet plus *C. ferrugineus* (DCR), which was like the diet without *C. ferrugineus,* but in this case 50 randomly chosen adults were added to the odour source. *Cryptolestes ferrugineus* adults (ADU), which consisted of a mixture of both males and females of the rusty grain beetle. Mixed larvae (LRM), which consisted of 50 randomly chosen larvae of *C. ferrugineus*. First and second larval instars (1-2 L) and third and fourth larval instars (3-4 L), which were obtained separating by a stereomicroscope Carl Zeiss Stemi DRC 50, larvae for each group based on body length. During all the experiments experienced females were used and at least five females were tested at each time for each odours combination.

4.3 Bioassays

The experiments were carried out in a four-chamber olfactometer (Fig. 1) (Steidle and Schöller, 1997). It consists of an opaque Plexiglas® cylinder (height 4 cm, diameter 19 cm) divided by vertical plates into four chambers. On the top of the cylinder, a walking arena (height 1 cm, diameter 19 cm) consisting of plastic gauze (mesh 0.2 mm) with a rim of heat resistant Plexiglas® (0.9 cm high) was covered with a glass plate. For each test a Petri dish (5.5 x 1.5 cm) containing the odour sample was placed beneath the arena in one chamber and the opposite chamber either contained an empty Petri dish or a Petri dish with another odour sample. The other two chambers remained empty as transition zones. The experiments were conducted in a dark room except for the olfactometer, which was illuminated from above. At the beginning of each experiment, a wasp was placed at the centre of the walking arena and for a period of 600 s its behaviour (walking, resting) and the position of the wasp were registered using the computer software "The Observer 5.0" (Noldus, Wageningen, The Netherlands). The olfactometer was rotated after each observation and no airflow was generated during all the experiments. The times that each wasp spent walking upon the odours or control field when provided in the experiment, were compared by the Wilcoxon-matched pairs test by using the software package Statistica for Windows 6.0 (StatSoft Inc. 2003).

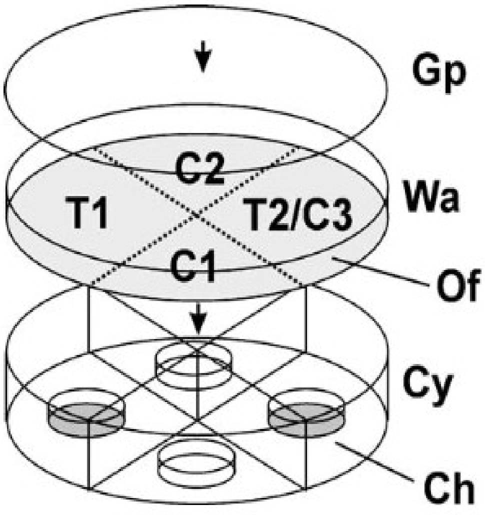

Fig. 1 Olfactometer for testing the response of wasps towards different odour sources. Height 5 cm, diameter 19 cm. Abbreviations: Gp-glass plate; Wa-walking arena, Of-odour fields; Cy-cylinder; Ch-chambers, T1 test field above odour sample, C1 and C2; transition zones above empty petri dishes; T2/C3: field opposite of T1 with odour sample in Petri-dish or with empty dish.

4.4 Results

The dust arrested wasps when it was compared to healthy grains (Fig. 2A), infested grains (Fig. 2B) and empty (Fig. 3A) but not when it was compared to diet plus *C. ferrugineus* (Fig. 4A). The dust from Hohenheim, obtained keeping *S. granarius* on grains, arrested *C. waterstoni* females when it was compared to the empty chamber (Fig. 3B). The diet containing *C. ferrugineus* adults arrested females when it was compared to diet without adults (Fig. 4B). Third and fourth instar larvae arrested the females when they were compared to first and second instar (Fig. 6A), but not when they were compared to adults (Fig. 5A). Mixed larvae arrested females when they were compared to the empty chamber (Fig. 6B).

