

UNIVERSITÀ DEGLI STUDI DI NAPOLI “FEDERICO II”



Consiglio per la Ricerca e sperimentazione in
Agricoltura
Centro di Ricerca per l'Agrobiologia e la Pedologia

**DOTTORATO INTERNAZIONALE DI RICERCA
“INSECT SCIENCE AND BIOTECHNOLOGY” XXVI CICLO**

TESI DI DOTTORATO

**HYMENOPTERAN PARASITOIDS: LABORATORY REARING AND
EVALUATION OF STORAGE TECHNIQUES**

Coordinatore

Prof. Francesco Pennacchio

Candidato

Francesco Binazzi

Relatore

Prof. Pio Federico ROVERSI

Co-Relatore

Dr. Giuseppino Sabbatini Peverieri

Anno accademico 2013-2014

INDEX

INTRODUCTION

A. Biological control in agriculture: Limits and opportunities	4
B. Biological control in forest environments	7
C. Predators as pest control agents in forest environments	8
D. Biological control by parasitoids in forest environments	9
E. The role of insect cold storage in biological control programs	14
F. Endogenous factors involved in parasitoid cold tolerance	15
F.1. Age and Stage	15
F.2. Dormancy status	16
G. Exogenous factors involved in parasitoid cold tolerance	18
G.1. Temperature	18
G.2. Rates of cooling and heating	19
H. The focus of the research	21

MATERIALS AND METHODS

Host origin	22
1. Biological parameters of <i>O. pityocampae</i> reared on alternative host eggs	25
2. Identification and biological development of <i>O. pityocampae</i> larvae within eggs of the alternative host <i>N. viridula</i>	26
3. Effects of host egg age on <i>O. pityocampae</i> parasitization	26
3.a. Parasitoid origin	26
3.b. Host egg aging	27
3.c. Microscopical observations	28
3.d. Data analysis and statistics	28
4. Temperature-dependent egg to adult development of <i>O. pityocampae</i> within eggs of the alternative host <i>Graphosoma lineatum</i>	28
4.a. Data analysis and statistics	29
5. Cold tolerance evaluation of <i>O. pityocampae</i> immatures stored for 10 days at low temperatures from 15°C to 0°	29
5.a. Data analysis and statistics	30
6. Evaluation of the effects of cooling and heating rates on the survival of <i>O. pityocampae</i> immatures stored at different temperatures (15°C to 0°C):	31
6.a. Data analysis and statistics	32

RESULTS AND DISCUSSION

1. Biological parameters of <i>O. pityocampae</i> reared on alternative host eggs	33
2. Identification and biological development of <i>O. pityocampae</i> larvae within eggs of the alternative host <i>N. viridula</i>	36
3. Effects of host egg age on <i>O. pityocampae</i> parasitization	38
4. Temperature-dependent egg to adult development of <i>O. pityocampae</i> within eggs of the alternative host <i>Graphosoma lineatum</i>:	43
5. Cold tolerance evaluation of <i>O. pityocampae</i> immatures stored for 10 days at low temperatures from 15°C to 0°	46
6. Evaluation of the effects of cooling and heating rates on the survival of <i>O. pityocampae</i> immatures stored at different temperatures (15°C to 0°C):	51
7. Time to adult development.	56
Final conclusions	62

REFERENCES	64
-------------------	-----------

ACKNOWLEDGEMENT	71
------------------------	-----------

INTRODUCTION

Biological control can be defined as the use of an organism to decrease the population density of another organism. It is an old technique that has been applied for about two millennia but became a widespread strategy for pest management only at the end of the nineteenth century (DeBach 1964; van Lenteren and Godfray 2005; van Lenteren 2011). The main kinds of biological control are natural, conservation, inoculative (=classical) and augmentative biological control (van Lenteren 2011). In natural biological control the reduction of pest organisms is carried out by their natural enemies. It is typical of all the ecosystems and since it does not require human intervention represents from an economic point of view the most relevant contribute to agriculture (Waage and Greathead 1988). On the contrary conservation biological control concerns human activities aimed at protecting and enhancing the performance of natural enemies (Gurr and Wratten 2000). In inoculative biological control instead, natural enemies are collected in specific areas that generally coincide with the geographical origin of the pest, and then released in different sites where the pest was accidentally introduced. The purpose is to allow the offspring of the introduced organism to build up populations large enough to control or eradicate the pests in the following years. Biological invasions that reach new areas without their natural enemies, are ideal targets for this type of control. On the other hand, Augmentative biological control involves mass rearing in biofactories of natural enemies and their subsequent release in the field in order to achieve an immediate effect.

A. Biological control in agriculture: Limits and opportunities

In the last 120 years commercial mass production and sale of natural enemies have played a key role in many areas of agriculture as an environmentally and economically sound effective alternative to chemical control (van Lenteren and Bueno 2003; van Lenteren 2011).

According to recent estimates, inoculative biological control is a technique employed on 10% of land under cultivation (Bale et al. 2008). Moreover in the last 120 years, 165 pest species were subjected to long-term control (Cock et al. 2010). On the other hand, in the last decades, augmentative biological control moved from small-scale production to professional production and farmers were provided with improved guidance. In addition quality control protocols as well as new methods for shipment and release were developed (van Lenteren 2003; Cock et al. 2010; van Lenteren 2011).

Conservation and inoculative biological control rely largely upon public funds, whereas augmentative biological control is often a business activity since mass production and regular large-scale field release of natural enemies are required for a successful pest management (van Lenteren 2011).

Nevertheless, this form of biological control, despite being regarded as the environmentally safest and the most cost-effective, is currently in a critical phase since its application is restricted to an impressively small acreage (e.g. DeBach and Rosen 1991; Cock et al. 2010). Moreover Warner and Getz (2008) argued that some of the criteria often employed to evaluate the viability of the commercial natural enemy industry, such as number of species suitable to be marketed as pest control agents or number of distributors of the same species, might be false metrics of the strength of the sector

The same authors highlight how the overall economic status of the industry (rearing, not distributing) appears static or in some sectors of production, even declining.

Several causes may contribute to this crisis. Non-chemical pest control, is often poorly endorsed or stimulated by governmental institutions. Moreover a consistent array of regulations regarding the application of biological control, limits, delays or even forbids its use (van Lenteren 2012)

In addition pesticide industry considers biological control as complex and of restricted use because pest control agents cannot be patented and are often difficult to be combined with chemical control.

Furthermore the industry shows little interest in sustainable long-term solutions for pest control, since sales personnel and farmers working on biological control often require additional training. Because patent periods on pesticides are limited

the main goal of industries is simply to develop and market new insecticides.

On the other hand, companies involved in commercial natural enemy production often face major challenges. In the USA commercial production is usually carried out at a small scale by operations that are usually insufficiently capitalized. Moreover trade of commercially sold species often suffers constraints and limitations due to the restrictive policies regarding international and intercontinental transfer (Warner and Getz 2008).

Augmentative biological control seems particularly challenging when predators or parasitoids have to be deployed in outdoor crops. Some biological agents are required only seasonally and their window of effective use is often narrow. Nonetheless, the producer has to sustain the costs of production before to exploit that narrow market opportunity. Furthermore if alternative pest control strategies become available or climatic factors delay or eliminate the problem, then the industry does not realize any profit for the efforts carried out in producing a specific natural enemy. By contrast labor, in either the phase of development or production, is expensive for the producer and production on a large scale of natural enemies depends often upon the maintenance of three trophic levels: the host plant or medium, the prey, and the predator or parasitoid. All the three levels must be suitable for production at commercial scales.

Natural enemy industry is generally supported by revenue generated by a few largely marketed products, and any resulting profit sponsors specialty or minor-use products specifically developed to fit the needs of customers (Hale and Elliott 2003)

Additional difficulties as losses in shipment and costs of transportation may lead small companies to shift their sales policies from direct selling to supplying larger companies. Furthermore collaboration between researchers, producers, and end users should be implemented in order to achieve an efficient and successful augmentative biological control (Hoffmann et al., 1998). The history of augmentative biological control in the USA suggests how successful projects were mainly those where a combination of factors was present: the biology of natural enemies was adequately investigated, laboratory rearing protocols were developed, know-how transferred to an industry for production at commercial scale, and wide-scale trials carried out jointly with end users inclined to

participate with their crops (Hale & Elliott 2003)

Despite the threat to biological control posed by pesticide industries and the crisis of natural enemy industry, the International Organization for Biological Control (IOBC) succeeded to achieve a European Union (EU) demand of testing side effects on natural enemies before releasing new pesticides on the market. The tests originally developed by the IOBC and the European Plant Protection Organization (EPPO) to evaluate side effects of chemicals, are currently necessary for the EU registration procedure of pesticides (EPPO 2003). Moreover some scholars highlight that chemical industry is rapidly shifting its goals in terms of production and that some companies are even starting to produce natural enemies. A reasonable explanation for this change in their policies is that biological control is becoming necessary in circumstances where chemical control alone fails to control all pests (Merino-Pachero 2007).

Moreover pesticides are often regarded as unfairly economical since their indirect costs such as diseases, pollution, elimination of non-target organisms and interference with the ecosystems end up to be paid by society (Costanza et al. 1997; Pimentel 2009).

The increasing resistance of pests to pesticides, the new requests of residue free food by supermarkets and consumers and the endorsement of integrated pest management from governmental institutions like the European Union are all factors likely to stimulate biological control in agriculture in the very next future (van Lenteren 2012).

B. Biological control in forest environments

In forest areas, biological control represents an ideal strategy for pest suppression since the very same resources of nature can often be successfully exploited. In the past this strategy was often erroneously regarded as a panacea for all problems. Nevertheless further investigations showed that in such a context several difficulties may arise during the planning and the execution of pest management programs (Tiberi, 2009). In natural habitats biological control, far from being a

universal solution for pest management, may represent yet a valid tool to control phytophagous insects. In fact, in the protection of this ecosystems it is often impossible to achieve with a single intervention the same level of efficacy required or expected in the context of agriculture (Covassi and Masutti, 1999).

In forest areas relevant interventions often require to re-establish a previously altered natural equilibrium or in case of biological invasion to favor a proper balance between the introduced phytophagous and its natural enemies. These objectives can be fulfilled either by the manipulation of interspecific relationships or by modifying intraspecific interactions. In the first case inoculative biological control can be performed by the introduction of natural enemies transferred from high-density to low-density areas.

In the second instance instead, the very same intraspecific behavior of a phytophagous is artificially altered in a way that may benefit the entire ecosystem (Tiberi, 2009).

C. Predators as pest control agents in forest environments

For what concerns predators as pest control agents, research efforts focused on biological control of the lepidopteran defoliator Pine Processionary Moth (PPM) (*Thaumetopoea pityocampa* Denis et Schiffermüller) (Lepidoptera Notodontidae) by the employment of *Formica rufa* (L.) (Hymenoptera Formicidae). Entire colonies of this generalist predator of lepidopteran larvae were collected in their alpine natural sites and transferred to pine stands scattered in the Appenines and other mediterranean habitats. However, despite the efforts carried out throughout the last 20 years only modest results were achieved (Pavan, 1959). Some authors pointed out that the lack of optimal climatic conditions and the absence in the new habitats of honeydew from lachnid aphids may partially explain the failure of these insects as pest control agents (Covassi and Masutti 1999).

More recently, in Turkey, the potential of *Calosoma sycophanta* (Coleoptera Carabidae) L. as a biological control agent was evaluated in a program aimed at targeting the same PPM. To this end this beetle was mass reared in laboratory using larvae of *T. pityocampa*, *T. solitaria* Freyer ed *Ephestia kuehniella* Zeller

(Lepidoptera Pyralidae)(Kanat and Özbolat, 2006).

Likewise, some predators of *Matsucoccus* Cock (Homoptera Margarodidae) were laboratory reared in Italy. These control agents were the hemipteran anthocorid *Elatophilus nigricornis* (Zetterstead), and *E. pini* (Bärensprung) and the coccinellid beetle *Rhyzobius chrysomeloides* (Herbst) (Coleoptera Coccinellidae), a species particularly active on eggs and young larvae of *Matsucoccus feytaudi* (Ducasse). These beetles were then regularly recorded in *Pinus pinaster* stands infested by *M. feytaudi* (Covassi *et al.*, 1991)

D. Biological control by parasitoids in forest environments

In the last decades ooparasitoids have been thoroughly investigated for their mass rearing potential on natural and factitious hosts and for their capacity to target pests in forest environments.

In 1961 the potential role of parasitoids in the control of *Thaumetopoea wilkinsoni* Tams, a major coniferous pest in Israel, was investigated by Halperin (1990). In a first survey aimed at evaluating the occurrence of *T. wilkinsoni* egg parasitoids, *Ooencyrtus pityocampae* (Mercet) (Hymenoptera Encyrtidae) was detected in most of the infested sites; a parasitization peak of 5.9% was recorded in the most heavily infested stands.

Afterward, a field trial was carried out in which 600 egg batches of *T. wilkinsoni* were collected from pine stands with high peaks of *O. pityocampae* parasitization and subsequently exposed in nine stands at an altitude of 200-750 m where a low level of parasitization had been previously recorded

At the end of the breeding season egg batches were collected again in the same stands in order to estimate possible variations in the parasitization rate. An average increment of parasitization by 17-fold was recorded in the treated pine stands compared with a 3-fold one in control stands. However further studies showed that such a level of control could only be achieved in years with mild and rain free autumns. Despite these drawbacks the same author argues that *O. pityocampae* release may be cost-effective even when long-term forecasts of temperature and precipitation are not available.

In a 1973 field experiment, *Ooencyrtus ennomophagus* Yoshimoto, was released in two forests of Connecticut in order to control the elm spanworm *Ennomos subsignarius* L., (Lepidoptera Geometridae) a polyphagous defoliator of hardwoods. This parasitoid proved to be particularly efficient in the upper strata of the forest where most of the elm spanworm eggs are laid, succeeding to parasitize almost all host eggs (Anderson and Kaya, 1973)

In 1997, in a coniferous stand close to Florence (Italy) infested by the Pine Processionary Moth, 800 females of the Eulophid ooparasitoid *Baryscapus servadeii* (Domenichini) (Hymenoptera Eulophidae) were released in order to control the pest. In the following years a higher parasitization rate was recorded in the same area as a consequence of the population increase of the eulophid. Nevertheless after the release a parallel decrease of *O. pityocampae* (Mercet) was recorded in the same site (Tiberi, 1980). Good results in term of pest control were achieved in a similar biological control program carried out in Sicily where a population of *Ooencyrtus kuwanae* (Howard) was released in oak stands colonized by *Lymantria dispar* (L.) (Lepidoptera Erebidae) (Longo *et al.*, 1994).

Pine Processionary Moth, in several countries of the Mediterranean basin, is responsible for serious damages to tree canopies of both natural and artificial coniferous stands. Its main targets are *Pinus* ssp. but *Cedrus* spp. but *Pseudotsuga menziesii* can be attacked as well. Furthermore larvae because of their urticating hairs represent in urban settings a major public health concern. Therefore, in last the decades, the potential of hymenopteran endoparasitoids as pest control agents against this lepidoptera were extensively evaluated in integrated pest management contexts. Among these parasitoids *Ooencyrtus pityocampae* because of its wide distribution, capability to adapt to host density fluctuations and to develop on alternative hosts plays a key role in bio-control strategies of PPM caterpillars (Battisti *et al.*, 1990; Halperin 1990; Masutti *et al.*, 1991).

Several interventions were recently performed in Tuscany and Lazio (Italy) in order to control the PPM. These programs were carried out by using egg batches of *Nezara viridula* (L.) (Heteroptera Pentatomidae) as oviposition substrate for the mass rearing of *O. pityocampae*.

Populations of this parasitoid were then released in specific sites synchronizing their activity with the egg stage of PPM. In the summer of 1991 in a young pine stand close to (Fondi, LT) 19000 individuals of *O. pityocampae* were released during the flight period of PPM. Moreover in the following years similar biological control programs were performed in Monte Senario and Consuma (FI) where respectively 26000 and 17000 individuals of *O. pityocampae* were released in infested pine stands during the flight period of the PPM. The analysis of PPM batches collected in the three different stands after parasitoid population enhancement showed a dramatic increase of parasitized eggs compared with those collected and examined in the previous years (Tiberi, 2009).

Among the successful programs of biological control in Italy, the introduction of the parasitoid *Anaphes (Patasson) nitens* (Girault)(Hymenoptera Mymaridae) deserves particular attention. This species, native of Australia, was introduced in 1978 with the purpose of protecting *Eucalyptus* from damages produced by *Gonipterus scutellatus* (Gyllenhal) (Arzone, 1985) (Coleoptera Curculionidae). Excellent results were achieved and the parasitoid alone was capable to successfully control the curculionid. Likewise in 1987 *Neodryinus typhlocybae* (Ashmead)(Hymenoptera Dryinidae) was introduced to many Italian regions in order to control *Metcalfa pruinosa* Say (Hemiptera Flatidae). The parasitoid spread rapidly in the environment showing good searching capabilities (Girolami and Camporese, 1994). Similarly *Closterocerus chamaeleon* (Girault) (Hymenoptera Eulophidae) was employed in Sicily and Sardegna for the control of *Ophelimus maskelli* (Ashmead) (Hymenoptera Eulophidae) (Caleca *et al.*, 2009).

In recent times the scientific community has focused on ooparasitoids of lepidopteran larvae responsible for severe defoliation in forest environments. The lepidoptera *T. pityocampa*, *T. processionea* L., *Tortrix viridana* (Lepidoptera Tortricidae), *L. dispar* and *Euproctis chrysorrhoea* L. (Lepidoptera Erebidae) deserve particular attention for their role as marker species for climatic change. The influence of climate on these species is investigated by the analysis of their prevalence and distribution in space and time. Variations in parasitoid distribution were highlighted for *Baryscapus servadeii* that was recorded for the first time in pine stands on Etna and for *Trichogramma embryophagum* (Hartig) (Hymenoptera Trichogrammatidae) whose prevalence shows a gradual

generalized decrease in hill areas of the Italian peninsula (Tiberi, 2009).

The relationship between climate change and insect outbreaks in forest environments was recently investigated. When temperatures increase, forest insects tend to exploit immediately the new environmental conditions by accelerating their biological cycle and enhancing their offspring production. In particular, an expansion in the range of *Thaumetopoea pityocampa* (Lepidoptera Notodontidae) was recently observed and linked to an increase of winter temperatures that improved survival of larval colonies. Moreover, in the new areas of expansion, the efficacy of natural enemies in targeting the host is substantially reduced leading to a rapid population growth and consequent severe damages to forest ecosystems. (Battisti and Faccoli, 2007)

That being said, the goals of scientific community have currently shifted towards the investigation and control of biological invasions. Efforts are often focused on the introduction of natural enemies of the invasive species with the purpose of re-establishing the host-parasitoid association in the infested areas. Particularly good results were achieved by the introduction from Japan of *Torymus sinensis* Kamijo (Hymenoptera Torymidae) a species native of China capable to target *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera Cynipidae) whose activity endangers *Castanea sativa* stands all over the Italian peninsula. *Torymus sinensis* in Italy was obtained from plant material imported from Japan and maintained in specific sites until parasitoid emergence from galls produced by the same cynipid. Moreover new associations between *Dryocosmus kuriphilus* and the native parasitoid *Torymus flavipes* (Walker) were investigated in the Bologna (Italy). In fact, this parasitoid is known to target many species of gall-maker host larvae that lives on different species of plants included oaks (Santi and Maini, 2011). Another biological invasion recently investigated in Tuscany is *Leptoglossus occidentalis* Heideman (Hemiptera Coreidae). This North American coreid is a polyphagous cone and seed feeder known to target many coniferous species causing cone abortion and seed loss and thus endangering seed production of many pine species (Koerber, 1963; Hedlin *et al.*, 1981; Cibrian-Tovar *et al.*, 1986; Bates *et al.*, 2000, 2002; Strong *et al.*, 2001; Strong, 2006). The pest, accidentally introduced into Europe, was firstly recorded in 1999 in Italy (Tescari, 2001) where a few

years later severe losses in commercial pine nut production due to feeding on *Pinus pinea* L. were reported (Roversi, 2009; Santini, 2010). The recorded spreading history of *L. occidentalis* in Europe indicates that this insect had a rapid expansion, moving from the mediterranean basin, to the United Kingdom, Scandinavia and Ukraine (Eppo, 2010; Mjos *et al.*, 2010; Werner, 2011; Hizal and Inan, 2012; Gapon, 2012; Zhu *et al.*, 2013).

