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Enriched artificial diets for the predatory bug *Orius thripoborus* and the parasitoid fly *Exorista larvarum*

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FOREWORD

This is it. The very last words I write to top off the work I have done for the last 10 months. The last words in a seemingly neverending series of writing, correcting, writing, correcting, adjusting and correcting again. But also, the last words of the entire 4 and a half years I got to spend in the beloved 'Boerekot', and half year I got to spend in the vibrant city of Bologna.

There are some people I want to thank before I reach the absolute end. First of all, prof. dr. ir. Patrick De Clercq and prof. dr. Maria Luisa Dindo, together with prof. dr. Davide Viaggi, Hilde Vandecasteele, and the Universities of Ghent and Bologna in general, for giving me the opportunity to do a part of my thesis work in beautiful Italy, getting to know the ways and common uses of other laboratories and gaining experiences I will never forget.

I want to express even more gratitude towards prof. De Clercq and prof. Dindo for their help in correcting the thesiswork and helping me adjust to the new environment. You have both used the best of your capabilities to guide me in this foreign adventure, and to help me reach the deadline by correcting the texts during free time. Thank you.

I would like to thank all the people I got to work with during this entire year in both labs for making this whole ride a real pleasure. I will not name them all, but I want to point out three of them: Maurizio and Elisa from Bologna, for jumping in to do the lab work when I could not make it due to classes. You have made my time in Bologna so much more comfortable by giving up your own time. And Jochem from Ghent, for your neverending efforts in getting me through rough days, helping me with articles and statistics and giving up free time to help me as fast as you could. You managed to end every positive or negative message with a witty joke, and helped me with my English with hair on. You are the tutor everyone wishes for. The only thing I regret is that we weren't able to celebrate the championship of Club Brugge. Maybe next year!

Lastly, I want to thank my girlfriend Tina. In good and in bad times, you were always there to listen to me when something was going on with my thesis. The way you supported me towards taking this big step of going on Erasmus is unimaginable, I can't thank you enough for that. You have made my life so much brighter in so many ways. Before getting to sentimental, I will end with this: Team 27 forever! That's true, right?

Jonas Vandicke, June 2015

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INTRODUCTION

Today, most beneficial insects are still being mass reared on natural or factitious hosts/prey. This way the insect learns how to search for its victim in a natural and efficient way. However, maintaining these live hosts leads to high costs for labor involved in their production. Reducing costs of production could lower the market price of beneficial insects and increase the number of growers using biological solutions for pest management. Therefore there has been a trend towards eliminating the presence of live host insects in mass rearing cultures. Alternatives have been proposed as artificial diets/media devoid of insect components. These artificial diets can strongly reduce costs and facilitate automation of the production. Much research has been done towards developing artificial diets for a variety of predators and parasitoids.

However, the quality of an insect reared on an artificial diet devoid of insect components is often not as high as the quality of an insect reared on a natural or factitious host. Artificial diets may lack some specific growth factors or feeding stimulants typically found in natural hosts. Adding insect components, like hemolymph, to the artificial diet could solve this issue.

The aim of this thesis is to test whether the quality of arthropod natural enemies reared on an artificial diet may be increased by enriching the artificial diet with insect hemolymph. Two different types of hemolymph were tested: hemolymph of the black soldier fly *Hermetia illucens* L., and hemolymph of the Chinese oak silk worm *Antheraea pernyi* (Guérin-Méneville). *Hermetia illucens* has great potential for industrial-scale production due to several beneficial traits (e.g., short development cycle, high conversion rate, ability to develop on a variety of food sources,…)(Nguyen et al., 2013). *Antheraea pernyi* hemolymph is a byproduct of the silkproduction in China, but in recent years it has become less easily available and prices have increased (Lü et al., 2013). The hemolymph of these two insects was added to the artificial diet of a predator and a parasitoid.

Orius thripoborus (Hesse) was chosen as the predator to be studied in this thesis. *Orius* species are omnivores and can attack a variety of soft-bodied arthropods such as aphids, whiteflies, mites, lepidopterous larvae and small arthropod eggs. They are however mostly known for their worldwide use in the control of different thrips pests. *Orius thripoborus* is a southern African species that has been suggested as a biological control agent of several thrips pests, such as the western flower thrips, *Frankliniella occidentalis* (Pergande) and the sugarcane thrips, *Fulmekiola serrata* Kobus. Experiments on *O. thripoborus* were performed in the Laboratory of Agrozoology, Department of Crop Protection, **Ghent University**. In this part of the study, the artificial diet

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used to be enriched by insect hemolymph was an egg yolk diet developed by Arijs & De Clercq (2002).