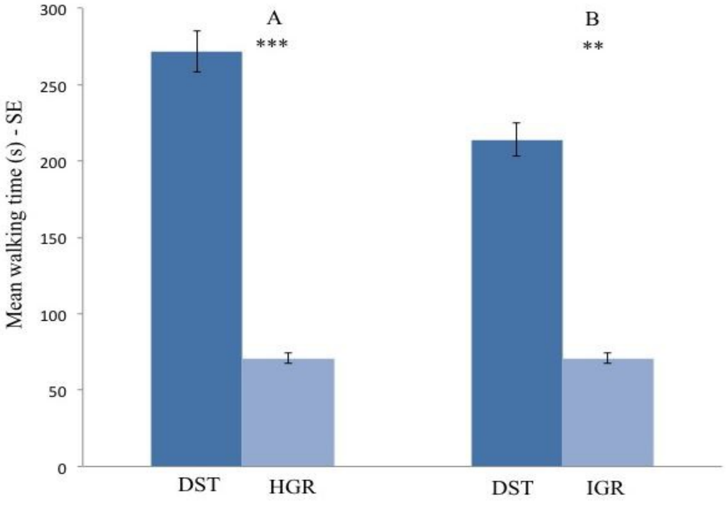

Fig. 2 Abbreviations: DST-dust; HGR-healthy grain; IGR-infested grains. *** $P < 0.0005$; ** $P < 0.005$ (Wilcoxon-matched pairs test).

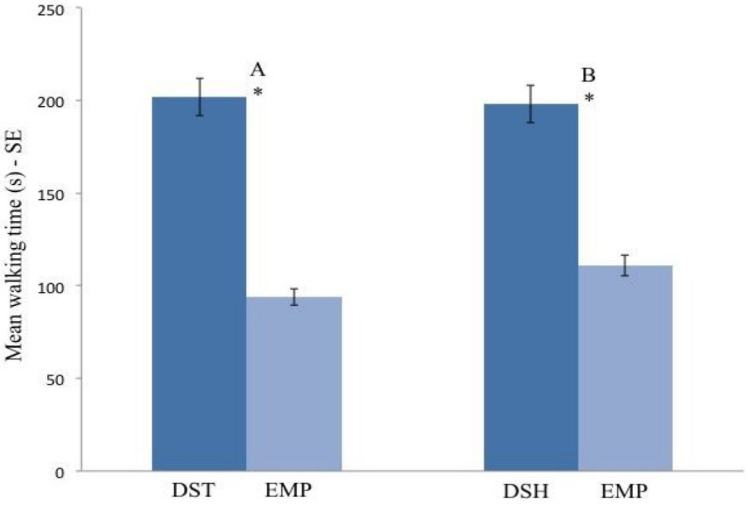

Fig. 3 Abbreviations: DST-dust; EMP-empty; DSH-dust from Hohenheim. * $P < 0.05$ (Wilcoxon-matched pairs test).

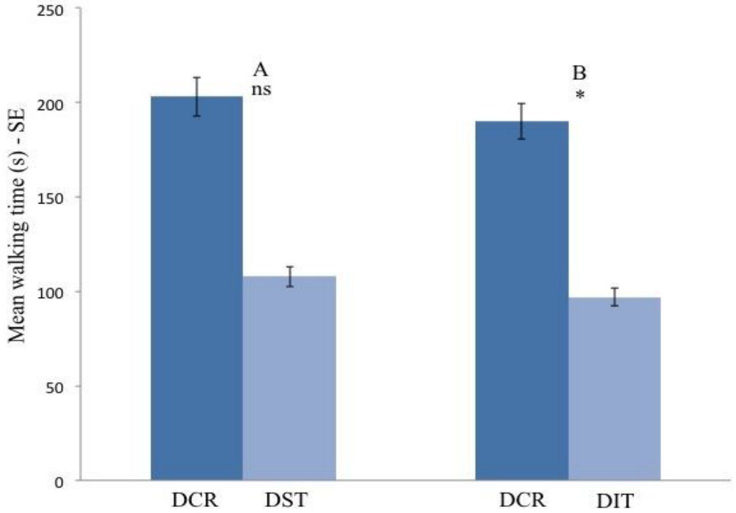

Fig. 4 Abbreviations: DCR-diet plus *C. ferrugineus*; DST-dust; DIT-diet without *C. ferrugineus*. * $P < 0.05$; ns-not significant (Wilcoxon-matched pairs test).