In Italy pest control by chemicals is restricted because *P. pinea* woods are primarily located in protected areas along the coasts (parks, reserves and other natural habitats). Therefore, the use of natural biological control agents for targeting *L. occidentalis* is highly advisable. In North America, the platygastriid hymenoptera *Gryon pennsylvanicum* (Ashmead) represents the predominant egg parasitoid of *L. occidentalis* (Masner, 1983; Bates and Borden, 2004; Maltese *et al.*, 2011). This egg parasitoid was introduced in Italy in 2010 under quarantine conditions and its potential as a biocontrol agent is under investigation in laboratory conditions (Roversi *et al.*, 2011a; Sabbatini Peverieri *et al.*, 2012; Sabbatini Peverieri *et al.*, 2013; Paoli *et al.*, 2013). Recent studies are particularly focusing on *G. pennsylvanicum* host range in order to detect potential negative effects of this hymenoptera on the native fauna (Roversi *et al.*, 2013). Since *G. pennsylvanicum* introduction in the field is still a possibility under evaluation, an augmentative biological control program based on mass production for field release of indigenous natural enemies may represent a valid alternative. Studies were thus focused on indigenous parasitoids in order to examine their potential adaptation to the new host. In Italy, two indigenous generalist egg parasitoids *Anastatus bifasciatus* (Geoffroy) (Hymenoptera Eupelmidae) and *O. pityocampae* (Mercet) (Hymenoptera Encyrtidae) were recently found to parasitize *L. occidentalis*. Nevertheless parasitization rates reported for these two hymenoptera were very low (Camponogara *et al.*, 2003; Niccoli *et al.*, 2009; Santini, 2010, Roversi *et al.*, 2011b). Despite this, *O. pityocampae*, deserves yet a particular attention for its flexibility and capacity to adapt to different hosts including pentatomids and coreids (Heteroptera) (Battisti *et al.*, 1988).

E. The role of insect cold storage in biological control programs

Mass production of beneficial insects is an essential step of biological control programs, particularly of those depending on augmentative field releases (van Lenteren and Tommasini, 2002). Some of the political and economic obstacles to the successful implementation of inundative releases in agriculture have already been debated in previous sections. However another major limiting factor in mass production of natural enemies may be represented by the difficulty of storing them before the release. The organisms used in pest control programs often have a relatively short shelf life. For that reason they have to be produced just before their use. Therefore the development of adequate storage procedures can limit the cost of biological control by spreading the production period over several months (Colinet and Boivin 2011).

Cold storage plays a key role as a method for extending the shelf life of biological control agents and provides an adequate supply of insects for pest management programs. Moreover low temperature storage allows a precise synchronization between the release of natural enemies and the most critical phases of pest outbreaks (McDonald and Kok, 1990; Venkatesan *et al.*, 2000). Other methods of pest control as Sterile insect technique (SIT) may also benefit of cold storage of mass-reared insects in the phases of collection, distribution and release. Moreover storage at low temperature proved to be not only essential for insect industrial production but also useful for maintaining colonies under laboratory conditions for research purpose. Furthermore the rescue of endangered species may also take advantage of improvements in cold storage techniques (Leopold, 2007).

Because parasitoids are widely employed in pest management programs, studies on their low-temperature conservation started over 75 years ago; since then, a large body of literature dealing with parasitoid use in biological control has been developed due to their scientific and economic relevance (Colinet and Boivin, 2011).

Every parasitoid species has a peculiar evolutionary history responsible for its current adaptation. Therefore the variability in the capacity of parasitoid species to withstand low temperatures is often very high. In such a context any taxonomic

generalization of insect response to cold storage seems doomed to failure (Leopold *et al.*, 1998). Moreover parasitoid cold tolerance may be affected not only by genotypic-based plasticity but also by variability at the phenotypic level (Hawes and Bale, 2007).

Cold storage under sub-ambient temperature above 0°C reduces insect development and metabolic rate and it is usually significant in order to increase parasitoid availability, providing efficiency and flexibility in mass-rearing, extending shelf-life and improving synchronization for deployment in inundative release programs (Colinet and Boivin, 2011; Leopold, 2007). Unfortunately, cold-induced extension of parasitoid shelf life is often associated with major fitness costs even above the freezing temperature. Mortality often represents the consequence of a progressive increase up to an ultimate level of sub-lethal perturbations occurred during chilling. Nonetheless fitness-related traits may also decrease in survived parasitoids not only immediately after storage or in the subsequent development stages but also in the following generations. In fact parasitoid cold tolerance depends ultimately on the interaction of **exogenous** and **endogenous** factors affecting insect response to low-temperature stress.

F. Endogenous factors involved in parasitoid cold tolerance

F.1. Age and stage.

Ontogeny-related variation in insect cold tolerance was reviewed by Bowler and Terblanche (2008). However generalizing a pattern of ontogeny-related cold tolerance seems very complex because marked variations between different taxonomic groups often occur. Therefore it is necessary to determine which developmental stage is the most suitable for cold storage. In general, conservation of adults appears to a decrease parasitoid performance whereas storage of immature is often more appropriate (Colinet and Boivin 2011). There is some evidence that pupae are more cold resistant than larvae or adults (Nakama and Foerster, 2001). In particular pupae seem more suitable for short-term

conservation (van Lenteren and Tommasini, 2002). Nevertheless studies have been reported in which adults were more cold tolerant than juvenile stages (Bayram *et al.*, 2005). Another source of variability in parasitoid cold tolerance is represented by within-life-stage effects (Bowler and Terblanche, 2008). Furthermore the relationship between metabolic/developmental rate and cold tolerance was investigated in housefly pupae by Leopold *et al.* (1998); results suggest an association between lower metabolic activity and insect cold tolerance. However because variability in cold tolerance depends on a combination of different factors like species, temperature and duration of cold exposure, general guidelines are not available (Bayram *et al.* 2005)

F.2. Dormancy status

Dormancy was defined by (Kostal, 2006) as a “generic term covering any state of suppressed development (developmental arrest), which is adaptive (that is ecologically or evolutionarily meaningful and not just artificially induced), and usually accompanied with metabolic suppression”. Dormancy includes diapause, and quiescence defined by (Colinet and Boivin 2011) respectively as a “programmed obligatory or facultative interruption in development” and “immediate interruption in development in response to adverse conditions”.

Cold storage of insect dormant stages is potentially useful provided that dormancy start and termination may be artificially regulated (Boivin, 1994).

Unfortunately low temperature conservation of insect dormant stages is a valuable tool for storage only in few circumstances. In fact mortality is often reported to occur during attempts to artificially induce diapause (van Lenteren and Tommasini, 2002). The relevant influence of photoperiod on the induction of diapause in the Hymenoptera Chalcidoidea was underlined by Anderson and Kaya (1974). Moreover the same authors investigated the overwintering strategies of *Ooencyrtus ennomophagus* Yoshimoto (Hymenoptera Encyrtidae) in Connecticut. Diapausing larvae of this parasitoid are able to survive under adverse climatic conditions inside its host egg until they emerge as adults at the end of June. Diapause is facultative and occurs at the end of larval development soon before pupation. The photoperiodic induction takes place in the maternal generation and

the adult is likely to be the most sensitive stage even though pupae are sensitive too. On the other hand even low temperatures independent from photophase may also induce diapause in *O. ennomophagus* by acting directly on the developing larvae. The ecological significance of this phenomenon is still unknown but may represent an additional mechanism aimed at insuring the parasitoid enter diapause in the late autumn (Anderson and Kaya 1974). In Israel mass breeding of egg parasitoids of *T. wilkinsonii* was investigated by Halperin (1990) with particular emphasis on *O. pityocampae*. When mean daily temperatures fell below 22°C a sharp decrease in parasitoid developmental rate was observed. The same author stressed that mature larvae entered diapause when night temperatures reached 14°C or dropped below.

Other observations on *O. pityocampae* immatures overwintering within egg batches of *T. pityocampa* showed that winter diapause of *O. pityocampae* larvae may be interrupted by simply warming them up to a temperature of 28-30°C (Masutti, 1964). In the following decades new experiments were performed by Battisti et al. (1990) in order to test the influence of temperature on *O. pitocampae* larvae. Egg clusters of *T. pityocampa* were collected in the autumn of 1987 in a coniferous stand close to Padova (Italy). Thereafter they were divided in groups and cooled down to 5°C by a rate of 2°C/day. Each group was then warmed up to 29°C using the same gradient after different time lags. *O. pityocampae* response to the increased cooling intervals was: a reduced mortality of juveniles, a reduction of the time spent for emergence and a higher synchronism in adult hatching. This trial showed that long cooling periods might be ideal for storing diapausing larvae to be used in mass rearing programs. The same authors suggested it would be worth to test whether diapausing immatures can be stored long enough to synchronize parasitoid adult emergence with the egg stage of *T. pityocampa*.

More recently Rivers et al., (2000) showed that *N. vitripennis* (Hymenoptera Pteromalidae) diapausing larvae are more cold tolerant than non-diapausing ones. Likewise, in *A. rhopalosiphi* and *A. ervi* (Hymenoptera Braconidae), increased survival rates of diapausing mummies after cold storage were reported by Langer and Hance, (2000). The few examples available in scientific literature highlight how diapause is only rarely a possible solution for cold storages since the capacity

of entering diapause is not characteristic of all species (Rundle et al., 2004). Nonetheless when diapause is controlled it can be then extended even for long time lags in order to improve shelf life of insects (Foerster and Doetzer, 2006).

G. Exogenous factors involved in parasitoid cold tolerance

G.1. Temperature

A way to conserve parasitoids is to store them at low temperatures from 0 to 15 °C. However even at these relatively low temperatures the majority of the species tends to show a certain degree of reduction in their survival rates (Leopold *et al.*, 1998; van Lenteren and Tommasini, 2002). As a general rule survival declines when temperature decreases (Lopez and Botto, 2005; Pandey and Johnson, 2005; Luczynski et al., 2007; Bernardo et al., 2008; Colinet and Boivin 2011).

Temperature is an abiotic factor responsible for affecting parasitoid survival during low-temperature conservation. The use of specific thermal regimes and the determination of the lower threshold temperature for development (T_0) are generally necessary prerequisites for an efficacious design of cold storage protocols (Leopold, 2007). When a temperature is selected for cold conservation care should be taken to achieve a right balance between the reduction of metabolic rate and/or development and the potential risk of accumulating chilling injuries. High temperatures may conversely produce undesirable outcomes such as precocious emergence during insect storage (Hofsvang and Hagvar, 1977; Pitcher *et al.*, 2002). Threshold temperature is another relevant parameter to be determined when cold storage protocols are designed. Colinet & Boivin (2011) define this parameter as the “temperature at or above which permanent detrimental effects will not occur” Threshold temperature is often reported to be around development threshold T_0 .

Cold storage of *Anagyrus ananatis* (Gahan) (Hymenoptera Encyrtidae) juvenile stages below and above T_0 (12.6 °C) was evaluated by Pandey and Johnson (2005). Immatures were subjected to storage for more than eight weeks at 14.8 °C with no effect on emergence whereas storage at 10.1°C produced a dramatic drop in parasitoid hatching even after less than two weeks. Cold tolerance can be heavily influenced even by small variations in storage temperature particularly if it is

close to T_0

Gonatocerus ashmeadi (Girault) (Hymenoptera Mymaridae) ($T_0 = 3.8\text{ °C}$) exposure at 4 °C produced a remarkable decrease in parasitoid survival while at 4.5 °C immatures could withstand up to 20 days of cold storage (Leopold, 2004)

Lethal chilling injuries are often reported to be the consequence of the arrest of juvenile development below the T_0 . Nonetheless other studies suggest that successful cold storage can be carried out even at temperatures below T_0 . *Encarsia formosa* (Gahan) (Hymenoptera Aphelinidae) ($T_0 = 12.7\text{ °C}$) was reported to withstand storage up to 28 days at 4.5 °C (Lopez and Botto, 2005). There is evidence that parasitoid cold storage tolerance as a function of temperature is highly variable. For this reason optimal temperature zone for each species should be empirically ascertained before to proceed with storage trials. Moreover the thermal history of the parental generations of parasitoids submitted to treatment should be analyzed in order to evaluate its influence on the following generations. This peculiar type of phenotypic plasticity was described in *Drosophila melanogaster* (Diptera Drosophilidae) by Magiafoglou and Hoffmann (2003) but has never been extensively evaluated in parasitoids. A maternal effect was described in *Anaphes* sp. by Hance and Boivin (1993): In fact the progeny of this parasitoid displayed an increased cold hardiness when its parental generations had been previously exposed to a combination of low temperature and short photoperiod.

G.2. Rates of cooling or heating

Other relevant factors affecting insect survival during storage, are the cooling and heating rates. Slower cooling rates are generally reported to be more ecologically relevant and thus more likely to produce cold hardening. Tolerance to temperature changes can be similarly affected by heating rates (Chown and Nicolson, 2004). Experiments carried out on *D. melanogaster* adults showed that rapid cold hardening of *D. melanogaster* could be achieved by cooling at rates that naturally occur, and that the level of protection reached by these thermal regimes may improve insect survival at ecologically relevant temperatures. In fact when *D. melanogaster* specimens were cooled at natural rates (0.05 and 0.1 °C min^{-1}), an

increased survival, after one hour of exposure to -7 and -8°C, was observed. In contrast flies directly transferred to these temperatures or cooled at faster rates such as 0.5, or 1.0°C min⁻¹ were more severely affected by the storage period (Kelty and Lee, 1999). Nevertheless in recent studies cold tolerance was reported to decrease when slow rates of temperature change were applied. When *D. melanogaster* larvae were directly transferred to room temperature after cold exposure an increased survival was observed whereas slow or stepwise re-warming failed to achieve the same result (Sinclair and Rajamohan, 2008).

In a recent trial, groups of 1-d-old and 3-d-old *Aphidius rhopalosiphi* (De Stefani-Peres), mummies were transferred to -5°C and stored for 10 d. Different thermal regimes were applied including direct transfer and five different acclimation treatments. Mummies that were subjected to direct transfer from room temperature to storage temperature showed a higher reduction in their survival rates compared with those submitted to thermal treatments by using step temperature decrease (Levie *et al.*, 2005). Parasitoid cold tolerance might thus be influenced by the rate of temperature change. However in the vast majority of parasitoid species the role of cooling and heating rates has not been thoroughly investigated yet (Colinet and Boivin, 2011)

H. The focus of the research

The present work focused on *O. pityocampae* (Mercet) (Hymenoptera Encyrtidae), an indigenous and polyphagous egg-parasitoid that might play a role in biological control programs against *Thaumetopoea pityocampa* (Den. & Schiff.) (Lepidoptera Notodontidae). Therefore biological parameters of this encyrtid reared on alternative host eggs were analyzed by testing its behavior and reproductive parameters. Time between oviposition and progeny emergence, sex ratio of offspring, parasitization rate and offspring production were recorded. Thereafter, in a second set of experiments, the stages of *O. pityocampae* juveniles developing within eggs of *Nezara viridula* (L.) (Heteroptera Pentatomidae) were also identified by morphological analysis and measurements. Egg-to-adult temperature-dependent development of *O. pityocampae* within eggs of the alternative host *Graphosoma lineatum* (L.) (Heteroptera Pentatomidae) was also investigated in order to determine development times. Moreover, because parasitoid development within host eggs is often influenced by host egg age, trials were carried out to determine the capacity of this encyrtid to accept and exploit eggs from different age classes. Since maintenance of living organisms is particularly useful in mass rearing programs, the second part of the project was aimed at evaluating cold storage potential of *O. pityocampae* for long periods. For this reason cold tolerance of this parasitoid was evaluated by storing different juvenile stages within eggs of *G. lineatum* at several low temperatures for 10 days. The effect of cooling and heating rates on the survival of stored immatures was also assessed for each thermal treatment.

MATERIALS AND METHODS

Host origin

a) *Nezara viridula*

Populations of *N. viridula* were collected in tomato plantations located in different regions of Italy including Lombardia, Tuscany and Sicily. New colonies were then established by the introduction in each cage of about 100 individuals (sex ratio 1:1). The cages were cubic structures (100x35x35cm) whose top and lateral sides were made by plastic nets of 1mm mesh. One of the lateral sides could be opened for colony feeding and maintenance. A plastic layer was then set on the bottom of the cage serving as a solid easy-cleaning ground surface. Five 90 mm petri dishes were placed on the bottom of the cage and filled with a food source made by mixed soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*) dry seeds. Moistened cotton was provided and refreshed and/or replaced every 48 h whereas seeds were weekly added and /or replaced. Moreover a mixed host system consisting of tomato (*Lycopersicon esculentum*) soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*) plants was provided as an additional food source for the pentatomids. Since *N. viridula* is particularly susceptible to inbreeding depression, ideal rearing conditions often imply annual recolonization with field-collected specimens in order to increase genetic variability and limit sibling mating.

All colonies were reared in environmentally controlled rooms (26°C, 70 ± 5% RH, 16:8 L:D).

b) *Leptoglossus occidentalis* individuals were initially collected in pine stands in Central Italy. The insects were then reared in cages located in rearing rooms at 26±1°C, 40±10% RH and 16:8 L:D using young potted *P. pinea* as an oviposition substrate and seeds of *Pinus nigra* Arnold *s.l.* as a food source.

Moreover each colony cage was provided with moistened cotton as a water source. This technique allowed egg production all year round

- c) *Graphosoma lineatum* (L.) specimens were collected from fennel plants *Foeniculum vulgare* in four geographically distinct locations of Tuscany (Italy) during the summer of 2012 and reared in a climatized room at $28 \pm 1^\circ\text{C}$, $45 \pm 5\%$ relative humidity (RH) and a photoperiod of 16:8 (L:D)h. The standard rearing technique for *Graphosoma semipunctatum* Voegele' (1966), was initially adopted. After 5 generations a pool of young adult specimens (<24h) (1:1 sex ratio) were separated from the original colony and moved into new cages in order to establish different rearing conditions. Afterward *Graphosoma lineatum* was reared with a new technique for 20 weeks (5 generations). The new cage was a light wood structure (100x35x35cm) whose bottom and lateral short sides were plywood sections of 5mm height whereas the top and lateral long sides were made by metal nets of 1mm mesh. One of the long lateral sides functioned as a door fixed to the structure by metal hinges. A polystyrene layer set on the bottom of the cage served as a robust and easy-cleaning inner ground surface. Five 140 mm petri dishes were placed on the bottom of the cage and filled with a food source of mixed *Foeniculum vulgare* and *Anethum graveolens* (Apiaceae) dry fruits (Ca' dei fiori SRL) in a proportion 8:2. Moreover the polystyrene sheet forming the cage floor was fully covered by the same mixture of dry fruits that formed a 5mm layer. Dry fruits of Apiaceae were stored in large baskets in a climatized and ventilated room at $10 \pm 1^\circ\text{C}$, $30 \pm 10\%$ (RH) and were weekly added in equal proportions into the petri dishes. Furthermore the floor of the cage was cleaned every two weeks and dry fruits added again to cover the entire surface. On the short sides of the cage absorbent paper strips (Linea Roll) 20x10cm were attached in order to form a substrate for insect oviposition. Below the paper strips four thick and hard cardboard tubes (10 cm long and 8 cm diameter) were placed on each side of the cage. The inner surface of each tube was fully paved by disks of pressed cotton

(SISMA S.p.A) protruding 3cm out of both edges. Thereafter a 20x10 cm stripe of absorbent paper was loosely folded into each tube in order to fill the inner volume leaving crevices for the insect to rest and oviposit. Two longer tubes (25 x 8 cm) similarly arranged were likewise placed along the lateral sides. On the bottom of the cage two potted young plants of *Foeniculum vulgare* were introduced to improve the quality of the rearing providing the insects with their natural host plant. These plants were cultivated in a greenhouse at standard conditions of $25 \pm 1^\circ\text{C}$, $50 \pm 10\%$ relative humidity (RH) and a photoperiod of 16:8 (L:D) and represented both an extra food source and a more natural resting area for pentatomids. Closed to the pots a small automatic irrigation system was set up to water the two plants. The irrigation system included a 2 L tank and seepage control sprinklers made of a porous porcelain head part that was completely inserted into the soil and plastic pipes whose end was fully immersed in the water container. The same automatic irrigation devices were then employed to provide the insect with constant fresh water. To this end the porous porcelain head parts of the irrigators were first cleaned and then completely enveloped with wet cotton disks of the type mentioned above. This system allowed the cotton to remain constantly moistened ensuring a permanent water source for the pentatomids. The sprinklers were then placed on a plastic net of 1mm mesh covering a glass container of (15x15x5cm) that served to collect the extra water preventing unwanted liquid spillage on the bottom of the cage. The extra water was eliminated from the glass container when required whereas the big 2L tank was weekly cleaned and refilled with fresh new water. Once a week the cages were opened and all paper stripes and cotton disks removed and inspected in order to collect egg batches. When paper and cotton were found to be dirty or no more fit to deploy as oviposition substrate they were eliminated and replaced with new ones. Clusters of eggs were cut out with scissors and placed in 140 mm petri dishes whose bottom had been previously covered by a 5 mm layer of dry fruits mixture. Nymph cages were Plexiglas transparent containers (40x30x30cm) whose lateral long sides were made of plastic nets of 0.5 mm mesh. The same

mixture of dry fruits used in the adult cage covered the bottom of the structure. A metal rack (35x25x10 cm) was inserted along one lateral side of the cage while on the opposite side two small potted young fennel plants were placed. A plant irrigation system and water supply for the insects were arranged as previously described. Petri dishes full of egg batches gathered from the adult rearing were then transferred into the metal rack in the nymph cages to allow egg hatching and the starting of new generations. Food was weekly added and cages completely cleaned every two weeks. As soon as new adults hatched they were transferred to the larger adult cages to increase the population.