In the second part of this thesis the parasitoid *Exorista larvarum* L. was studied. *Exorista larvarum* is a tachinid with a wide geographical range. Many of the natural hosts of this parasitoid are pest species. Promising results have been obtained in research aiming to use *E. larvarum* as a biological control agent of several caterpillar species such as *Peridroma saucia* (Hübner), *Spodoptera littoralis* (Boisduval), and *Lymantria dispar* L.. Experiments on *E. larvarum* were performed in the laboratories of Entomology of DipSA (Dipartimento di Scienze Agrarie, *Alma Mater Studiorum* **Università di Bologna, Italy**) in the framework of an **exchange program**. The basic artificial medium used in this part of the research was an egg yolk and skimmed milk medium described by Farneti et al. (1998).

Development and reproduction of both studied species were tested on their basic artificial diet and on the artificial diet enriched with either *H. illucens* hemolymph or *A. pernyi* hemolymph. For *O. thripoborus*, these results were compared with the performance of individuals reared on *Ephestia (Anagasta) kuehniella* Zeller eggs. *Ephestia kuehniella* eggs have proven to be a food of high quality for *O. thripoborus*, and are widely used in the laboratory culture of this predator. For *E. larvarum*, results for developmental and reproduction parameters were compared with those of individuals reared on *Galleria mellonella* L. larvae observed by Dindo et al. (2006). The larvae of *G. mellonella* are currently being used in the laboratory culture of *E. larvarum* in the laboratories of Entomology of DipSA.

PART I: LITERATURE STUDY

1. Foods for rearing arthropod natural enemies

1.1. Introduction

Due to the rising interest in biological control for crop pest management, the number of companies producing natural enemies, including predators and parasitoids, has increased (Riddick, 2009). These companies try to mass-produce natural enemies at the lowest price, while maintaining a high quality and field performance against the target pest (Thompson & Hagen, 1999; De Clercq, 2004). For each natural enemy an optimal rearing food has to be sought, all quality parameters taken into consideration. The potential foods are natural hosts/prey, factitious hosts/prey, or artificial diets/media.

1.2. Natural prey/hosts

The most obvious choice is to rear the insect on its natural host or prey (further called host in a general context), often being a herbivorous species. The natural host is usually being maintained on its host plant One advantage of rearing on natural hosts is that the predator/parasitoid will develop an efficient and natural way of searching for its host on the host plant (De Clercq, 2009). This means that this type of production contains three trophic levels (the natural enemy, the host and the host's food plant), which require separate spaces and equipment (and sometimes even whole facilities). This results in high investment and labor costs. (Etzel & Legner, 1999; De Clercq, 2004; Riddick, 2009; De Clercq et al., 2014).

Many natural hosts used in parasitoid rearing are being cultured on an artificial diet (Cohen, 2004). The tachinids *Lydella minense* (Townsend) and *Paratheresia claripalpis* van de Wulp for instance are reared on their natural host *Diatraea* spp., which can be reared on an artificial diet instead of its natural food, sugarcane (Rossi & Fowler, 2003). Successful rearing has been achieved with many tachinid natural hosts but also with other dipteran parasitoid hosts (Dindo & Grenier, 2014). For some species a natural rearing system is economically viable, e.g., for the

parasitoid *Encarsia formosa* Gahan on tobacco plants infested with the greenhouse whitefly, *Trialeurodes vaporarorium* (Westwood) (De Clercq, 2004).

Host food is a key factor in a successful parasitoid rearing system, since it may influence parasitoid survival and quality (Thompson & Hagen, 1999). Much of the natural hosts are generalists, which means there can be a large variety in nutritional quality of the prey they attack in their natural habitats. This leads to variation in the nutritional quality of the natural host itself. Therefore, when rearing a parasitoid on natural hosts, there should be variation in the prey offered to the natural host to create an optimal diet. (Richman & Whitcomb, 1978; Evans et al., 1999; Lundgren, 2011).