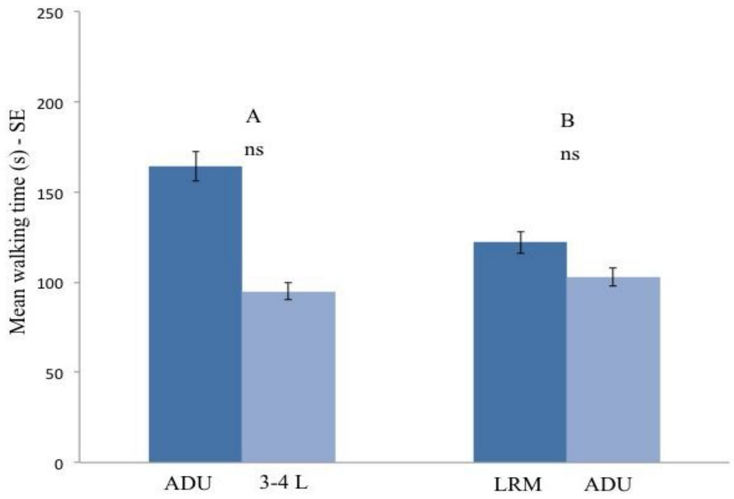

Fig. 5 Walking time (mean ± s.e.) of female *C. waterstoni* Abbreviations: ADU-adults of *C. ferrugineus*; 3-4 L-third and fourth instar larvae; LRV-mixed larvae. ns-not significant (Wilcoxon-matched pairs test).

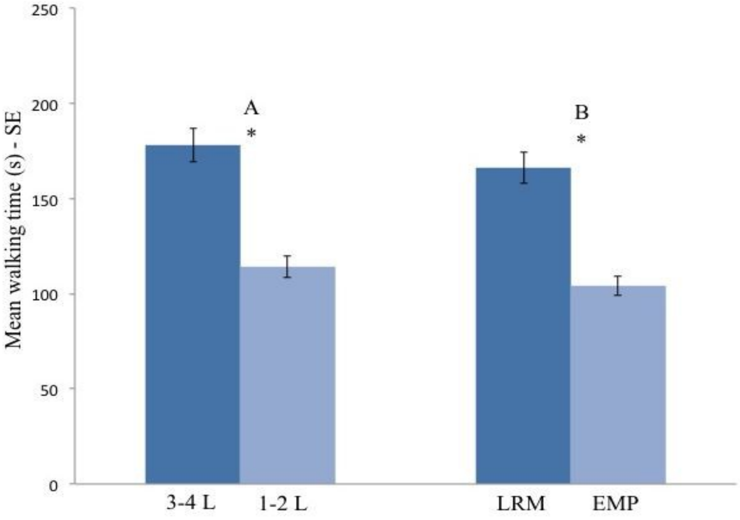

Fig. 6 Abbreviations: 3-4 L third and fourth instar larvae; 1-2 L first and second instar larvae; LRM-mixed larvae; EMP-empty. * $P < 0.05$ (Wilcoxon-matched pairs test).