1. Biological parameters of *O. pityocampae* reared on alternative host eggs

Newly hatched females of *O. pityocampae* were individually isolated in glass tubes with moistened cotton. Parasitoids were fed with honey ad libitum and followed up until natural death in a climatic chamber at standard rearing conditions (26°C, 75% RH and 16:8 L:D). Fresh egg batches of *Nezara viridula* (< 24h) were placed inside the tubes (batches of \pm 40 eggs for each tube) and exposed to adult females for 24h parasitization. Forty eggs per day were offered since preliminary tests, carried out at the same rearing conditions, showed that this quantity exceeded the parasitization rate per day. Parasitized eggs were identified by the presence of egg stalks protruding from the chorion. Host eggs were then daily replaced until parasitoid natural death. 30 *N. viridula* egg batches were isolated in separate tubes as controls. Parasitized batches were then stored at standard conditions and daily checked for adult emergence. Time between oviposition and progeny emergence, sex ratio of offspring, parasitization rate and offspring production were recorded for each egg mass. The test was replicated 10 times and females drowned in honey droplets were excluded from analysis. Descriptive statistics were provided.

2. Identification and biological development of *O. pityocampae* larvae within eggs of the alternative host *N. viridula*

Egg batches of *N. viridula* were placed in tubes and exposed to *O. pityocampae* females for 4h parasitization at a 1:5 parasitoid/host ratio (26°C, 75± 5% RH and 16:8 L:D). Parasitized egg batches were then identified by the presence of an egg stalk protruding from the chorion. After parasitization batches were removed from tubes and reared in a climatic chamber at standardized conditions (26°C, 75 ± 5% RH, 16:8 L:D). Thereafter parasitized eggs were separated and dissected and *O. pityocampae* specimens observed under a stereomicroscope (Stereoscopic Zoom Microscope SMZ1500) at 8h intervals in order to investigate larval development. Once individuals were detected they were gently removed by forceps and repeatedly washed by PbS. After that living immature individuals were transferred into small tanks with Rhodamine to be stained for 30 minutes. Repeated washing by PBS was then carried out to remove all the dye. Afterwards stained individual were mounted in slides with hoyer and observation and measurements carried out by Nikon A1 microscope and NIS-Elements AR 3.10 software (modified from Doane *et al.*, 1989). Juvenile stages were then identified by the following parameters: mandible length, head width, body length and segmentation. Descriptive statistics were provided and larval instars identified by frequency distribution analysis.

3. Effects of host egg age on *O. pityocampae* parasitization

3.a Parasitoid origin

Parasitized egg clusters of *T. pityocampa* hosting diapausing juveniles of *O. pityocampae* were originally collected from coniferous stands (300 m asl) in Gargano region (South East of Italy). Batches were subsequently set into glass tubes (15 cm long and 2 cm diameter closed on both ends by a cotton net of 250 micron mesh) and placed in a climatized room at 30±1°C, 40±10% RH and 16:8 L:D in order to break parasitoid diapause (Halperin, 1990; Battisti *et al.*, 1990). After three weeks parasitoids hatched and a new laboratory colony was

established.

Parasitoids were reared for over 15 generations on of *L. occidentalis* host eggs in a climatic chamber (Binder KBWF 720, Tuttlingen, Germany) at standard condition of $26\pm 1^{\circ}\text{C}$, $75\pm 5\%$ RH and 16:8 L:D before the start of the trial. Females were provided with pure honey drops *ad libitum* as food source. Drops were then refreshed three times per week.

3.b. Host egg aging

L. occidentalis females oviposit their eggs in a single row on pine needles. Clusters consist of about 3-20 eggs each. Since under laboratory conditions eggs of *L. occidentalis* are reported to hatch in 8.9 d. on average, host eggs from seven age classes were used to carry out the trials: fresh eggs (< 24h) and 2, 3, 4, 5, 6, 7 days old (Nechols *et al.*, 1989; Hirose *et al.*, 2003; Sabbatini Peverieri *et al.*, 2013; Binazzi *et al.*, 2013).

The process of oogenesis of *O. pityocampae* is not complete at the emergence. In fact the number of mature eggs after adult hatching is low and starts to increase only after several days (Battisti *et al.*, 1990). Our preliminary findings on *O. pityocampae* biological traits agree with these results showing that parasitization peak of females reared on *Graphosoma lineatum* (L.) lies between days 4-7 of parasitoid life (Binazzi and Roversi, unpublished data). For that reason in the present experiment newly eclosed specimens were housed one per each tube, maintained at standard rearing conditions and fed with honey *ad libitum* for 5 days before the trial. The eggs of *L. occidentalis* were collected every 24h from the colony by removing needles with an egg cluster from the pines. Clusters of 15 eggs (*ad libitum*) were then aged in a climatic chamber at standard conditions and then exposed for parasitization to *O. pityocampae* females, after fixing the egg-bearing pine needle by wire paper clips onto paper strips (Bates and Borden, 2004, 2005; Sabbatini Peverieri *et al.*, 2012).

A host egg cluster of, all in one of the seven egg age classes, was exposed to each female for a 24 h. *L. occidentalis* eggs were considered parasitized when the presence of at least one egg stalk on the host chorion was verified (Maple, 1947; Battisti *et al.*, 1988; Nechols *et al.*, 1989). After the exposure, host egg batches were removed and transferred to a climatic chamber at standard conditions. Egg

batches were then daily checked until parasitoids hatched or non parasitized eggs hatched. All tests were replicated 13 times and the following parameters were recorded: no. of parasitized host eggs per batch, no. of egg stalks per batch, offspring production (no. of females emerged per batch), sex ratio (% females), and female development time.

3.c. Microscopical observations

After parasitoid hatching all parasitized host eggs were dissected under a stereomicroscope (Stereoscopic Zoom Microscope SMZ1500) with the purpose of describing the host egg content.

3.d. Data analysis and statistics

Data were tested for normality with the Shapiro-Wilk test. Transformations were then applied but failed to normalize the data. Consequently Kruskal–Wallis test followed by the Mann–Whitney U-test was performed to compare the recorded parameters in the seven age classes. For multiple comparisons, the significance level ($\alpha = 0.05$) was adjusted with Bonferroni correction, α/n , where n was the number of pairs in the multiple comparison. Furthermore the correlation of each parameter with host egg age was analyzed by nonparametric Spearman's rho test (Zar, 2010). Statistical procedures were performed by the statistical software SPSS 20.0.0 (2011).

4. Temperature-dependent egg to adult development of *O. pityocampae* within eggs of the alternative host *Graphosoma lineatum*:

Fresh egg batches of *G. lineatum* (< 24h) were collected from colonies housed in glass tubes and exposed to *O. pityocampae* females for 4h random parasitization at a 1:5 parasitoid/host ratio and standard rearing conditions ($26\pm 1^\circ\text{C}$, $75\pm 5\%$ RH and 16:8 L:D). Parasitization was ascertained for all egg batches by checking for egg stalks protruding from host chorion. After parasitization egg batches were then randomly assigned to each of the following constant-temperature treatment 12, 14, 19, 26, 30, 36, 38 °C selected to test *O. pityocampae* development. In the

following six moths parasitized eggs were daily checked from the time of parasitization until adult emergence and then sexed. For each treatment 15 replicates were carried out and the following parameters recorded: No. of parasitized host eggs per batch, no. of egg stalks per batch, offspring production (no. of females emerged per batch), sex ratio (% females), and days (d) required for each parasitoid to complete development from egg to adult.

4.a. Data analysis and statistics

Data were tested for normality with the Shapiro-Wilk test. Since transformations failed to normalize the data, Kruskal–Wallis test followed by the Mann–Whitney U-test was performed to compare the recorded parameters in the groups. For multiple comparisons, the significance level ($\alpha = 0.05$) was adjusted with Bonferroni correction, α/n , where n was the number of pairs in the multiple comparison. Statistical procedures were performed by the statistical software SPSS 20.0.0 (2011).

5. Cold tolerance evaluation of *O. pityocampae* immatures stored for 10 days at low temperatures from 15°C to 0°C: Fresh egg batches of *G. lineatum* (< 24h) were isolated in glass tubes (one per each tube) and exposed to *O. pityocampae* females for 24h parasitization at a 1:5 parasitoid/host ratio and standard rearing conditions (26±1°C, 75±5% RH and 16:8 L:D). One group of parasitized batches were subjected to the trials immediately after parasitization (24h) whereas other two group were held at the standard conditions until the 5th and 11th days. This provided three different development stages in parasitized eggs: immature larvae, mature larvae, and pupae. After each storage treatment, batches from every development stage were divided in two groups, transferred to separate climatic

chambers and held respectively at 26°C and 30 °C (75±5% RH and 16:8 L:D) until hatching. Two groups of parasitized batches were transferred to the same chambers at 26°C and 30 °C and served as controls. Treatments included:

- Direct transfer (DT) to 15 °C storage for 10 days and then DT to 26/30°C
- DT to 10 °C storage for 10 days and then DT to 26/30°C
- DT to 5 °C storage for 10 days and then DT to 26/30°C
- DT to 0 °C storage for 10 days and then DT to 26/30°C
- Control at 26°C
- Control at 30°C

This gave a total of 24 storage treatments. For each treatments 15 replicates (i.e., tubes) were carried out and the following parameters were recorded: no. of parasitized host eggs per batch, no. of egg stalks per batch, offspring production (no. of females emerged per batch), sex ratio (% females), and female development time.

5.a. Data analysis and statistics

Data distribution was analyzed by the Shapiro-Wilk test. Transformations were applied but failed to normalize the data. Kruskal–Wallis test followed by the Mann–Whitney U-test was then performed to compare the recorded parameters in the groups. For multiple comparisons, the significance level ($\alpha = 0.05$) was adjusted with Bonferroni correction, α/n , where n was the number of pairs in the multiple comparisons (Zar, 2010). All statistical procedures were performed by the statistical software SPSS 20.0.0 (2011).

6. Evaluation of the effects of cooling and heating rates on the survival of *O. pityocampae* immatures stored at different temperatures (15°C to 0°C):

Fresh egg batches of *G. lineatum* (< 24h) were isolated in glass tubes (one per each tube) and exposed to *O. pityocampae* females for 24h parasitization at a 1:5 parasitoid/host ratio and standard rearing conditions (26±1°C, 75±5% RH and 16:8 L:D). One group of parasitized batches underwent the trials immediately after parasitization (24h) whereas other two groups were held at the standard conditions until the 5th and 11th days. This provided three different development stages in parasitized eggs: immature larvae, mature larvae, and pupae. This time batches were randomly assigned to storage treatments that included specific cooling and heating rates applied before and after the storage period:

- A1) Cooling rate (CR1) of 0.05 min⁻¹ down to **10 °C**; Storage for 10 days; Heating rate (HR1) of 0.05 min⁻¹ up to 26°C; Hatching at 26°C and 30 °C
- A2) Cooling rate (CR2) of 0.005 min⁻¹ down to **10 °C**, storage for 10 days and heating rate (HR2) of 0.005 min⁻¹ up to 26°C; Hatching at 26°C and 30 °C

- B1) Cooling rate (CR1) of 0.05 min⁻¹ down to **5 °C**, storage for 10 days and heating rate (HR1) of 0.05 min⁻¹ up to 26°C; Hatching at 26°C and 30 °C (Only immature larvae)
- B2) Cooling rate (CR2) of 0.005 min⁻¹ down to **5 °C**, storage for 10 days and heating rate (HR2) of 0.005 min⁻¹ up to 26°C; Hatching at 26°C and 30 °C

- C1) Cooling rate (CR1) of 0.05 min⁻¹ down to **0 °C**, storage for 10 days and heating rate (HR1) of 0.05 min⁻¹ up to 26°C; Hatching at 26°C and 30 °C (Only immature larvae)
- C2) Cooling rate (CR2) of 0.005 min⁻¹ down to **0 °C**, storage for 10 days and heating rate (HR2) of 0.005 min⁻¹ up to 26°C; Hatching at 26°C and 30 °C

After each storage treatment, batches from every development stage were divided in two groups, transferred to separate climatic chambers and held respectively at 26°C and 30 °C (75±5% RH and 16:8 L:D) until hatching. Two groups of

parasitized batches were transferred to the same chambers at 26°C and 30 °C (75±5% RH and 16:8 L:D) and served as controls. A total of 28 storage treatments was carried out; for each treatment 15 replicates (i.e., tubes) were carried out and the following parameters recorded: no. of parasitized host eggs per batch, no. of egg stalks per batch, offspring production (no. of females emerged per batch), sex ratio (% females), and female development time.

6.a. Data analysis and statistics

Data were tested for normality with the Shapiro-Wilk test. Transformations were then applied but failed to normalize the data. Consequently Kruskal–Wallis test followed by the Mann–Whitney U-test was performed to compare the recorded parameters in the groups. For multiple comparisons, the significance level ($\alpha = 0.05$) was adjusted with Bonferroni correction, α/n , where n was the number of pairs in the multiple comparisons (Zar, 2010). Statistical procedures were performed by the statistical software SPSS 20.0.0 (2011).

Results & Discussion

1. Biological parameters of *O. pityocampae* reared on alternative host eggs

Biological parameters of *Ooencyrtus* spp. have often been investigated in the context of biological control. (Aung *et al.* 2010b) focused on the influence of temperature on egg maturation and longevity of *O. nezare*, a gregarious egg parasitoid of several hemipterans including the bean bug *Riptortus clavatus* (Hemiptera Alydidae). Results indicated that egg maturation increased with increasing female age. This trend reached a peak between day 5 and 10 days but then gradually declined with female aging. Egg maturation also increased with the raising of temperature and stopped only at 35C. The decline in egg production generally recorded at temperature extremes may be explained by the lower number of ovarioles produced at high temperatures and the decreased rates of oogenesis at low temperatures (Aung *et al.* 2010b, Huey *et al.*,1995)

In another study the relationship between *O. nezare* female age and parameters such as progeny production and sex ratio was evaluated under laboratory conditions. The outcomes highlighted how the percentage of parasitism and the number of offspring were significantly lower in old females. Moreover the optimal age for *O. nezarae* parasitization appeared to range between 1–4 days, while sex ratio and clutch size were not affected by female age. According to the same findings the reproductive potential of 4–day–old and 1–day–old parasitoids was significantly higher than 20–day–old ones and an increased number of progeny could be produced by 4-days-old females within short periods (Aung, *et al.* 2010a).

Some authors made a more general point, stressing that the oogenesis of *Ooencyrtus* spp. is not complete at adult emergence (Hinton 1981, Battisti *et al.*,1990). In fact when this process was investigated in *O. johnoni* and *O. kuwanae* by Maple (1937) and Weseloh (1986) respectively, evidence was provided that the quantity of mature eggs soon after the emergence is low. Only after several days the number of eggs was reported to increase .

In *O. pityocampae* fecundity depends on the process of oogenesis and oosorption. This mechanism investigated by Battisti *et al.* (1990) is likely to help parasitoid females when facing harsh environmental conditions. The same authors, analyzing the variation in *O. pityocampe* egg number, found out that it was consistent with the one described by Weseloh (1986) for *O. kuwanae*. From the results appeared evident that the number of mature eggs peaked in 10-days old females. Moreover the presence of host eggs clearly affected the mechanism of oogenesis and oosorption. This phenomenon is of key importance in parasitoid host searching strategies allowing the wasps to remain fertile for long times even in the absence of their hosts (Battisti *et al.*, 1990).

Our results (Table 1), while not exhaustive, tend to confirm the findings on *Ooencyrtus* spp. In fact, female life span was about 45 days on average and parasitoid development took about 16 days. Data showed an evident trend of parasitization and offspring production between day 4 and 5 of parasitoid life and a gradual decline until day 28. As in the previous studies, the peak of parasitization occurred several days after parasitoid hatching. Overall, the results of parasitization and progeny production were particularly coincident with those of *O. nezarae* whose reproductive potential was higher in 4-day-old females than in 20-day-old ones (Fig.1. and Fig.2) .

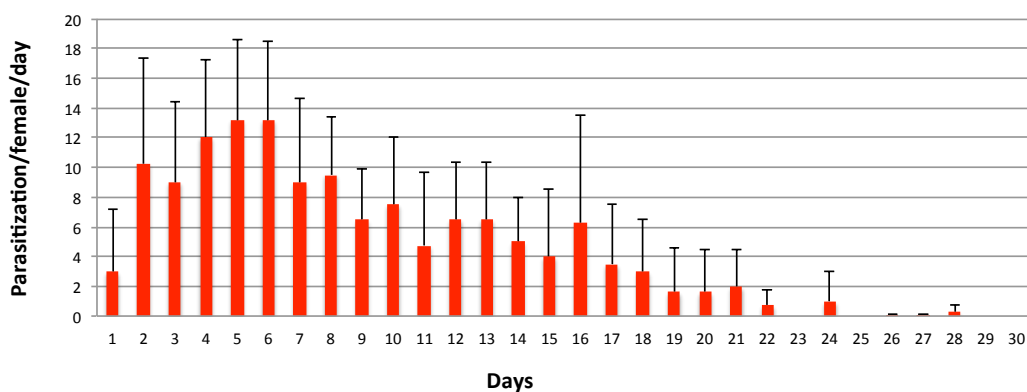


Fig .1. Number of parasitized eggs per female per day (means \pm S.D.) at standard rearing conditions (26°C, 75% RH and 16:8 L:D).

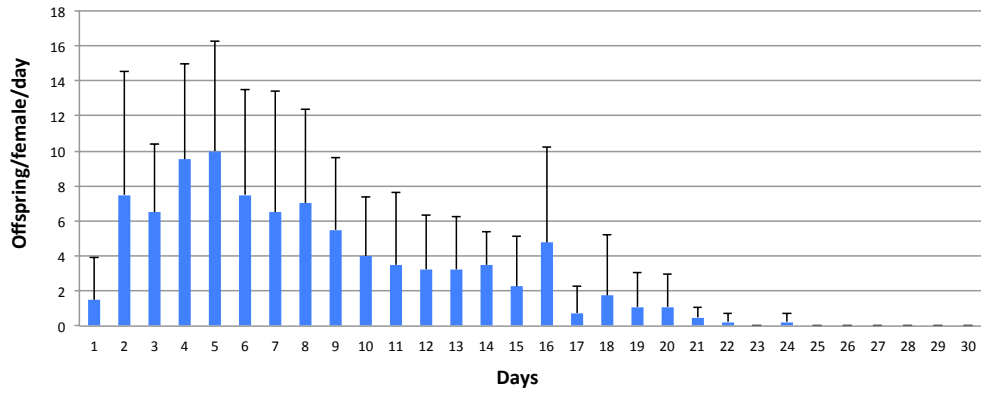


Fig .2. Number of offspring produced from eggs parasitized by each single female in a day (means \pm S.D.) at standard rearing conditions (26°C, 75% RH and 16:8 L:D).