1.3.   Factitious prey/hosts

Factitious prey/hosts are organisms that normally would not be attacked by the insect (mostly because they do not occur in its natural habitat), but do sustain its development. The main reason for including these factitious hosts in rearing systems is because they are easier and less expensive to rear than the natural ones (De Clercq, 2004). The adaptability of a beneficial insect to a factitious host depends on its feeding habits. A polyphagous species is more likely to be able to survive and reproduce on a factitious host, whereas oligophagous or monophagous species are less likely to do so (Riddick, 2009).

When searching for a potential factitious host, the first experiments should always apply rearing conditions that are similar to the natural conditions of the insect, using a natural host, to be able to obtain information about its basic biology, physiology and behavior. When this information is obtained, a factitious host may be considered (Dindo & Grenier, 2014). An example of this working structure is the research done on the dipteran parasitoid *Phryxe caudata* (Rondani), which was first reared on its natural host *Thaumetopoea pityocampa* (Denis and Schiffermuller) (Billiotti, 1956) and later on *G. mellonella,* which was easier and safer to rear (Delobel & Laviolette, 1969). For several parasitoids, the larvae of *G. mellonella* have been used as a factitious host. *Galleria mellonella* larvae offer many of the nutritional requirements needed for full development of many tachinids and hymenopteran parasitoids (Campadelli, 1988), and entomopathogenic nematodes (Ehlers & Shapiro-Ilan, 2005). They can be reared on an artificial diet without water, making it less susceptible to become contaminated with molds, thus eliminating the use of fungicides in the diet (Mellini & Coulibaly, 1991).

Many examples are known of factitious prey being used to rear predators instead of natural prey. Riddick (2009) and De Clercq et al. (2014) discussed many of these examples. In summary, lepidopteran eggs (*E. kuehniella*, *Plodia interpunctella* (Hübner), *Corcyra cephalonica* (Stainton), *Sitotroga cerealella* Olivier), decapsulated brine shrimp cysts (*Artemia franciscana* Kellogg), yellow mealworm pupae (*Tenebrio molitor* L.), and house fly larvae (*Musca domestica* L.), used as factitious prey generally showed positive effects on the development and survival rate of predators, in comparison to natural prey. Fecundity and body size even increased in some cases. These results lead to the conclusion that factitious prey can be effectively used in the production of predators. Factitious prey can be offered fresh, but often they are frozen, irradiated or lyophilized for storage purposes.

Several studies (e.g., Fauvel et al., 1987; Cocuzza et al., 1997a; Specty et al., 2003; De Clercq et al., 2005a and Bonte & De Clercq, 2008) show that some insect predators perform better when reared on *E. kuehniella* eggs than on some of their natural prey, leading to the conclusion that this factitious food is nutritionally superior. Vangansbeke et al. (2013) found that the predatory mite *Amblydromalus limonicus* Garman and McGregor can be efficiently reared on lepidopteran eggs and brine shrimp cysts, however further research over multiple generations was needed. In some cases factitious prey has even become the standard food, e.g., *E. kuehniella* eggs for the production of *Orius* species (Richards & Schmidt, 1995; van den Meiracker, 1999; Tommasini et al., 2004; Kakimoto et al., 2005; Bueno et al., 2006; Bonte & De Clercq, 2008; Venkatesan et al., 2008).

A disadvantage of the continuous use of lepidopteran eggs as a factitious prey is their high cost, which is caused by high investments in mechanization of rearing procedures, healthcare for the workers, and climate management. Due to these costs, market prices have risen up to €1000 per kg of *E. kuehniella* eggs (Koppert, 2015). Another problem is that the continuous demand and occasional rearing problems have led to periodic shortages (Arijs & De Clercq, 2001a; De Clercq et al., 2014).

1.4. Artificial diets/media

Artificial diets/media (further called diets in a general context) can be an alternative for natural or factitious hosts for the mass rearing of insects, reducing costs and facilitating automation of the production. To be able to create these diets, the feeding mechanism and digestive system of the insect should be known, as well as the nutritional requirements of the insect and the biochemical composition of its natural food . However, much of the successes with artificial diets

were based on a simple trial-and-error approach. An artificial diet should contain all basic nutrients (proteins or amino acids, lipids, carbohydrates) in balanced proportions. Besides, some minor components can be added as growth factors (sterols, vitamins, minerals, nucleic acids,...). The diet should be presented in a way that makes it acceptable for the insect to feed or oviposit on it (see 1.4.4) (De Clercq, 2004; Dindo & Grenier, 2014).