4.5 Discussion

The goal of this study was to evaluate the olfactometric response of experienced *C. waterstoni* females. According to Doutt (1959) three phases can be distinguished in the host searching and host selection behaviour of parasitoids: host habitat location, host location and host selection. This study reveals that *C. waterstoni* females are attracted in the olfactometer by odours deriving from host habitat, especially from dust. In mills, under practical conditions dust is produced by the wheat processing (Martin, 1981) but also by the oviposition and feeding behaviours of primary pests such as *S. granarius* and *R. dominica.* The reproductive biology of some primary pests involves that they have to bore a hole in the kernel prior to laid the egg. Afterwards in *S. granarius*, the hole will be sealed producing a gelatinous secretion. When the new adult emerges, it leaves the kernel making a new hole. Our results suggest that the action of the primary pests involves the release of herbivore-induced plant volatiles (HIPVs) (Dicke, 1999; Turlings et al., 1995) and these newly produced molecules elicited the olfactometric response of *C. waterstoni.* The literature reports evidence about the increased attractiveness of attacked plants in respect to the non attacked ones (Guerrieri et al., 1993; Mattiacci et al., 1994). When herbivorous insects attack a plant, additional compounds are released but this process often involves the production and release of specific molecules (Turlings and Tumlinson, 1990; Williams et al., 1988). The dust from mass rearing, obtained by the action of three different pests for a six months period, contained a high quantity of pheromones and faeces, which have significantly contributed to enhancing the attractiveness of this odour source. Until now nothing is known about the chemical composition of the dust produced by pests but Martin (1981) reported that dusts produced during grain processing in mills contain more fibre than entire grains. The results from the comparison of healthy grains and infested grains against dust showed a lower P-value compared to the experiments with dust against empty chamber and dust from Hohenheim rearing against empty chamber. These results indicate that when grains and dust are contemporarily present the searching behaviour of this natural enemy is enhanced. The wasp was not able to make a choice when the dust and the diet containing its favourite host were offered as odour sources. This indicates that when the wasp is in the proper environment, inside which all the desired olfactometric stimuli (*i.e.* substrate and the host) are recognized, new behaviours are elicited like antennation or mouth touching of the host larvae. A similar behaviour was observed when the wasp had to make a choice between adults and third fourth instar larvae and between mixed larvae and adults. In all these cases no significant difference was detected. This can be due to the fact that the stages tested during these two sets of experiments emit the same molecules and probably in the same quantity. On the contrary a significant difference was detected when mixed larvae where compared to empty and when third and fourth instars were compared to first and second instars. In the latter case the attraction can be due to the fact that third and fourth instars larvae emit different molecules or emit more than the first and second instars. This finding agrees with those of Howard and Flinn (1990) who observed that *C. ferrugineus* fourth instar larvae marked their pathway dragging the abdomen while they walked among the kernels. The authors also suggested that this larval instar leaves a kairomonal cue, which represents a strong species-specific cue for *C. waterstoni* females.

In this study the capacity of the parasitoid to recognize stimuli deriving both from the host environment and from the host itself is reported. The literature offers examples of *Cryptolestes* spp. found in empty bins and elevators when grain residues are present. These colonies can rapidly multiply and spread among the kernels when the new harvested wheat is stored (Arthur, 2006; Reed et al., 2003). The high preference of *C. waterstoni* females toward the dust represent a further useful element toward the enhancement of the biological control of *C. ferrugineus* especially when this pest is present in empty bins or elevators. Furthermore, as suggested by Steidle and van Loon (2002), artificial chemical cues deriving from odours, which have significantly arrested females in olfactometers should be taken into account in integrated pest management programs. These cues produce synomonic effects, attracting the natural enemies toward the habitat of pest species and enhancing their action. If on one hand the use of natural enemies is important to satisfy the growing request of organic food, on the other hand there is an urgent request of new solutions to solve the problem of the growing resistance to the common insecticides showed by pests. In this direction, recently Kaur and Nayak (2015) reported how the Australian grain industry is facing a problem of highly resistant *C. ferrugineus* surviving currently registered rates of phosphine used in bulk grain storage. Considering the urgent necessity to find methods and tools to enhance the activities of wasps in biological control programs, the data here reported offer new elements toward the optimisation of the use of *C. waterstoni* in integrated pest management programs.

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