Parameters	Mean value (\pm S.D.)
Adult female longevity (days)	48.25 \pm 9.32
Ovipositional period (days)	27.50 \pm 11.70
Post-reproductive period (days)	20.75 \pm 14.82
Total progeny	90.25 \pm 41.82
Mean development time female (days)	16.23 \pm 0.48
Mean development time male (days)	15.66 \pm 0.47
Sex ratio (% females)	99.05 \pm 1.42

Table 1. Summary of life history traits (means \pm S.D.) of *O. pityocampae* adult females reared on *N. viridula* eggs at 26 \pm 1 °C, 75 \pm 5% RH and 16:8 L:D and fed with honey.

2. Identification by morphology and measurements of *O. pityocampae*

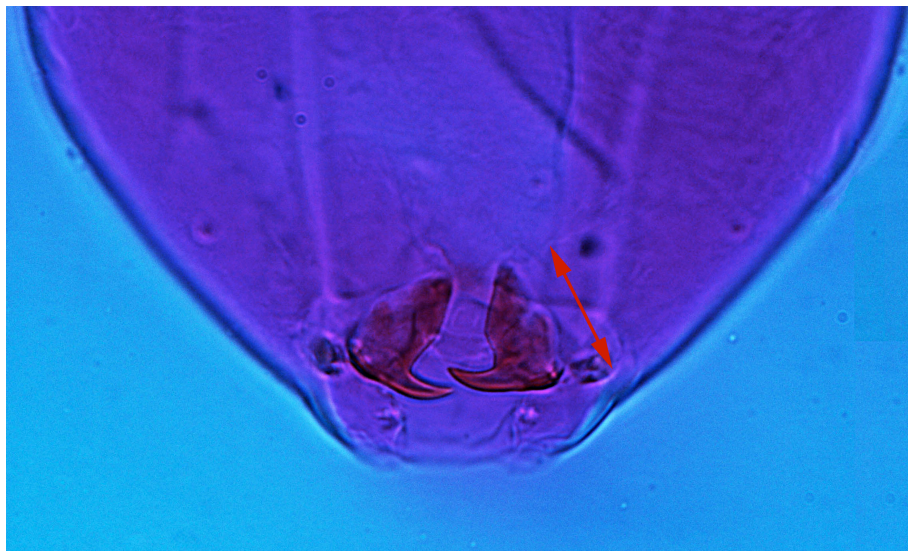
immature stages:

Larval instars of *Ooencyrtus spp.* have been thoroughly investigated in the last decades but the number of identified stages is not always consistent in published literature. *Ooencyrtus kuvanae* was initially reported to have three larval instars by Crossman (1925). However a more detailed investigation of mandibles revealed the presence of five distinct stages Parker (1933). In a 1937 study on the biology of *O. johnsoni*, Maple identified three larval instars by analyzing a series of parameters including the shape of mandibles and their sclerotized teeth. Number of larval stages were also investigated in *O. trinidadensis* Crawford and *O. patriciae* Subba Rao by Gerling et al., (1976) and Matteson (1981) respectively, and three distinct larval instars observed. However their findings were not based on mandible analysis. By contrast Takasu and Hirose (1989) focused exclusively on mandible analysis for the detection of *O. Nezarae* larval stages claiming that other criteria such as shape and size of larval body were not sufficiently reliable for a correct identification. Our results are consistent with the findings of Takasu and Hirose showing that some of the parameters selected for the identification of immatures such as body length and width, head width and segmentation were inadequate to define all larval instars. In fact at the conditions tested in our analysis only mandible length could successfully define five larval stages that were identified by frequency distribution analysis. Descriptive statistics of mandible length are reported in Table 2. In *Ooencyrtus* larvae, mandibles of previous instars are generally reported to remain on their skin embedded in anal shields after each molt is completed (Parker, 1933; Maple 1937; Takasu and Hirose, 1989). In our investigation 3rd instar mandibles were often visible, attached on their exuviae, in the middle of fourth instar larval body. However anal shields with embedded mandibles from preceding instars were never clearly observed as they were described by Maple (1937) for *O. johnsoni*. After the staining procedure all mandibles can be easily detected by microscopic observation except for those of the first instar whose measurements cluster around 3 μ and are thus more likely to be overlooked. Therefore our findings, though not conclusive, tend to confirm the hypothesis of Takasu and Hirose who

argued that the difficulty of detecting first instar mandibles may have led scientists in the past to draw misleading conclusions on the real number of stages. Therefore, according to the same authors the presence of 5 larval instars might represent a general rule in all *Ooencyrtus* spp.

Instar	Mean value ($\mu \pm$ S.D.)
1 st Instar	3.2 \pm 0.6
2 nd Instar	10.1 \pm 0.7
3 rd Instar	14.2 \pm 0.6
4 th Instar	19.4 \pm 0.9
5 th Instar	25.5 \pm 0.5

Table 2. Mandible length of each *O. pityocampae* larval instar



4th instar mandibles: mandibles were the most suitable character for measurements and analysis. The red arrow measures 20.05 μ

3. Evaluation of the effects of the host egg age on *O. pityocampae*

parasitization:

In laboratory tests *O. pityocampae* accepted and parasitized *L. occidentalis* host eggs of each age class and completed its development either in newly oviposited host eggs (<24h) or in eggs containing pharate nymphs close to hatching. No statistically significant differences among the seven age classes were observed in the no. of parasitized host eggs per batch (Kruskal-Wallis: $H = 9.21$; $df = 6$; $n = 89$; $P > 0.05$), no. of egg stalks per batch (Kruskal-Wallis: $H = 9.87$; $df = 6$; $n = 89$; $P > 0.05$), offspring production (Kruskal-Wallis: $H = 10.74$; $df = 6$; $n = 89$; $P > 0.05$) and sex ratio (Kruskal-Wallis; $H = 12.44$; $df = 6$; $n = 86$; $P > 0.05$). In contrast development time of immature *O. pityocampae* females was significantly affected by host egg age and increased in older *L. occidentalis* eggs from $14.85 \pm 0.09SE$ days on 24h eggs to $17.02 \pm 1.25 SE$ days on 7-days eggs (Kruskal-Wallis: $H = 15.24$; $df = 6$; $n = 62$; $P < 0.05$) (Table 1). In addition host egg age was not significantly correlated with no. of parasitized host eggs per batch (Spearman's $\rho = -0.009$, $n = 89$ $P = 0.930$), no. of egg stalks per batch (Spearman's $\rho = 0.049$, $n = 89$, $P = 0.651$), offspring production per batch (Spearman's $\rho = 0.050$, $n = 89$ $P = 0.644$) and sex ratio (Spearman's $\rho = -0.161$, $n = 84$, $P = 0.142$). On the contrary a significant correlation was detected between host egg age and female development time (Spearman' $\rho = 0.301$, $n = 62$, $P = 0.017$).

After parasitoid emergence, the residues remaining inside the egg shell of *L. occidentalis* were analyzed and some differences noticed (Fig. 3a). When the coreid eggs had been parasitized soon after the oviposition only few fecal pellets could be detected (Fig. 3a.2). On the contrary when parasitization had occurred at the end of embryo development a high quantity of fecal pellets could be observed (Fig. 3a.4). In figure 3a.3, on a 7-days old dissected host egg, the remains of a *L. occidentalis* nymph adherent to the inner side of the chorion can be observed in transparency on the right side of an *O. pityocampae* hatching hole.

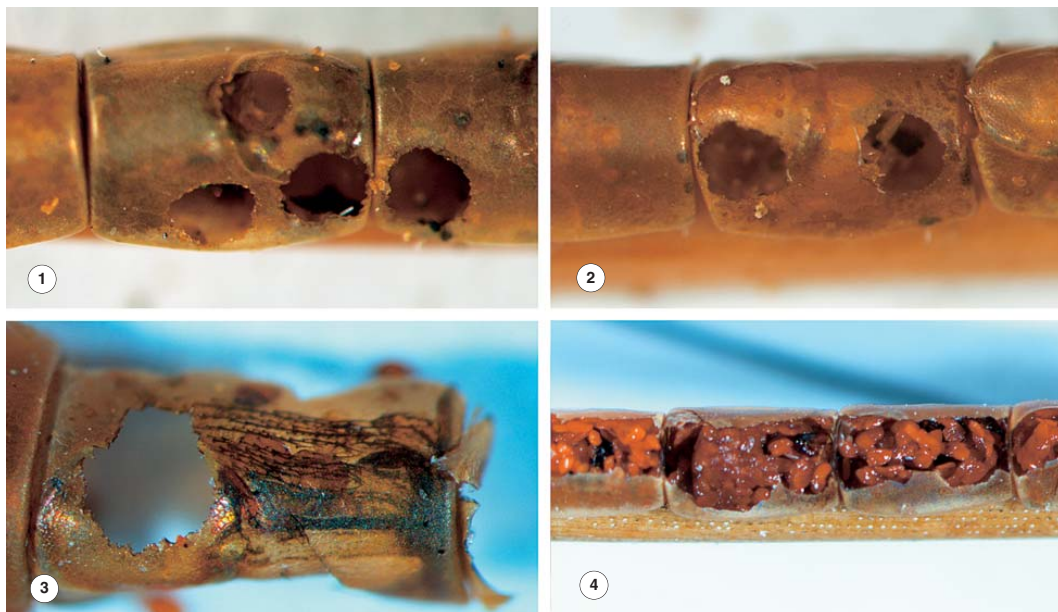


Fig. 3a. *Leptoglossus occidentalis* Heidemann eggs parasitized by *Ooencyrtus pityocampae* (Mercet): (1, 2) 1-day old parasitized eggs with parasitoid hatching holes and few fecal pellets visible inside the egg shell; (3) 7-days old parasitized eggs with sclerotized parts of a *L. occidentalis* nymph impressed on the inner side of the egg chorion; (4) 7-days old parasitized eggs dissected to show the several fecal pellets produced by developing parasitoids (parasitization occurred when host eggs contained pharate nymphs close to hatching).

Table 1. Mean no. ($\pm SE$) of parasitized eggs/batch, egg stalks/batch (means $\pm SE$), offspring production (means $\pm SE$), female development time (days, means $\pm SE$) and sex ratio (% of females $\pm SE$) of *Ooencyrtus pityocampae* on *Leptoglossuss occidentalis* host eggs of different ages.

Parameter	Host egg age						
	< 24h	2 d	3 d	4 d	5 d	6 d	7 d
No. of parasitized eggs	3.85 (± 0.79)a	3.2 (± 0.94)a	3.87 (± 1.01)a	3.27 (± 1.22)a	5.77 (± 0.77)a	4.55 (± 1.46)a	2.5 (± 1.04)a
No. of egg stalks	4.35 (± 1.05)a	4.33 (± 1.43)a	4.27 (± 1.32)a	4.45 (± 1.78)a	7.85 (± 1.22)a	5.64 (± 1.72)a	4.2 (± 2.04)a
Offspring production	4.35 (± 1.05)a	4.33 (± 1.43)a	3.87 (± 1.31)a	4.45 (± 1.78)a	7.77 (± 1.23)a	5.64 (± 1.72)a	3.8 (± 1.77)a
Female development time (d)	14.85 (± 0.09)a	14.67 (± 0.16)ab	15.01 (± 0.28)ab	15.85 (± 0.68)ab	14.79 (± 0.10)ab	15.20 (± 0.22)ab	17.02 (± 1.25)b
Sex ratio (% of females)	100a	100a	100a	84.53 (± 8.90)a	96.95 (± 2.57)a	100a	95.83 (± 4.16)a
No. of parasitized eggs	3.85 (± 0.79)a	3.2 (± 0.94)a	3.87 (± 1.01)a	3.27 (± 1.22)a	5.77 (± 0.77)a	4.55 (± 1.46)a	2.5 (± 1.04)a

Means in the same row followed by the same letters are not significantly different (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/21$).

Egg-parasitoid strategies for exploiting variable host resources, including variation in the quality of a host egg over time, were described by Vinson (1998). The parasitization pattern of encyrtids on old host eggs was investigated by Takasu and Hirose (1993) for *Ooencyrtus nezarae* Ishii. After exposing *Riptortus clavatus* Thunberg (Heteroptera Coreidae) eggs to females of *O. nezarae*, a decrease in number of laid eggs with increasing host age was observed. By contrast progeny survival, development time and size of emerged adults did not show significant differences among host ages except for a reduced *O. nezarae* survival rate in 7-days old eggs. The quantity of host material suitable for *O. nezarae* juvenile development seemed to decrease with increasing host age because larvae can not feed on sclerotized parts that remain in the host after adult hatching.

A similar parasitization pattern was also observed by Nechols *et al.* (1989) for another undetermined *Ooencyrtus* species and, to a lesser extent, for *Ooencyrtus anasae* Ashmead, whose responses to *Anasa tristis* (DeGeer) (Heteroptera Coreidae) host egg age were more variable. Nevertheless in the oldest (9-days old) *A. tristis* host eggs, lower rates of parasitization, longer development times, increased immature mortality and female offspring reduction were recorded for both parasitoid species.

In other studies the combined effects of parasitoid age and host egg age on female performance were highlighted by Hofstetter and Raffa (1998) who carried out a trial in which egg masses of different ages oviposited by the lepidopteran *Limantria dispar* (L.) were exposed for parasitization to females of *Ooencyrtus kuvanae* (Howard) (Hymenoptera Encyrtidae). As a result offspring production and proportions of females decreased when *O. kuvanae* immatures developed in older *L. dispar* eggs.

O. pityocampae showed a marked flexibility in the exploitation of the new host as most of the parameters considered were not influenced by the age of *L. occidentalis* eggs that ranged from fresh eggs to eggs presenting completely developed nymphs close to hatching. Infact data such as the number of parasitized eggs and sex ratio were not significantly affected by differences in host egg age. Only *O. pityocampae* females developing in older eggs took longer in

reaching adulthood, highlighting how in older host eggs the conversion of free nutrients into a more complex substrate (e.g., chitinized tissues) may partially impair both food quality and food availability (Vinson, 1998).

Our data on *O. pityocampae* parasitization of *L. occidentalis* eggs suggest a departure of this species from *Ooencyrtus* sp. general trend except for juvenile development time where results were more consistent with those recorded for *O. anasae*.

These findings may represent an adaptation to effective exploitation of older eggs in egg-parasitoids that target hosts whose eggs remain viable in nature only for a short period or show a rapid embryonic development followed by a more or less long lasting larval and pharate nymph stage. This phenomenon is evident in both *L. occidentalis* and *Thaumetopoea processionea* (L.) (Bin and Tiberi, 1983).

In conclusion, recent studies on egg-parasitoids have focused on host egg age as a key factor for parasitization and in several works a reduced parasitoid acceptance of older batches and a decreased suitability of older eggs over fresh ones were often reported. This trend despite being widespread among many different egg-parasitoid families (see Nechols *et al.*, 1989; Vinson, 1998) did not apply to *O. pityocampae* reared on *L. occidentalis* at the conditions tested in our experiment. On the contrary *O. pityocampae* parasitization pattern was more consistent with the trend described by Sabbatini Peverieri *et al.*, (2013) for *G. pennsylvanicum* parasitization of *L. occidentalis*.

4. Temperature-dependent egg to adult development of *O. pityocampae* within eggs of the alternative host *Graphosoma lineatum*:

The impact of several constant temperatures on survival, development, and adult longevity of *Venturia canescens* Gravenhorst (Hymenoptera: Ichneumonidae) parasitizing larvae of *Plodia interpunctella* Hubner (Lepidoptera: Pyralidae) were recently investigated by (Spanoudis & Andreadis 2011). After the exposure, parasitized larvae of *P. interpunctella* were transferred to incubators set at the following temperatures: 15, 17.5, 20, 22.5, 25, 27.5, 30, and 32.5°C. Percentage of parasitoid emergence at each temperature and time to adult eclosion were recorded. Results showed a significantly increased survival of *V. canescens* at 25 and 27.5°C compared to 17.5, 20, 30, and 32.5°C whereas no *V. canescens* specimen was able to complete development at 15°C. The increasing temperature resulted in a significant decrease of development time within the range of 17.5–27.5°C while maximum and minimum threshold temperatures for total development were estimated at 36.2 and 13.2°C, respectively.

In a similar study (Voss et al. 2010) evaluated the influence of temperature and host on the development of the forensically relevant parasitoid *Tachinaephagus zealandicus* Ashmead (Hymenoptera: Encyrtidae). *T. zealandicus* was able to complete development between 15°C and 27°C on five species of diptera Calliphoridae, *Calliphora albifrontalis* Malloch, *Calliphora dubia* Macquart, *Lucilia sericata* Meigen, *Chrysomya rufifacies* Macquart and *Chrysomya megacephala* Fabricius whereas parasitoids failed to hatch from any of the host species reared at 30°C. Temperature and host species significantly affected development time, emergence rate and size of progeny. Developmental time significantly increased in *Ch. megacephala* and *Ch. rufifacies* at 18–24°C and in *Ch. rufifacies* and *C. albifrontalis* at 15°C and 27°C while adult emergence rate was higher at 21°C, decreased at the temperature extremes (15°C and 27°C) and significantly declined on *Ch. megacephala* and *Ch. Rufifacies*.

The effect of temperature on the overwintering and development of *Anagyrus pseudococci* (Girault) was also studied by (Daane et al. 2004) in the context of pest management strategies against *Planococcus ficus* (Signoret), an insect responsible for serious damages to Californian vineyards. The following eight constant temperatures were tested (12, 14, 17, 22, 27, 32, 34, and 36 °C), and results showed that *A. pseudococci* managed to complete development (egg to adult hatching) from 14 to 34 °C. Developmental times ranged from 79.1 ± 1.0 days (14 °C) to 10.2 ± 0.3 days (34 °C). The same authors compared such laboratory-derived temperature relationships to *A. pseudococci* populations monitored in the field. The outcomes of these studies suggest that cues different from temperature are employed to synchronize *A. pseudococci* adult eclosion with the field presence of vine mealybug.

More recently, the influence of temperature on the development of *Ooencyrtus kuwanae*, an egg parasitoid of *L. dispar*, was investigated by (JianJun et al. 2012) in order to improve its mass rearing for biological control. To this end the following constant rearing temperatures were selected: 16,20,28,32°C ($\pm 0.5^\circ\text{C}$). After that *Antherea pernyi* Guerine Meneville (Lepidoptera Saturniidae) was chosen as alternative host for testing *O. kuwanae* physiological response. The quality of emerged offspring was then compared with other groups of individuals hatched from the natural host *L. dispar*. The results indicated that the developmental threshold was $10.50 \pm 1.41^\circ\text{C}$ and that offspring produced on the alternative host eggs was suitable for field release as effective pest control agents against *L. dispar*.

Though not conclusive, the results of our experiment on *O. pityocampae* reared within host eggs of *G. lineatum* indicated trends consistent with those recorded in literature. The analysis of *O. pityocampae* emergence data showed no statistically significant differences between groups except for the one reared at 36°C that was consistently affected by the increased temperature (K-W: $H=20.01$; $df=3$; $n=118$; $P=0.0001$) Fig. 3. On the other hand, data concerning the relationship between temperature and *O. pityocampae* developmental times seem to agree particularly with trends recorded for *Anagyrus* spp. In fact evaluation of egg-to-adult temperature-dependent development showed that

parasitoid developmental times gradually decreased reaching about 9 day at 36°C. By contrast an increase up to more than 30 days could be observed at 19°C (K-W: $H= 78.39$; $df= 3$; $n= 89$; $P= 0.0001$) Fig.4.

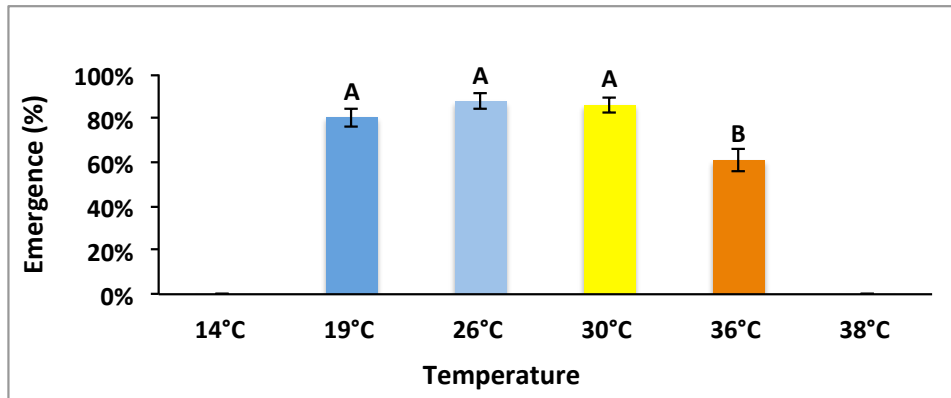


Fig. 3. Percentage of emergence (means \pm S.E.) of *O. pityocampae* immatures developing at different constant temperatures within eggs of *Graphosoma lineatum* (75 \pm 5% RH and 16:8 L:D) Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/6$).

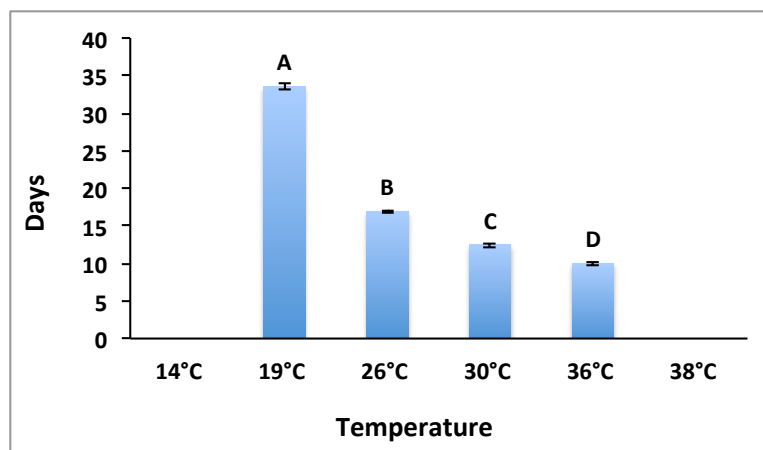


Fig. 4. Days of development (means \pm S.E.) of *O. pityocampae* immatures developing at different constant temperatures within eggs of *Graphosoma lineatum*. (75 \pm 5% RH and 16:8 L:D). Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/6$).

5. Cold tolerance evaluation of *O. pityocampae* immatures stored for 10 days at low temperatures from 15°C to 0°C:

As it was previously described, a general pattern in parasitoid storage is that mortality tends to increase when conservation temperature decreases (e.g. Bueno and Van Cleave, 1997; Lacey et al., 1999; Lysyk, 2004; Lopez and Botto, 2005; Pandey and Johnson, 2005; Luczynski et al., 2007; Bernardo et al., 2008).

Since a wide variability in cold tolerance is reported among different life stages, a generalization of this pattern to parasitoid genera/species is often impossible as exceptions often occur. However the determination of the most cold-tolerant developmental stage is of key importance in the design of cold storage protocols (Colinet and Boivin, 2011). Low temperature conservation of adults may produce a marked reduction in parasitoid fitness. Thus, storing juvenile stages is often reported to be a better strategy, particularly when short-term storage is planned (van Lenteren and Tommasini, 2002).

Because pupae are an immobile and often well-protected stage, they are generally selected for low temperature conservation. Nevertheless care must be taken to organize a proper storage protocol, since pupae, though theoretically inactive, are yet a metabolically very active stage. In fact during this period larval tissues undergo histolysis to be then reassembled in the adult shape. This phenomenon is responsible for the wide variability in cold storage tolerance within this peculiar stage (Colinet & Boivin 2011)

Our results generally agree with trends described in literature. In fact statistically significant differences were observed in the percentage of emergence of *O. pityocampae* held at 26°C, 75 ± 5% RH and 16:8 L:D after 10-days storage of **immature larvae** at the following constant temperatures: 15 °C, 10 °C, 5 °C, 0 °C (75 ± 5% RH and 16:8 L:D) (Kruskal-Wallis: $H = 72.29$; $df = 4$; $n = 111$; $P = 0.0001$) (Fig 5.1). Multiple comparisons showed a marked significant decline in adult emergence between parasitized batches stored at 15°C and all the other temperature treatments. Immature larvae were particularly affected by

the storage of batches at 5°C. By contrast no significant difference were found between the 15°C treatment and the control group where egg-to-adult development occurred at **26°C**, 75 ± 5% RH and 16:8 L: D.

Similar results were obtained when parasitoids were transferred to 30°C after being subjected to the same treatments (Kruskal-Wallis: $H = 75.97$; $df = 4$; $n = 113$; $P = 0.0001$) (Fig 5.2). Even in this case comparisons showed a pronounced decline in adult emergence between batches stored at 15°C and all the other temperature treatments. On the contrary no significant difference was found between the 15°C treatment and the control group where egg-to-adult development occurred at **30°C**, 75 ± 5% RH and 16:8 L: D.

Mature larvae and **pupae** were stored at the conditions described above and then each stage divided and transferred to 26°C and 30°C.

After storage, **mature larvae** held at **26°C** showed significant differences in emergence (Kruskal-Wallis: $H = 52.55$; $df = 4$; $n = 93$; $P = 0.0001$) (Fig 5.3).

Multiple comparisons revealed a decline in parasitoid survival that dropped dramatically for *O. pityocampae* stored at 0°C. No significant differences were recorded between control group and parasitoids stored at 15°C, 10°C.

Likewise mature larvae transferred to **30°C** were significantly different (Kruskal-Wallis: $H = 59.26$; $df = 4$; $n = 92$; $P = 0.0001$) (Fig 5.4). Multiple comparisons defined a trend in parasitoid survival similar to that described above.

Similarly, **pupae** reared either at **26°C** or **30°C** after storage, presented significant differences in emergence (Kruskal-Wallis: $H = 26.53$; $df = 4$; $n = 94$; $P = 0.0001$) (Fig 5.5) and (Kruskal-Wallis: $H = 37.26$; $df = 4$; $n = 91$; $P = 0.0001$) (Fig 5.6) respectively. This time the reduction in parasitoid survival associated with temperature decrease was less pronounced. At the conditions tested in our experiments pupae resulted to be the most cold tolerant stage confirming the findings and general trend of published literature.

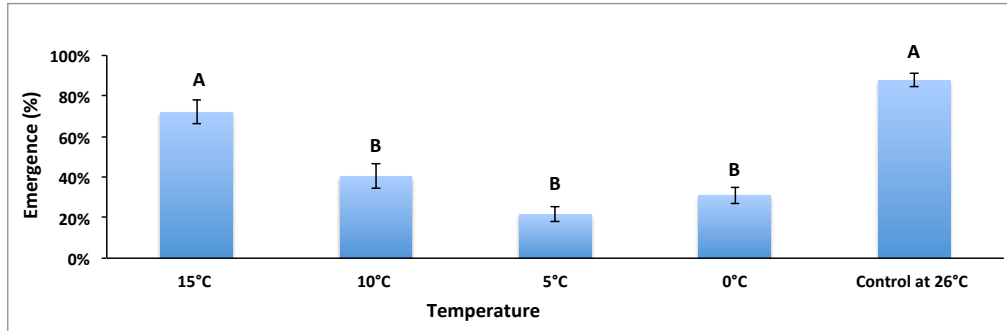


Fig.5.1 . Percentage of emergence (means \pm S.E.) of *O. pityocampae* adults at 26°C, 75 \pm 5% RH and 16:8 L:D after 10 days storage of **immature larvae** at the following constant temperatures 15 °C, 10 °C, 5 °C, 0 °C (75 \pm 5% RH and 16:8 L:D) within eggs of the host *Graphosoma lineatum*.

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; P < 0.05/10).

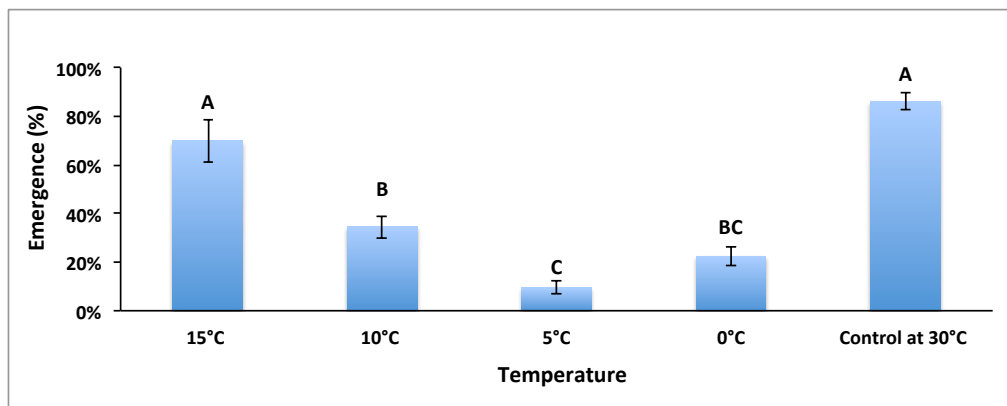


Fig.5.2 . Percentage of emergence (means \pm S.E.) of *O. pityocampae* adults at 30°C, 75 \pm 5% RH and 16:8 L:D after 10 days storage of **immature larvae** at the following constant temperatures 15 °C, 10 °C, 5 °C, 0 °C (75 \pm 5% RH and 16:8 L:D) within eggs of the host *Graphosoma lineatum*.

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; P < 0.05/10).

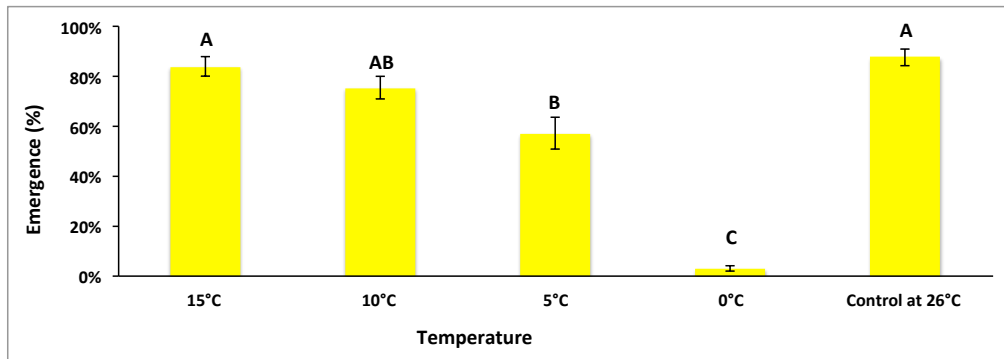


Fig. 5.3. Percentage of emergence (means \pm S.E.) of *O. pityocampae* adults at 26°C, 75 \pm 5% RH and 16:8 L:D after 10 days storage of **mature larvae** at the following constant temperatures 15 °C, 10 °C, 5 °C, 0 °C (75 \pm 5% RH and 16:8 L:D) within eggs of the host *Graphosoma lineatum*.

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/10$).

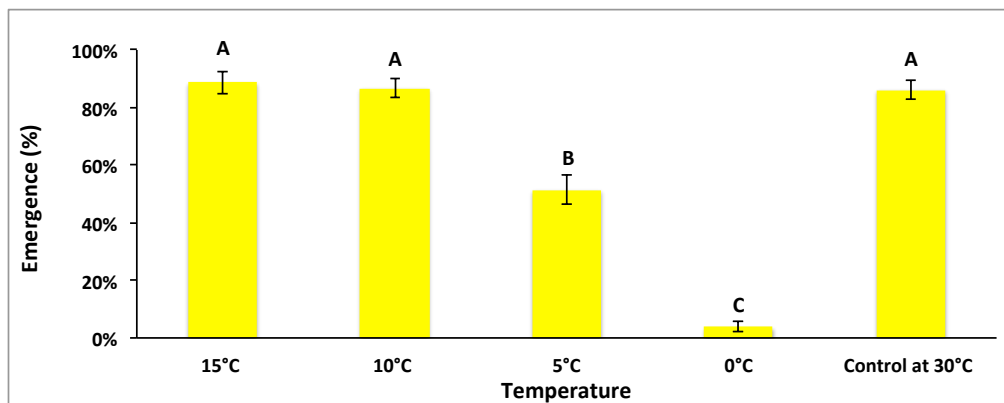


Fig. 5.4. Percentage of emergence (means \pm S.E.) of *O. pityocampae* adults at 30°C, 75 \pm 5% RH and 16:8 L:D after 10 days storage of **mature larvae** at the following constant temperatures 15 °C, 10 °C, 5 °C, 0 °C (75 \pm 5% RH and 16:8 L:D) within eggs of the host *Graphosoma lineatum*.

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/10$).

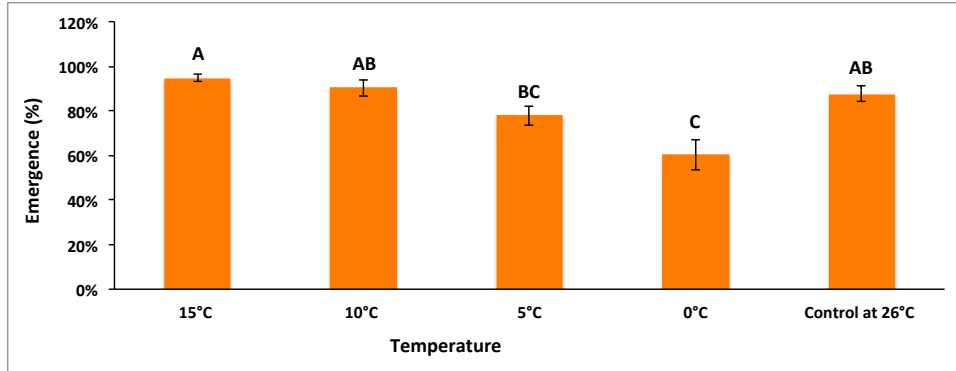


Fig. 5.5. Percentage of emergence (means \pm S.E.) of *O. pityocampae* adults at 26°C, 75 \pm 5% RH and 16:8 L:D after 10 days storage of **pupae** at the following constant temperatures 15 °C, 10 °C, 5 °C, 0 °C (75 \pm 5% RH and 16:8 L:D) within eggs of the host *Graphosoma lineatum*.

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/10$).

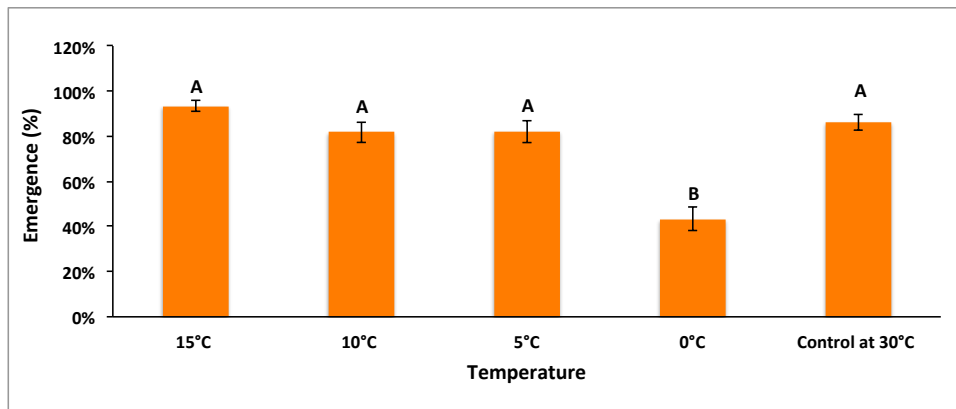


Fig. 5.6. Percentage of emergence (means \pm S.E.) of *O. pityocampae* adults at 30°C, 75 \pm 5% RH and 16:8 L:D after 10 days storage of **pupae** at the following constant temperatures 15 °C, 10 °C, 5 °C, 0 °C (75 \pm 5% RH and 16:8 L:D) within eggs of the host *Graphosoma lineatum*.

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/10$).

6. Evaluation of the effects of cooling and heating rates on the survival of *O. pityocampae* immatures stored at different temperatures (15°C to 0°C):

In *D. melanogaster* (Kelty & Lee 1999) found out that cooling at ecologically relevant rates (0.05 and 0.1°C min⁻¹) significantly increased cold hardiness compared with cooling at higher rates (0.5, 1.0, 1.5°C min⁻¹) and direct transfer. The improved cold tolerance of *D. melanogaster* cooled at slower rates is likely due to the increased time spent by these flies at protection-inducing temperatures. These results were consistent with the findings of Coulson and Bale (1990) who showed that survival of house fly (*Musca domestica*) pupae cooled at constant rates from 27 to -7°C increased with decreasing cooling rate. Levie *et al.*, (2005) showed that mummies of *A. rhopalosiphi* that were subjected to direct transfer from room temperature to -5 presented a higher mortality rates compared with those submitted to temperature treatments by using step temperature reduction. Nevertheless cold storage negatively influenced parasitoid fitness compared with the control that developed at room temperature. In fact, after temperature treatments, fecundity was reported to decrease for 3-d-old mummies in all treatments and for 1-d-old mummies with no previous acclimation. This fact may lead to reduced control efficiency after field release of cold-stored parasitoids. However, juvenile acclimation generally improved survival and fecundity as recorded by Polgar (1986) for the same parasitoid. In *Aphidius matricariae* Haliday stored at 8°C after acclimation, emergence rate was reported to increase by Shalaby and Rabasse (1979). Similar findings were observed by Bueno and van Cleave (1997) for *Aphelinus perpallidus* Gahan (Hymenoptera Aphelinidae) and by Singh and Srivastava (1988) for *Trioxys indicus* Subba Rao & Sharma (Hymenoptera Braconidae).

Our results showed statistically significant differences in the emergence of *O. pityocampae* transferred to 26°C, 75 ± 5% RH and 16:8 L:D after **immature larvae** were subjected for 10 days to the following temperature treatments: Storage by direct transfer (DT) at 10°C, storage at 10°C and cooling/heating rate of 0.05 min⁻¹ (CR1/HR1), storage at 10°C and cooling/heating rate of 0.005 min⁻¹ (CR2/HR2) (Kruskal-Wallis: H = 58.62; df = 3; n = 88; P = 0.0001) (Fig 6.a). Likewise similar results were obtained when immature larvae were transferred

to 30°C after being subjected to the same treatments (Kruskal-Wallis: $H = 62.15$; $df = 3$; $n = 89$; $P = 0.0001$) (Fig 6.b). These findings suggest that when immature larvae were submitted directly to 10°C for the 10 days storage, a sharp decline in adult emergence compared with the control took place. On the contrary, a positive significant effect of the slower 0.005 min⁻¹ CR2/HR2 on parasitoid survival was observed whereas the steady 0.05 min⁻¹ CR2/HR2 did not provide any significant benefit. When parasitized batches with immature larvae were transferred to 26°C, the CR2/HR2 group resulted to be lower in emergence than the control (Fig.6a). On the other hand when batches from the same treatment were moved to 30°C no significant difference in emergence was observed compared with the control (Fig.6.b).

These outcomes seem consistent with published research highlighting the positive effect exerted by acclimation on insect survival after conservation at low temperatures. However as Colinet and Boivin, (2011) pointed out, care must be taken to draw conclusions since the effects of acclimation might be partly counterbalanced by a development effect occurring when individuals during acclimation reach stages that display different temperature tolerance. When thermal acclimation concerns parasitoid juvenile stages, a combination of reversible and developmental acclimation might actually affect phenotypic changes (Anguilletta, 2009)

In the same set of experiments **Mature larvae** and **pupae** were stored at the conditions described above and then each stage divided and transferred to 26°C and 30°C.

Mature larvae reared at 26°C showed significant differences in emergence (Kruskal-Wallis: $H = 13.27$; $df = 3$; $n = 76$; $P = 0.004$) (Fig 6.c). This time results presented no significant differences between treatment groups and only the control group resulted to be significantly higher than the direct transfer group. Similarly, batches containing mature larvae moved to 30°C presented significant difference in hatching (Kruskal-Wallis: $H = 12.95$; $df = 3$; $n = 78$; $P = 0.004$) (Fig 6.d). However multiple comparisons evidenced a significant variation in adult emergence only between CR2/HR2 group and the control.

On the other hand, **pupae** reared at **26°C** showed no significant difference in emergence (Kruskal-Wallis: $H = 0.489$; $df = 3$; $n = 77$; $P = 0.924$) (Fig 6.e) whereas those reared at **30°C**, presented significant variations (Kruskal-Wallis: $H = 8.53$; $df = 3$; $n = 76$; $P = 0.034$) (Fig 6.f). Pupae subjected to storage without acclimation presented a borderline significant reduction in survival compared with those acclimated by (CR1/HR1), but no difference with the (CR2/HR2), group and the control.

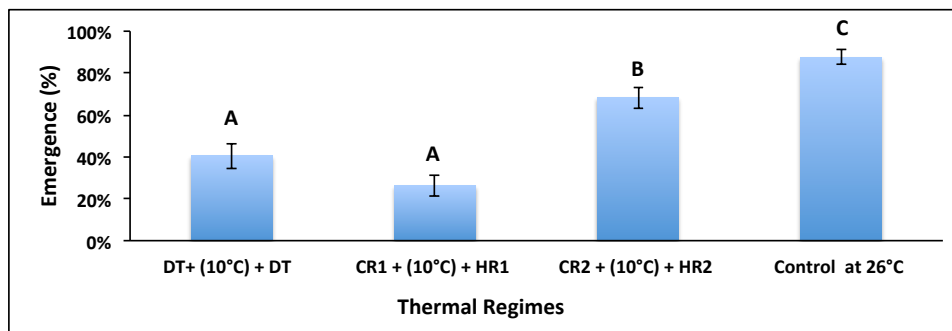


Fig. 6.a. Percentage of emergence (means \pm S.E.) of *O. pityocampae* after 10 days storage of **immature larvae** within eggs of the host *Graphosoma lineatum* ($75 \pm 5\%$ RH and 16:8 L:D). Immature larvae were subjected to the following temperature treatments: Storage by direct transfer (DT) at 10°C, storage at 10°C and cooling/heating rate of 0.05 min^{-1} (CR1/HR1), storage at 10°C and cooling/heating rate of 0.005 min^{-1} (CR2/HR2). In the control group egg-to-adult development occurred at standard conditions (26°C and 30°C, $75 \pm 5\%$ RH and 16:8 L: D).

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/6$).

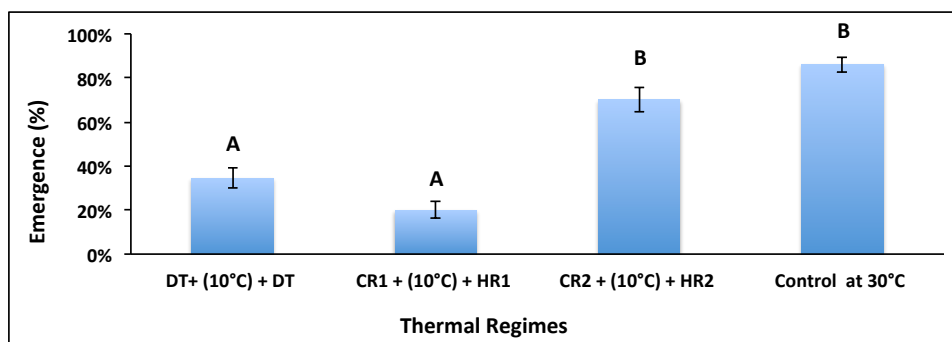


Fig. 6.b. Percentage of emergence (means \pm S.E.) of *O. pityocampae* adults after 10 days storage of **immature larvae** within eggs of the host *Graphosoma lineatum* ($75 \pm 5\%$ RH and 16:8 L: D). Larvae were subjected to the same temperature treatments described in Fig. 6.a. In the control **immature larvae** were reared at standard conditions (30°C, $75 \pm 5\%$ RH and 16:8 L: D).

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/6$).

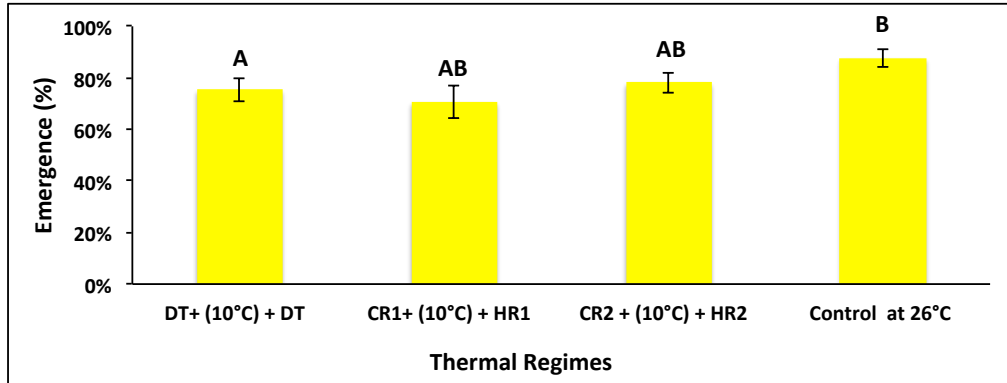


Fig.6.c. Percentage of emergence (means \pm S.E.) of *O. pityocampae* adults was analysed after 10 days storage of **mature larvae** within eggs of the host *Graphosoma lineatum* (75 \pm 5% RH and 16:8 L: D). Mature larvae were subjected to the following temperature treatments: Storage by direct transfer (DT) at 10°C, storage at 10°C and cooling/heating rate of 0.05 min⁻¹ (CR1/HR1), storage at 10°C and cooling/heating rate of 0.005 min⁻¹ (CR2/HR2). In the control **mature larvae** were reared at standard conditions (26°C, 75 \pm 5% RH and 16:8 L: D).

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; P < 0.05/6).

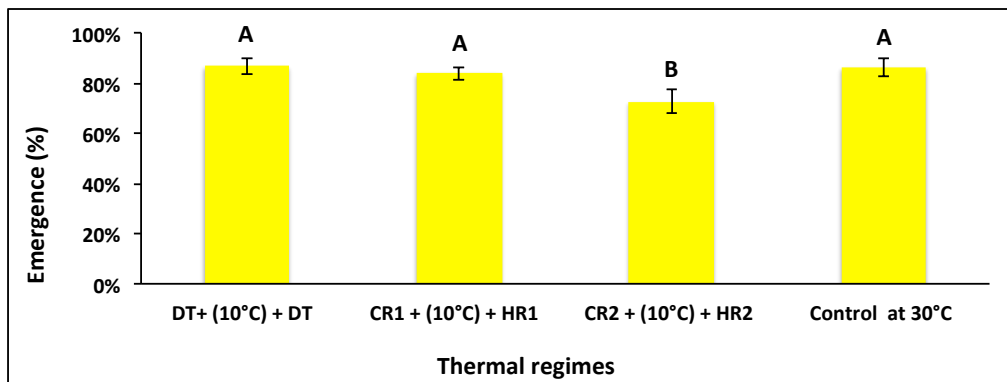


Fig. 6.d. Percentage of emergence (means \pm S.E.) of *O. pityocampae* adults was analysed after 10 days storage of **mature larvae** within eggs of the host *Graphosoma lineatum* (75 \pm 5% RH and 16:8 L:D). Mature larvae were subjected to the same temperature treatments described in Fig. 6.c. In the control **mature larvae** were reared at standard conditions (30°C, 75 \pm 5% RH and 16:8 L: D).

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; P < 0.05/6).

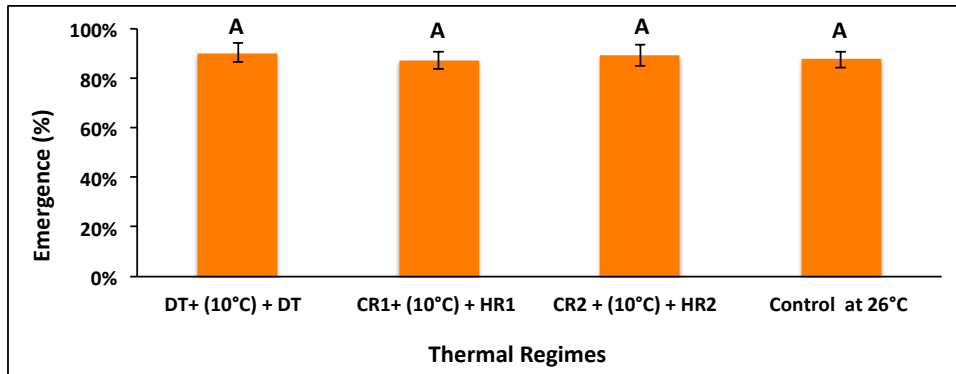


Fig. 6.e. Percentage of emergence (means \pm S.E.) of *O. pityocampae* adults after 10 days storage of **pupae** within eggs of the host *Graphosoma lineatum* (75 \pm 5% RH and 16:8 L: D). Pupae were subjected to the following temperature treatments: Storage by direct transfer (DT) at 10°C, Storage at 10°C and cooling/heating rate of 0.05 min⁻¹ (CR1/HR1), Storage at 10°C and cooling/heating rate of 0.005 min⁻¹ (CR2/HR2). In the control **pupae** were reared at standard conditions (26°C, 75 \pm 5% RH and 16:8 L: D).

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; P < 0.05/6).

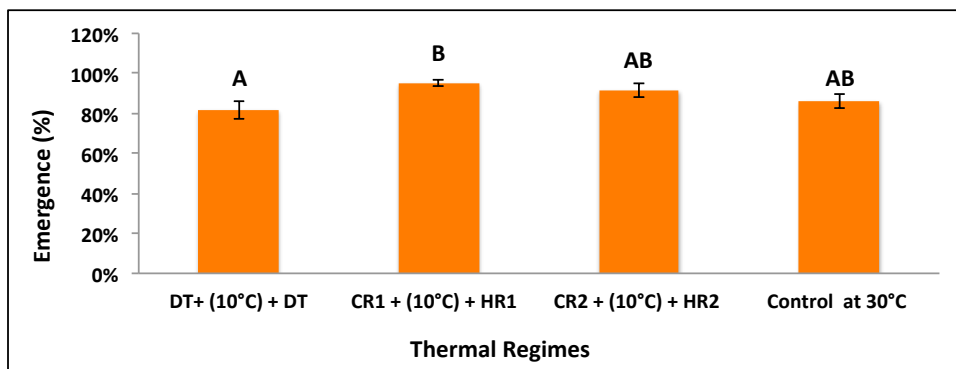


Fig. 6.f. Percentage of emergence (means \pm S.E.) of *O. pityocampae* adults after 10 days storage of **pupae** within eggs of the host *Graphosoma lineatum* (75 \pm 5% RH and 16:8 L: D). **Pupae** were subjected to the following temperature treatments described in Fig. 6.e. In the control **pupae** were held at standard rearing conditions (30°C, 75 \pm 5% RH and 16:8 L: D).

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; P < 0.05/6).

7. Time to adult development.

Time lag before adult emergence was analyzed as well. Statistically significant differences were recorded in the development time of *O. pityocampae* held at **26°C**, 75 ± 5% RH and 16:8 L:D after 10 days storage of **immature larvae** at the following constant temperatures: 15 °C, 10 °C, 5 °C, 0 °C (75 ± 5% RH and 16:8 L:D) (Kruskal-Wallis: $H = 32.20$; $df = 3$; $n = 68$; $P = 0.0001$). Moreover a positive significant correlation between development time and reduction of storage temperature was observed (Spearman's $\rho = 0.561$; $n=68$; $P = 0.0001$)(Table 7.1. and Fig. 7.a). Analogous results were obtained when parasitoids were transferred to **30°C** after being submitted to the same treatments (Kruskal-Wallis: $H = 33.87$; $df = 3$; $n = 59$; $P = 0.0001$). Furthermore a positive significant correlation between development time and reduction of storage temperature was detected (Spearman's $\rho = 0.573$; $n=59$; $P = 0.0001$)(Table 7.2. and Fig. 7.b). In Fig 7.a,b correlation and multiple comparisons show a marked and significant increase of developmental time for groups of immature larvae stored at 10 °C, 5 °C, 0 °C. This fact may be partly explained by the role played by the lower developmental threshold, which is expected to lie between 15 and 10 °C. During storage at 10 °C or below, development may be temporarily arrested to re-start again only when temperature rises up to the standard hatching conditions tested in our experiments.

Mature larvae and pupae were subjected to the same temperature treatments and then moved to 26°C and 30°C.

After storage, **mature larvae** held at **26°C** showed significant differences in development time (Kruskal-Wallis: $H = 40.60$; $df = 3$; $n = 52$; $P = 0.0001$) (Table 7.1. and Fig. 7.c). Likewise those transferred to **30°C** were significantly different (Kruskal-Wallis: $H = 15.00$; $df = 3$; $n = 51$; $P = 0.0001$) (Table 7.2. and Fig. 7.d). Development time of mature larvae showed an evident peak for larvae stored at 15°C. In particular those transferred to 26°C after storage, presented on average a consistently delayed emergence. This phenomenon might be due to diapause induction, a physiological mechanism that is known in to occur in *O. pityocampae* mature larvae (Battisti et al., 1990; Halperin, 1990).

Pupae reared at **26°C** after storage, presented significant differences in development time (Kruskal-Wallis: $H = 18.33$; $df = 3$; $n = 62$; $P = 0.0001$). Furthermore a positive significant correlation between development time and reduction of storage temperature was observed (Spearman's $\rho = 0.534$; $n=62$; $P = 0.0001$) (Table 7.1. and Fig. 7.e). Likewise, pupae from the same treatment that were moved to **30°C**, presented significant differences in development time (Kruskal-Wallis: $H = 44.03$; $df = 3$; $n = 60$; $P = 0.0001$). Moreover a positive correlation between development time and reduction of storage temperature was detected (Spearman's $\rho = 0.534$; $n=62$; $P = 0.0001$) (Table 7.1. and Fig. 7.e). Trends for developmental time in pupae show a gradual increase in days required for emergence associated with storage treatments at progressively lower temperatures. This delay in hatching is particular evident for pupae stored for 10 days at 0°C that took almost 1 day more for reaching emergence compared with those stored for the same time at 15°C.

Table 7.1. Days (means \pm S.E.) required for *O. pityocampae* adult emergence after 10 days storage of **immature larvae, mature larvae and pupae** within eggs of *Graphosoma lineatum*. Juveniles were subjected to the following temperature treatments: Storage by direct transfer (DT) at 15°C, 10°C, 5°C, 0°C and then direct transfer to standard rearing conditions (26°C, 75 \pm 5% RH and 16:8 L: D).

Thermal regime	DT+ (15°C) + DT	DT+ (10°C) + DT	DT+ (5°C) + DT	DT+ (0°C) + DT
Hatching Temperature	26°C	26°C	26°C	26°C
Time to adult emergence for Pupae (d)	4,16 (\pm 0,14) a	4,40 (\pm 0,21) a	4,66 (\pm 0,07) a	5,39 (\pm 0,21) b
Time to adult emergence for Mature larvae (d)	30,1 (\pm 1,14) a	10,45 (\pm 0,11) b	11,48 (\pm 0,21) c	14,33C (\pm 1,33) bc
Time to adult emergence for Immature larvae (d)	13,21 (\pm 0,09) a	15,19 (\pm 0,25) b	15,25 (\pm 0,33) b	15,23 (\pm 0,12) b

Means in the same row followed by the same letters are not significantly different (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/6$).

Table 7.2. Days (means \pm S.E.) required for *O. pityocampae* adult emergence after 10 days storage of **immature larvae, mature larvae and pupae** within eggs of *Graphosoma lineatum*. Juveniles were subjected to the following temperature treatments: Storage by direct transfer (DT) at 15°C, 10°C, 5°C, 0°C and then direct transfer to 30°C, 75 \pm 5% RH and 16:8 L: D).

Thermal regime	DT+ (15°C) + DT	DT+ (10°C) + DT	DT+ (5°C) + DT	DT+ (0°C) + DT
Hatching Temperature	30°C	30°C	30°C	30°C
Time to adult emergence for Pupae (d)	2,72 (\pm 0,05) a	3,76 (\pm 0,06) b	3,67 (\pm 0,04) c	4,34 (\pm 0,12) b
Time to adult emergence for Mature larvae (d)	13,15 (\pm 4,23) a	9,29 (\pm 0,16) b	9,04 (\pm 0,14) b	10 (\pm 0,25) ab
Time to adult emergence for Immature larvae (d)	9,17 (\pm 0,09) a	11,41 (\pm 0,11) b	11,43 (\pm 0,36) b	11,42 (\pm 0,16) b

Means in the same row followed by the same letters are not significantly different (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/6$).

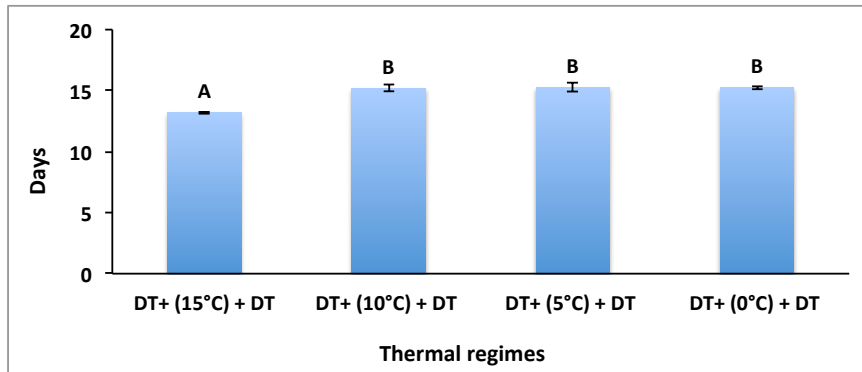


Fig. 7.a. Days (means \pm S.E.) required for of *O. pityocampae* adult emergence after 10 days storage of **immature larvae** within eggs of *Graphosoma lineatum*. Immature larvae were subjected to the following thermal regimes: Storage by direct transfer (DT) at 15°C, 10°C, 5°C, 0°C and then direct transfer to standard rearing conditions (26°C, 75 \pm 5% RH and 16:8 L: D).

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/6$).

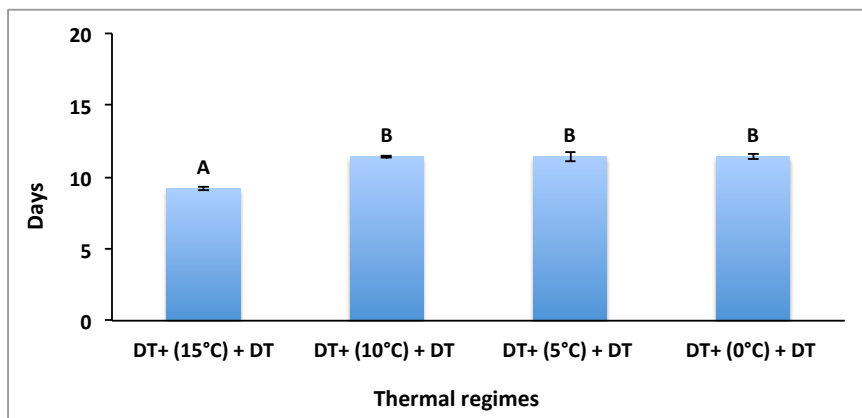


Fig. 7.b. Days (means \pm S.E.) required for of *O. pityocampae* adult emergence after 10 days storage of **immature larvae** within eggs of *Graphosoma lineatum*. Immature larvae were subjected to the following thermal regimes: Storage by direct transfer (DT) at 15°C, 10°C, 5°C, 0°C and then direct transfer to 30°C, 75 \pm 5% RH and 16:8 L: D).

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/6$).

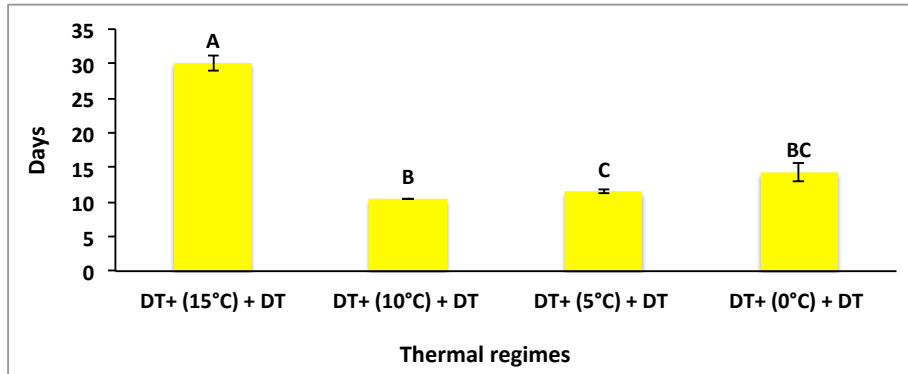


Fig. 7.c. Days (means \pm S.E.) required for of *O. pityocampae* adult emergence after 10 days storage of **mature larvae** within eggs of *Graphosoma lineatum*. Mature larvae were subjected to the following thermal regimes: Storage by direct transfer (DT) at 15°C, 10°C, 5°C, 0°C and then direct transfer to standard rearing conditions (26°C, 75 \pm 5% RH and 16:8 L: D).

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/6$).

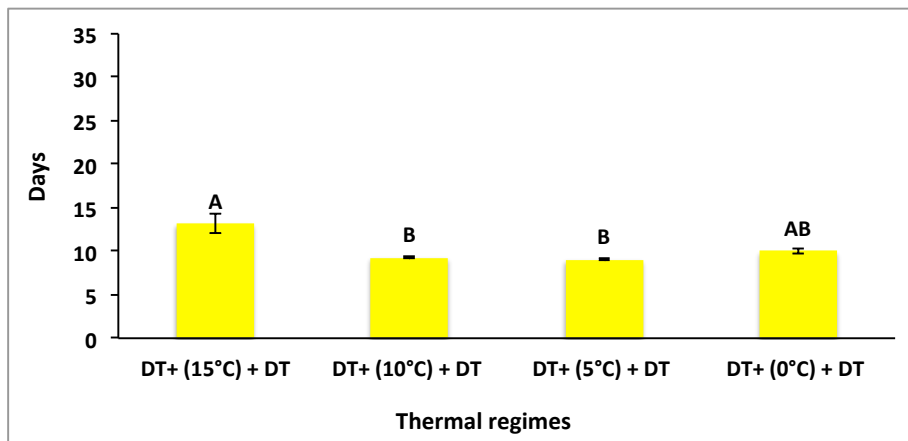


Fig. 7.d. Days (means \pm S.E.) required for of *O. pityocampae* adult emergence after 10 days storage of **mature larvae** within eggs of *Graphosoma lineatum*. Mature larvae were subjected to the following thermal regimes: Storage by direct transfer (DT) at 15°C, 10°C, 5°C, 0°C and then direct transfer to 30°C, 75 \pm 5% RH and 16:8 L: D).

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/6$).

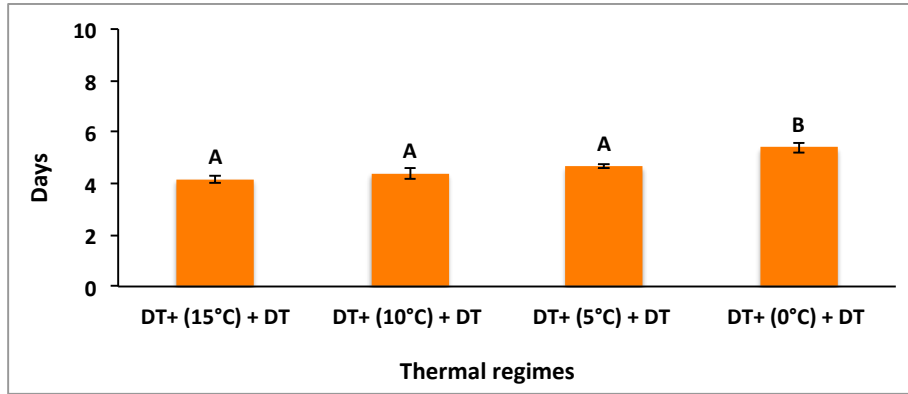


Fig. 7.e. Days (means \pm S.E.) required for of *O. pityocampae* adult emergence after 10 days storage of **pupae** within eggs of *Graphosoma lineatum*. Pupae were subjected to the following thermal regimes: Storage by direct transfer (DT) at 15°C, 10°C, 5°C, 0°C and then direct transfer to standard rearing conditions (26°C, 75 \pm 5% RH and 16:8 L: D).

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/6$).

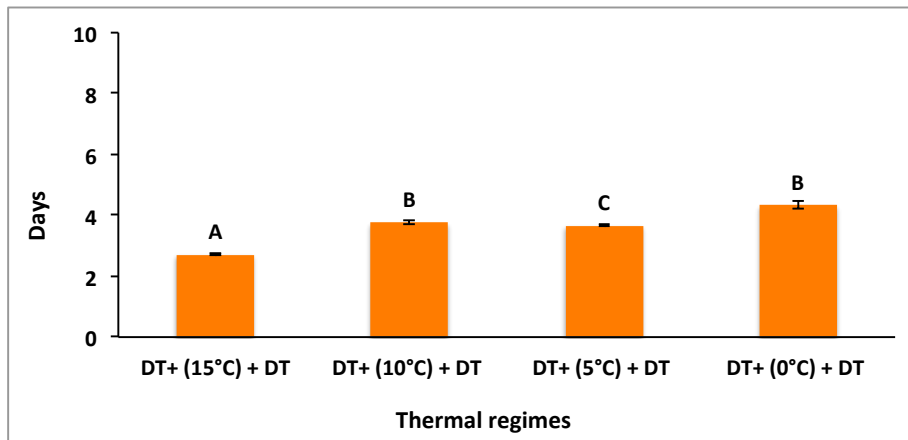


Fig. 7.f. Days (means \pm S.E.) required for of *O. pityocampae* adult emergence after 10 days storage of **pupae** within eggs of *Graphosoma lineatum*. Pupae were subjected to the following thermal regimes: Storage by direct transfer (DT) at 15°C, 10°C, 5°C, 0°C and then direct transfer to 30°C, 75 \pm 5% RH and 16:8 L: D).

Different letters imply significant differences (Kruskal-Wallis Test; Mann-Whitney Test; $P < 0.05/6$).

Final Conclusions:

1. Evaluation of biological parameters of *O. pityocampae* reared at (26°C, 75 ± 5% RH, 16:8 L:D) showed that female life span was about 48 days on average and parasitoid development time 16 days. Ovipositional period on average was 27.50 days while post reproductive period was around 21 days. The overall progeny produced in the trial resulted to be on average 90.25; however wide variability was recorded. Moreover highly female-biased sex ratio (99.05 % of females) was reported. Overall data display evident trends of parasitization and offspring production with peaks for both parameters reached when females were between 4 and 5 days old.
2. Mandible length, head width, body length and segmentation were some of the parameters analysed in order to define juvenile stages of *O. pityocampae*. The most suitable parameter for the identification of larvae was mandible length. The length of these structures allowed the identification of 5 larval instars by frequency distribution analysis. In contrast other parameters were not reliable in the identification of juveniles.
3. The experiment on the effects of aged *L. occidentalis* eggs on *O. pityocampae* parasitization showed that number of parasitized eggs and sex ratio were not significantly influenced by host egg age whereas female development time was longer in older eggs. This trial underlined that *O. pityocampae* was not only flexible to adapt to the new host but also cable to efficiently exploit eggs containing fully developed nymphs.
4. Evaluation of egg-to-adult temperature-dependent development of *O. pityocampae* within eggs of *G. lineatum* provided evidence that parasitoid development time gradually decreased reaching about 9 day at 36°C whereas it increased up to more than 30 days at 19°C. Data suggest that

minimum and maximum developmental thresholds are likely to cluster around respectively 14.5°C and 36.5°C. However additional experiments are currently in progress for a better identification of these limits.

5. In the set of experiments concerning *O. pityocampae* immature larvae stored for 10 days at different temperatures from 15°C to 0°C, a marked decline in parasitoid survival was recorded. In fact, emergence rate dramatically dropped when storage temperature passed from 15°C to 10°C while no significant differences in emergence were observed between parasitoid stored from 10° to 0°C. In contrast mature larvae and pupae showed a less pronounced reduction of parasitoid emergence associated with the decreasing of temperature. In fact a marked decline in adult hatching became evident only for mature larvae stored at 0°C whose emergence rate clustered around 4%. For what concerns cooling and heating rates (CR/HR), substantial benefits in terms of survival were only achieved when a CR/HR rate of 0.005 °C min⁻¹ was applied to immature larvae stored at 10°C for 10 days. By contrast survival of mature larvae and pupae was not particularly affected by CR/HR. Overall data suggest that mature larvae and pupae are the most cold-tolerant stages and thus the most suitable to be employed in cold storage techniques.

References

- Anguilletta, Jr., M.J. (2009). *Thermal Adaptation: A Theoretical and Empirical Synthesis*. Oxford University Press, Oxford.
- Arzone, A., (1985). Biological control of the *Eucalyptus* snout beetle in Italy. *Bulletin SROP*, 8 (1): 70-73.
- Aung, K.S.D., Takagi, M. & Ueno, T. (2010a). Effect of Female's Age on the Progeny Production and Sex Ratio of *Ooencyrtus nezarae*, an Egg Parasitoid of the Bean Bug *Riptortus clavatus*. *Journal of the Faculty of Agriculture, Kyushu University*, 55 (1): 83-85.
- Aung, K.S.D., Takasu, K., et al. (2010b). Effect of Temperature on Egg Maturation and Longevity of the Egg Parasitoids *Ooencyrtus nezarae* (Ishii) (Hymenoptera: Encyrtidae). *Journal of the Faculty of Agriculture, Kyushu University*, 55 (1): 87–89.
- Bale, J., van Lenteren, J.C., Bigler F. (2008). Biological control and sustainable food production. *Philos. Trans. R. Soc. Lond. B*, 363 (1492): 761-776.
- Bates, S. L., Borden, J. H. (2004). Parasitoids of *Leptoglossus occidentalis* Heidemann (Heteroptera: Coreidae) in British Columbia. *J. Entomol. Soc., B. C.*, 101: 143-144.
- Bates, S.L., Borden, J.H. (2005). Life table for *Leptoglossus occidentalis* Heidemann (Heteroptera: Coreidae) and prediction of damage in lodgepole pine seed orchards. *Agric. Forest Entomol.*, 7: 145-151.
- Bates, S.L., Borden, J.H., Kermode, A.R., Bennet, R.G. (2000). Impact of *Leptoglossus occidentalis* (Hemiptera: Coreidae) on Douglas-Fir seed production. *J. Econ. Entomol.*, 39: 1444-1451.
- Bates, S.L., Strong, W.B., Borden, J.H. (2002). Abortion and seed set in lodgepole and western white pine conelets following feeding by *Leptoglossus occidentalis* (Heteroptera: Coreidae). *Environ. Entomol.*, 31: 1023-1029.
- Battisti, A., Ianne, P., Milani, N. and Zanata M. (1990). Preliminary accounts on rearing of *Ooencyrtus pityocampae* (Mercet) (Hymenoptera: Encyrtidae). *Journal of Applied Entomology*, 110: 121-127.
- Battisti, A., Colazza, S., Roversi, P.F., Tiberi, R. (1988). Alternative hosts of *Ooencyrtus pityocampae* (Mercet) (Hymenoptera: Encyrtidae) in Italy. *Redia*, 71: 321-328.
- Battisti, A., Faccoli, M. (2007). Gli insetti forestali nel quadro del cambiamento climatico. *Atti Accademia Nazionale di Entomologia*, LV: 49-51
- Bayram, A., Ozcan, H., & Kornosor, S. (2005). Effect of cold storage on the performance of *Telenomus busseolae* Gahan (Hymenoptera: Scelionidae), an egg parasitoid of *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae). *Biological Control*, 35 (1): 68-77.
- Bernardo, U., Iodice, L., Sasso, R., Pedata, P.A. (2008). Effects of cold storage on *Thripobius javae* (= *T. Semiluteus*) (Hymenoptera: Eulophidae). *Biocontrol Science and Technology*, 18: 921-933.
- Bin, F., Tiberi, R. (1983). Notizie preliminary sui parassitoidi oofagi di *Thaumetopoea processionea* (L.) in Italia Centrale (Hym., Chalcidoidea; Lep., Thaumetopoeidae). *Redia*, 66: 449-459.
- Binazzi, F., Benassai, D., Sabbatini Peverieri, P., Roversi, P.F. (2013). Effects of *Leptoglossus occidentalis* Heidemann (Heteroptera: Coreidae) egg age on the indigenous parasitoid *Ooencyrtus pityocampae* Mercet (Hymenoptera: Encyrtidae). *Redia*, 96: 79–84.
- Boivin, G. (1994). Overwintering strategies of egg parasitoids. In: Wajnberg, E., Hassan, S.A. (Eds.), *Biological Control with Egg Parasitoids*. CAB International, Wallingford, UK: 219-244.
- Bowler, K., Terblanche, J.S. (2008). Insect thermal tolerance: what is the role of ontogeny, ageing and senescence? *Biological Review*, 83: 339-355.
- Bueno, R., Jr., Van Cleave H. W. (1997). The effect of cold storage on the emergence of *Aphelinus perpallidus* a parasitoid of *Monellia caryella*. *Southwest. Entomol.*, 22: 39-51.

- Caleca, V., Rizzo, M.C., Lo Verde, G., Rizzo, R., Buccellato, V., Luciano, P., Cao, O., Palmeri, V., Grande, S.B., Campolo, O. (2009). Diffusione di *Closterocerus chamaeleon* (Girault) introdotto in Sicilia, Sardegna e Calabria per il controllo biologico di *Ophelimus maskelli* (Ashmead) (Hymenoptera, Eulophidae), galligeno esotico sugli eucalipti. Atti del III Congresso Nazionale di Selvicoltura, Taormina, 16-19 Ottobre 2008. Atti 2: 638-642.
- Camponogara, P., Festi, M., Battisti, A. (2003). La cimice dei semi americana: un ospite indesiderato delle nostre conifere. Vita in Campagna: 7-8.
- Chen, W., Leopold, R.A., Morgan, D.J., Harris, W.M.O. (2006). Development and reproduction of the egg parasitoid, *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae), as a function of temperature. Environmental Entomology, 35 (5): 1178-1187.
- Chown, S. L., & Nicolson, S. (2004). Insect physiological ecology: mechanisms and patterns. Oxford University Press.
- Cibrian-Tovar, D.B., Hebel, B.H., Yates, H.O., Mendez-Mondiel, J.T (1986). *Leptoglossus occidentalis* Heidemann. In: Cibrian-Tovar, D.B., Hebel, B.H., Yates, H.O., Mendez-Mondiel, J.T. (eds.). Cone and Seed Insects of the Mexican Conifers. USDA Forest Science, Southeastern Forest Experiment Station Publishing, Asheville, NC: 55-58.
- Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., van den Belt, M. (1997). The value of the world's ecosystem services and natural capital. Nature, 387: 253-260.
- Coulson, S.J., Bale, J.S., (1990). Characterisation and limitations of the rapid cold-hardening response in the housefly *Musca domestica* (Diptera: Muscidae). Journal of Insect Physiology 36: 207-211.
- Covassi, M., Binazzi, A., Toccafondi, P., (1991). Studi sugli entomofagi predatori di cocciniglie del gen. *Matsucucco* Cock. in Italia. I - Note faunistico-ecologiche su specie osservate in pinete della Liguria e della Toscana. Redia, 74 (2): 575-598.
- Covassi, M., Masutti, L., (1999). La protezione del patrimonio forestale dagli artropodi dannosi. II Congresso Nazionale di Selvicoltura, Venezia, 24-27 Giugno 1998. Atti, 3: 301-333.
- Crossman, S. S. (1925). Two imported egg parasites of the gipsy moth, *Anastatus bifasciatus* (Fonsc.) and *Schedius kuvanae* (Howard). J. Agric. Res., 30: 643-675.
- Daane, K.M., Malakar-Kuenen, R.D., Walton, V.M. (2004). Temperature-dependent development of *Anagyrus pseudococci* (Hymenoptera: Encyrtidae) as a parasitoid of the vine mealybug, *Planococcus ficus* (Homoptera: Pseudococcidae). Biological Control, 31 (2): 123-132.
- DeBach, P. (1964). Biological control of insect pests and weeds. Chapman and Hall, London.
- DeBach, P., Rosen, D. (1991). Biological control by natural enemies, 2nd edn. Cambridge University Press, Cambridge.
- Doane, J. F., De Clerck-Floate, R., Arthur, A. P., Affolter, F. (1989). Description of the life stages of *Macroglenes penetrans* (Kirby) (Hymenoptera: Chalcidoidea, Pteromalidae), a parasitoid of the wheat midge, *Sitodiplosis mosellana* (Géhin) (Diptera: Cecidomyiidae). The Canadian Entomologist, 121 (12): 1041-1048.
- EPPO (2003). Environmental risk assessment scheme for plant protection products. Standard PP 3/9 of the European Plant Protection Organization. EPPO Bull., 33: 99-101.
- EPPO (2010). *Leptoglossus occidentalis*: an invasive alien species spreading in Europe. EPPO Reporting Service, 1: 8-12.
- Foerster, L.A., Doetzer, A.K. (2006). Cold storage of the egg parasitoids *Trissolcus basalis* (Wollaston) and *Telenomus podisi* Ashmead (Hymenoptera: Scelionidae). Biological Control, 36: 232-237.
- Gapon, D.A. (2012). First records of the western conifer seed bug *Leptoglossus occidentalis* Heid. (Heteroptera, Coreidae) from Russia and Ukraine, regularities in its distribution and possibilities of its range expansion in the Palearctic region. Ent. Obzrenie, 91: 559-568.

- Gerling, D., Conde, J. E., Rabinovich, J. E. (1976). The comparative development of two egg parasites of *Rhodnius prolixus* (Hemiptera, Reduviidae), vector of Chagas' disease in Venezuela. *The Canadian Entomologist*, 108: 427-432.
- Girolami, V., Camporese, P. (1994). Prima moltiplicazione in Europa di *Neodryinus typhlocybae* (Ashmead) (Hymenoptera: Dryinidae) su *Metcalfa pruinosa* (Say) (Homoptera: Flatidae). In atti del XVII Congresso Nazionale Italiano di Entomologia, Udine 13-18 Giugno 1994. Atti: 655-658.
- Gurr, G.M., Wratten, S.D. (2000). Measures of success in biological control. Kluwer Academic Publishers, Dordrecht.
- Hale, M.A., Elliott, D. (2003). Successes and challenges in augmentative biological control in outdoor agricultural applications: a producer's perspective. *Proceedings of the First International Symposium on Biological Control of Arthropods*, Honolulu, HI: 185-188.
- Halperin, J. (1990). Mass breeding of egg parasitoids (Hym., Chalcidoidea) of *Thaumetopoea pityocampa* Tams. (Lep., Thaumetopoeidae). *J. Appl. Ent.*, 109: 336-340.
- Hance, T., Boivin, G. (1993). Effect of parasitism by *Anaphes sp.* (Hymenoptera: Mymaridae) on the cold-hardiness of *Listronotus oregonensis* (Coleoptera: Curculionidae) eggs. *Canadian Journal of Zoology*, 71: 759-764.
- Hawes, T.C., Bale, J.S. (2007). Plasticity in arthropod cryotypes. *Journal of Experimental Biology*, 210: 2585-2592.
- Hedlin, A.F., Yates, H.O., Tovar, D.C., Ebel, B.H., Koerber, T.W., Merkel, E.P. (1981). Cone and seed insects of North American conifers. Canadian Forestry Service, Ottawa, USDA Forest Service, Washington, DC, and Secretaria de Agricultura y Recursos Hidraulicos, Mexico.
- Hirose, Y., Ehler, L.E., Hirose Y. (2003). Influence of host age on patch use by a quasi-gregarious egg parasitoid. *Environ. Entomol.*, 32: 789-796.
- Hinton, H. E. (1981). *Biology of insect eggs*. Volume I. Pergamon Press.
- Hizal E., Inan M. (2012). *Leptoglossus occidentalis* (Heidemann, 1910) is an invasive insect species. *Bartın Orman Fakültesi Dergisi*, 14: 56-61.
- Hoffmann, M. P., Ridgeway R. L., Show, E. D., and Matteoni J. (1998). Practical application of mass-reared natural enemies: selected case histories, pp. 268-293. In Ridgeway, R. L., M. P. Hoffmann, M. N. Inscoe, and C. S. Glenister (eds.). *Mass-Reared Natural Enemies: Application, Regulation, and Needs*. Thomas Say Publications in Entomology, Entomological Society of America, Lanham, Maryland, USA.
- Hofstetter, R.W., Raffa, K.F. (1998). Endogenous and exogenous factors affecting parasitism of gypsy moth egg masses by *Ooencyrtus kuvanae*. *Entomol. Exp. et Appl.*, 88 (2):123-135.
- Hofsvang, T., Hagvar, E.B. (1977). Cold storage tolerance and supercooling points of mummies of *Ephedrus cerasicola* Ståry and *Aphidius colemani* Viereck (Hymenoptera: Aphidiidae). *Norwegian Journal of Entomology*, 24: 1-6.
- Huey, R. B., Wakefield, C. W. D., Gilchrist, G. W. (1995). Within and between generation effects of temperature on early fecundity of *Drosophila melanogaster*. *Heredity*, 74: 216-223.
- JianJun, W. *et al.* (2012). Relationship between temperature and development of *Ooencyrtus kuvanae* (Hymenoptera: Encyrtidae), the egg parasitoid of *Lymantria dispar* (Lepidoptera: Lymantriidae), with an evaluation of its offspring quality reared from eggs of the substitute host *Antheraea pernyi* (Lepidoptera: Saturniidae). *Acta Entomologica Sinica*, 55 (5): 570-574.
- Kanat, M., Özbolat, M. (2006). Mass production and release of *Calosoma sycophanta* L. (Coleoptera: Carabidae) used against the Pine Processionary Moth, *Thaumetopoea pityocampa* (Shiff.) (Lepidoptera: Thaumetopoeidae), in Biological Control. *Turk. J. Zool.*, 30: 181-185.
- Kelty, J.D., Lee, R.E. (1999). Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*. *Journal of Insect Physiology*, 45 (8): 719-726.
- Koerber, T.W. (1963). *Leptoglossus occidentalis* (Hemiptera: Coreidae), a newly discovered

- pest of coniferous seed. *Ann. Entomol. Soc. Am.*, 56: 229-234.
- Kostal, V. (2006). Eco-physiological phases of insect diapause. *Journal of Insect Physiology*, 52 (2): 113-127.
- Lacey, L.A., Millar, L., Kirk, A.A., Perring, T.M. (1999). Effect of storage temperature and duration on survival of eggs and nymphs of *Bemisia argentifolii* (Homoptera: Aleyrodidae) and pupae of the whitefly parasitoid *Encarsia formosa* (Hymenoptera: Aphelinidae). *Annals of the Entomological Society of America* 92: 430-434.
- Langer, A., Hance, T., (2000). Overwintering strategies and cold hardiness of two aphid parasitoid species (Hymenoptera: Braconidae: Aphidiinae). *Journal of Insect Physiology*, 46: 671-676.
- Leopold, R.A., (1998). Cold storage of insects for integrated pest management. In: Hallman, G.J., Denlinger, D.L. (Eds.), *Temperature Sensitivity in Insects and Application in Integrated Pest Management*. Westview Press, Boulder, pp. 235-267.
- Leopold, R.A., (2007). Colony maintenance and mass-rearing: using cold storage technology for extending the shelf-life of insects. In: Vreysen, M.J.B., Robinson, A.S., Hendrichs, J. (Eds.), *Area-Wide Control of Insect Pests*. Springer, Netherlands: 149-162.
- Leopold, R.A., Chen, W.L., Yocum, G.D. (2004). Effects of using constant and cyclical stepwise-increasing temperatures on parasitized and unparasitized eggs of the glassy-winged sharpshooter during cold storage. In: Tariq, M.A., Oswald, S., Blincoe, P., Ba, A., Lorick, T., Esser, T., (Eds.), *Proceedings of the 2004 Pierce's Disease Research Symposium*, San Diego, CA: 124-127.
- Levie, A., Vernon, P., Hance, T. (2005). Consequences of acclimation on survival and reproductive capacities of cold-stored mummies of *Aphidius rhopalosiphii* (Hymenoptera: Aphidiinae). *Journal of Economic Entomology*, 98: 704-708.
- Longo, S., Palmieri, V., Siscaro, G., Spampinato, R.G. (1994). Rilievi sui lepidotteri defogliatori nei querceti. – Atti del Convegno MiRAAF «Innovazioni e prospettive nella difesa fitosanitaria», Ferrara 1994. Atti: 343-346.
- Lopez, S.N., Botto, E. (2005). Effect of cold storage on some biological parameters of *Eretmocerus corni* and *Encarsia formosa* (Hymenoptera: Aphelinidae). *Biological Control*, 33: 123-130.
- Luczynski, A., Nyrop, J.P., Shi, A. (2007). Influence of cold storage on pupal development and mortality during storage and on post-storage performance of *Encarsia formosa* and *Eretmocerus eremicus* (Hymenoptera: Aphelinidae). *Biological Control*, 40: 107-117.
- Lysyk, T.J. (2004). Effects of cold storage on development and survival of three species of parasitoids (Hymenoptera: Pteromalidae) of house fly, *Musca domestica* L. *Environmental Entomology* 33: 823-831.
- Magiafoglou, A., Hoffmann, A.A. (2003). Cross-generation effects due to cold exposure in *Drosophila serrata*. *Functional Ecology*, 17: 664-672.
- Maple, J. D. (1937). The biology of *Ooencyrtus johnsoni* (Howard), and the role of the egg shell in the respiration of certain encyrtid larvae (Hymenoptera). *Annals of the Entomological Society of America*, 30: 123-154.
- Masutti, L. (1964). Studies on the egg parasitoids of *Thaumetopoea pityocampa* Schiff. *Annali Centro Economia Montana Venetia*, 4: 205-271.
- Masutti L., Salvazza A., Battisti, A. (1991). Oviposition of *Ooencyrtus pityocampae* (Mercet) into artificial eggs (Hymenoptera: Encyrtidae). Insect parasitoid, 4th European workshop. Perugia 3-5 April 1991, *Redia*, 74 (3): 457-462.
- Maltese, M., Caleca, V., Strong, W.B., Guerrieri, E., Roversi, P.F. (2011). I parassitoidi di *Leptoglossus occidentalis* in Sicilia e nel suo areale di origine nordamericano. In: *Proceedings XXIII Congresso Nazionale Italiano di Entomologia*, 13-16 giugno 2011, Genova, Italy. Erredi Publishing, Genova: 350.
- Maple, J.D. (1947). The eggs and first instar larvae of Encyrtidae and their morphological adaptations for respiration. *Univ. California Press, Berkeley*: 122 pp.
- Masner, L. (1983). A revision of *Gryon* Haliday in North America (Hymenoptera: Proctotrupoidea: Scelionidae). *Can. Entomol.*, 115: 123-174.

- Matteson, P. C. (1981). Egg parasitoids of hemipteran pests of cowpea in Nigeria and Tanzania, with special reference to *Ooencyrtus patriciae* Subba Rao (Hymenoptera: Encyrtidae) attacking *Clavigralla tomentosicollis* Stål (Hemiptera: Coreidae). *Bulletin of Entomological Research*, 71: 547-554.
- McDonald, R.C., Kok, L.T., 1990. Post refrigeration viability of *Pteromalus puparum* (Hymenoptera: Pteromalidae) prepupae within host chrysalids. *Journal of Entomological Science* 25: 409-413.
- Merino-Pachero, M. (2007). Almeria finally forced to turn green. *Fruit Veg Technol.*, 7: 23-25.
- Mjøøs, A.T., Nielsen, T.R., Ødegaard, F. (2010). The Western Conifer Seed Bug (*Leptoglossus occidentalis* Heidemann, 1910) (Hemiptera, Coreidae) found in SW Norway. *Norwegian J. Entomol.*, 57: 20-22.
- Nakama, P.A., Foerster, L.S.A. (2001). Efeito da alternancia de temperaturas no desenvolvimento e emergencia de *Trissolcus basalis* (Wollaston) e *Telenomus podisi* Ashmead (Hymenoptera: Scelionidae). *Neotropical Entomology*, 30: 269- 275.
- Nichols, J.R., Tracy, J.L., Vogt, E.A. (1989). Comparative ecological studies of indigenous egg parasitoids (Hymenoptera: Scelionidae; Encyrtidae) of the squash bug, *Anasa tristis* (Hemiptera: Coreidae). *J. Kansas Entomol. Soc.*, 62: 177-188.
- Niccoli, A., Benassai, D., Croci, F., Roversi, P.F. (2009). *Anastatus bifasciatus* ooparasitoide di *Leptoglossus occidentalis*. In: Proceedings XXII Congresso Nazionale Italiano di Entomologia, 15-18 giugno 2009, Ancona, Italy, Coppini Publishing, Firenze: p. 337.
- Pandey, R.R., Johnson, M.W. (2005). Effects of cool storage on *Anagyrus ananatis* Gahan (Hymenoptera: Encyrtidae). *Biological Control*, 35: 9-16.
- Paoli, F., Gottardo, M., Marchini, D., Dallai, R., Roversi, P.F. (2013). Morphology of the male reproductive system and sperm ultrastructure of the egg parasitoid *Gryon pennsylvanicum* (Ashmead) (Hymenoptera, Platygastridae). *Arthrop. Struc. Dev.*, 42: 297-308.
- Parker, D. L. (1933). The interrelations of two hymenopterous egg parasites of the gypsy moth, with notes on the larval instars of each. *J. Agric. Res*, 46: 23-34.
- Pavan, M., (1959). Attività italiana per la lotta biologica con formiche del gruppo *Formica rufa* contro gli insetti dannosi alle foreste. Ministero Agricoltura e Foreste, Roma, Collana Verde, 4: 1-79.
- Peverieri, G. S., Furlan, P., Simoni, S., Strong, W. B., Roversi, P. F. (2012). Laboratory evaluation of *Gryon pennsylvanicum* (Ashmead) (Hymenoptera, Platygastridae) as a biological control agent of *Leptoglossus occidentalis* Heidemann (Heteroptera, Coreidae). *Biological Control*, 61 (1): 104-111.
- Pimentel, D. (2009). Environmental and economic costs of the application of pesticides primarily in the United States. In: Peshin R, Dhawan AK (eds) *Integrated pest management: innovation-development process*. Springer, Dordrecht, pp 89-111.
- Pitcher, S.A., Hoffmann, M.P., Gardner, J., Wright, M.G., Kuhar, T.P. (2002). Cold storage of *Trichogramma ostriniae* reared on *Sitotroga cerealella* eggs. *BioControl*, 47: 525-535.
- Polgar, L. (1986). Effect of cold storage on the emergence, sex ratio and fecundity of *Aphidius matricariae*: 255-260. In: I. Hodek [ed.], *Ecology of Aphidophaga*. Academia, Prague & Dr. W. Junk, Dordrecht, Holland.
- Quacchia, A., Moriya, S., Bosio, G., Scapin, I., Alama, A. (2008). Rearing, release and settlement prospect in Italy of *Torymus sinensis*, the biological control agent of the chestnut gall wasp *Dryocosmus kuriphilus*. *BioControl*, 53 (6): 829-839.
- Rivers, D.B., Lee, R.E., Denlinger, D.L., (2000). Cold hardiness of the fly pupal parasitoid *Nasonia vitripennis* is enhanced by its host *Sarcophaga crassipalpis*. *Journal of Insect Physiology*, 46: 99-106.
- Roversi, P.F. (2009). Adattamento di specie neointrodotte, *Leptoglossus occidentalis* Heidemann. In: Jucker C., Barbagallo S., Roversi P.F. and Colombo M. (eds.). *Insetti Esotici e Tutela Ambientale*. Arti Grafiche Maspero Fontana & C., Cermenate, Italy: 224-229.

- Roversi, P.F., Santini, L., Rossi, E., Loni, A. (2011b). Ooparassitoidi di *Leptoglossus occidentalis* in Toscana. In: Proceedings XXIII Congresso Nazionale Italiano di Entomologia, 13-16 giugno 2011, Genova, Italy. Erredi Publishing, Genova: 139.
- Roversi, P.F., Strong, W.B., Caleca, V., Maltese, M., Sabbatini Peverieri, G., Marianelli, L., Marziali, L., Strangi, A. (2011). Introduction into Italy of *Gryon pennsylvanicum* (Ashmead), an egg parasitoid of the alien invasive bug *Leptoglossus occidentalis* Heidemann (Heteroptera, Coreidae). OEPP/EPPO Bulletin 41: 72-75.
- Roversi, P. F., Sabbatini Peverieri, G., Maltese, M., Furlan, P., Strong, W. B., & Caleca, V. (2014). Pre-release risk assessment of the egg-parasitoid *Gryon pennsylvanicum* for classical biological control of *Leptoglossus occidentalis*. Journal of Applied Entomology, 138 (1-2): 27-35.
- Rundle, B.J., Thomson, L.J., Hoffmann, A.A. (2004). Effects of cold storage on field and laboratory performance of *Trichogramma carverae* (Hymenoptera: Trichogrammatidae) and the response of three *Trichogramma* spp. (*T. carverae*, *T. nr. brassicae*, and *T. funiculatum*) to cold. Journal of Economic Entomology, 97: 213-221.
- Sabbatini Peverieri, G., Furlan, P., Simoni, S., Strong, W.B., Roversi, P.F. (2012). Laboratory evaluation of *Gryon pennsylvanicum* (Ashmead) (Hymenoptera, Platygasteridae) as a biological control agent of *Leptoglossus occidentalis* Heidemann (Heteroptera, Coreidae). Biol. Control, 61: 104-111.
- Sabbatini Peverieri, G., Furlan, P., Benassai, D., Caradonna, S., Strong, S.W.B., Roversi, P.F. (2013). Host Egg Age of *Leptoglossus occidentalis* (Heteroptera, Coreidae) and Parasitism by *Gryon pennsylvanicum* (Hymenoptera, Platygasteridae). J. Econ. Entomol., 106 (2): 633-640.
- Santi, F., Maini, S. (2011). New association between *Dryocosmus kuriphilus* and *Torymus flavipes* in chestnut trees in the Bologna area (Italy): first results. Bulletin of Insectology, 64 (2): 275-278.
- Santini, L. (2010). La cimice americana delle conifere (*Leptoglossus occidentalis* Heidemann) (Heteroptera, Coreidae) e fruttificazione del pino domestico. In: Loreti F. (ed.). Proceedings, Insetti di recente introduzione dannosi alle pinete, 9 ottobre 2009, Pisa (Italy). I Georgofili, Quaderni 2009. IV sezione Centro-Ovest, supplemento Atti dell'Accademia dei Georgofili, anno 2009, serie VIII, vol. 6. Felici Publishing, Pisa: 15-36.
- Shalaby, F.F., Rabasse, J.M. (1979). Effect of conservation of the aphid parasite *Aphidius matricariae* Hal. (Hymenoptera; Aphidiidae) on adult longevity, mortality and emergence. Ann. Agric. Sci., 2: 59-71.
- Sinclair, B.J., Rajamohan, A. (2008). Slow or stepped rewarming after acute low- temperature exposure does not improve survival of *Drosophila melanogaster* larvae. Canadian Entomologist, 140: 306-311.
- Singh, R., Srivastava, M. (1988). Effect of cold storage of mummies of *Aphis craccivora* Koch subjected to different pre-storage temperature on per cent emergence of *Trioxys indicus* Subba Rao and Sharma. Insect Sci. Appl., 9: 655-657.
- Spanoudis, C.G., Andreadis, S.S. (2011). Temperature-dependent survival, development, and adult longevity of the koinobiont endoparasitoid *Venturia canescens* (Hymenoptera: Ichneumonidae) parasitizing *Plodia interpunctella* (Lepidoptera: Pyralidae). Journal of Pest Science, 85 (1): 75-80.
- Strong, W.B. (2006). Seasonal changes in seed reduction in lodgepole pine cones caused by feeding of *Leptoglossus occidentalis* (Hemiptera: Coreidae). Can. Entomol., 138: 888-896.
- Strong, W.B., Bates, S.L., Stoehr, M. (2001). Feeding by *Leptoglossus occidentalis* (Hemiptera: Coreidae) reduces seed set in lodgepole pine (Pinaceae). Can. Entomol., 133: 857-865.
- Takasu, Keiji, and Yoshimi Hirose (1989). The number of larval instars in *Ooencyrtus* species (Hymenoptera, Encyrtidae). Japanese Journal of Entomology 57 (2): 398-401.
- Takasu, K., Hirose, Y. (1993). Host Acceptance behavior by the host-feeding egg parasitoid, *Ooencyrtus nezarae* (Hymenoptera: Encyrtidae): Host age effects. Ann. Entomol. Soc.

- of Am., 86 (1): 117-121.
- Tescari, G. (2001). *Leptoglossus occidentalis*, Coreide nearctic rinvenuto in Italia (Heteroptera, Coreidae). *Lavori Soc. Ven. Sc. Nat.*, 26: 3-5.
- Tiberi, R. (1980). Modificazioni della distribuzione dei parassiti oofagi in ovature di processionaria del pino conseguenti al potenziamento artificiale di *Tetrastichus servadeii* Dom. (Hymenoptera: Chalcidoidea). *Redia*, 63: 307-321.
- Tiberi, R. (2009). Controllo biologico degli insetti in foresta: Passato presente e futuro. *Atti Accademia Nazionale Italiana di Entomologia*, Anno LVII, 2009: 107-116.
- van Lenteren J.C. (ed) (2003). *Quality control and production of biological control agents: theory and testing procedures*. CABI Publishing, Wallingford, UK.
- van Lenteren, J.C., Godfray, H.C.J. (2005). European science in the Enlightenment and the discovery of the insect parasitoid life cycle in The Netherlands and Great Britain. *Biological Control*, 32: 12-24.
- van Lenteren, J.C. (2012). The state of commercial augmentative biological control: plenty of natural enemies, but a frustrating lack of uptake. *BioControl*, 57 (1): 1-20.
- van Lenteren J.C., Bueno VHP (2003). Augmentative biological control of arthropods in Latin America. *BioControl* 48:123-139.
- van Lenteren, J., Tommasini, M. (2002). Mass production, storage, shipment and quality control of natural enemies. In: Albajes, R., Gullino, M.L., van Lenteren, J.C., Elad, Y. (Eds.), *Mass Production, Storage, Shipment and Quality Control of Natural Enemies, Integrated Pest and Disease Management in Greenhouse Crops*. Springer, Netherlands, pp. 276-294.
- Venkatesan, T., Singh, S.P., Jalali, S.K. (2000). Effect of cold storage on cocoons of *Goniozus nephantidis* Muesebeck (Hymenoptera: Bethyridae) stored for varying periods at different temperature regimes. *Journal of Entomological Research*, 24: 43-47.
- Vinson, S.B. (1998). The general host selection behavior of parasitoid hymenoptera and comparison of initial strategies utilized by larvaphagous and oophagous species. *Biol. Control*, 11: 79-96.
- Voegele, J. (1966). "Biology and morphology de *Graphosoma semipunctata* Fabricius (Heteroptera, Pentatomidae). *Revue de la Recherche Agronomique Marocaine*.
- Voss, S.C., Spafford, H., Dadour, I.R. (2010). Temperature-dependent development of the parasitoid *Tachinaephagus zealandicus* on five forensically important carrion fly species. *Medical and veterinary entomology*, 24 (2): 189-198.
- Waage, J.K., Greathead, D.J. (1988). Biological control: challenges and opportunities. *Philos. Trans. R. Soc. Lond., B*, 318: 111-128.
- Wang, J.J., Wei, J.R., Wang, Y.Z., Zhang, Y.C. (2012). Relationship between temperature and development of *Ooencyrtus kuwanae* (Hymenoptera: Encyrtidae), the egg parasitoid of *Lymantria dispar* (Lepidoptera: Lymantriidae), with an evaluation of its offspring quality reared from eggs of the substitute host *Antheraea pernyi* (Lepidoptera: Saturniidae). *Acta Entomologica Sinica*, 55 (5): 570-574.
- Warner, K.D., Getz, C. (2008). A socio-economic analysis of the North American commercial natural enemy industry and implications for augmentative biological control. *Biological Control*, 45 (1): 1-10.
- Werner, D.J. (2011). Die amerikanische Konifer-Samen-Wanze *Leptoglossus occidentalis* (Heteroptera: Coreidae) als Neozoon in Europa und Deutschland. *Ausbreitung und Biologie*. *Entomol. Heute*, 23: 31-68.
- Weseloh, R. M. (1986). Effect of photoperiod on progeny production and longevity of gypsy moth (Lepidoptera: Lymantriidae) egg parasite *Ooencyrtus kuvanae* (Hymenoptera: Encyrtidae). *Environmental entomology*, 15 (6): 1149-1153.
- Zar, J.H. (2010). *Biostatistical analysis*, 5th ed., Prentice Hall, Englewood Cliffs, NJ.
- Zhu, G.P., Redei, D., Kment, P., Bu, W.J. (2013). Effect of geographic background and equilibrium state on niche model transferability: predicting areas of invasion of *Leptoglossus occidentalis*. *Biological Invasions*: 1-13.

Acknowledgments

My first and sincere appreciation goes to Pio Federico Roversi and Giuseppino Sabbatini Peverieri of CRA/ABP (Florence) who have kindly supervised me with their assistance during my “Insect Science PhD”. I am also grateful to Sauro Simoni, Fabrizio Pennacchio and Jacopo de Silva (CRA/ABP) for their strong support. Many thanks also to my colleagues Daniele Benassai, Sarah Caradonna and Muhamad Abidalla, for their precious help during my experiments. Finally I would like to express my gratitude to Ferdinando Bin (University of Perugia) and Guy Boivin, Saint-Jean-sur-Richelieu, Quebec (Canada), for their worthwhile advices in planning and managing my research.