1.4.1. Definitions

Efforts have been made to classify artificial diets. One of these classifications defines three types of artificial diets. A holidic diet is one of which the chemical structure of all ingredients is known. A meridic diet has a holidic basis but at least one of the components has an unknown structure or purity. An oligidic diet consists of mainly crude organic materials (Dougherty, 1959).

The issue with this classification is that the distinction is often not clear, and that only a complete description of the composition of the diet would be able to characterize it. Therefore a new classification has been proposed by Grenier & De Clercq (2003), classifying a diet by the presence or absence of insect components (De Clercq, 2004; Riddick, 2009; De Clercq et al., 2014).

1.4.2. Different types of artificial diets/media

Many artificial diets have been tested for their nutritional value and the adaptiveness of the reared insect to this diet. De Clercq et al. (2014) gave an overview of several artificial diets for predators. Most of them contained vertebrate protein from beef (beef liver, ground beef), chicken (whole hen's egg or egg yolk, chicken liver), pig (pork liver), and in one case, tuna. Besides proteins, other nutrients can be important in the potential use of an artificial diet. For instance, beef liver contains high levels of vitamins (Arijs & De Clercq, 2001b), and egg yolk is rich in amino acids, phospholipids, minerals and vitamins (Stadelman, 1992).

A major drawback of artificial diets based on meat products is their perishability, caused by autolytic activity, growth of bacteria, and to a lesser extent growth of yeasts, fungi and viruses. This spoilage is even more a problem when the diet is exposed to the high rearing temperatures of many insects (20-25 °C) (Gill, 1983; Hernandez-Herrero et al., 1999). A possible solution is the addition of antibiotics. Arijs et al. (2002) found that a 0.05% addition of gentamycin sulfate to the artificial diet used for *Orius laevigatus* (Fieber) suppressed bacterial growth in the diet, without influencing the insect's development.

De Clercq et al. (2014) found that artificial diets were in many cases not optimal as a stand-alone food source for the rearing of predators. Development time and reproduction were overall lower compared to factitious prey or natural prey. However, several studies showed that the ability of a predator to switch to natural prey after long-term rearing on inanimate artificial diets was not affected compared to counterparts that were reared on nutritionally optimal factitious prey.

Dindo & Grenier (2014) summarized which nutritional and other requirements need to be fulfilled when developing an artificial medium for a dipteran parasitoid. Proteins, peptides and free amino acids can be provided as a nitrogen source. The medium should have a balanced composition to avoid loss of energy and time in conversion metabolism, since dipteran parasitoids grow fast during their larval stages (Grenier, 1980). Free fatty acids or triglycerides can be added as a lipid source, but require surfactants when added to a water-based medium. Adding lipids to the medium improves survival and fecundity. However, free fatty acids are toxic for some species (Thompson, 1977), and surfactants may affect larval respiration negatively by modifying the cuticle near spiracles (Grenier et al., 1994). Thus, an assessment needs to be made for each individual species. Trehalose or sucrose may be added to the medium as a source of carbohydrates (Cohen, 2004). Next to the three basic nutrient types (proteins, lipids and carbohydrates), also vitamins, organic acids, nucleic acids and minerals can be added. The requirements are similar to most insects, but are not well examined for dipteran parasitoids (Dindo & Grenier, 2014).

The artificial medium for endoparasitoids not only represents their food but also their environment for larval development. This means that compared to predators or ectoparasitoids more attention needs to be paid to factors such as osmotic pressure, oxygen supply and acidity of the medium (Grenier er al., 1994; De Clercq, 2004). For many tachinids, the pH of the medium should be fixed between 6,5 and 7,5 (Nettles, 1986), and the osmolarity of the medium is optimal between 350 and 450 mOsm (Grenier, 2012).

The only dipteran parasitoids that have been reared continuously *in vitro* with complete exclusion of the host are sarcophagid flies, such as *Sarcophaga aldrichi* Parker. Larvae were deposited directly on the medium containing pork liver and fish and were reared for several generations (Arthur & Coppel, 1953). No species of the Tachinidae have been reared continuously *in vitro* (Dindo et al., 2007; Dindo & Grenier, 2014).

1.4.3. Addition of insect components

The addition of insect components in an artificial diet can be useful for the insect to acquire certain growth factors or feeding stimulants typically found in their natural host. Adding material of the natural (or factitious or even non-permissive) host can strongly improve the yield and quality of several parasitoids reared *in vitro*. Nettles (1990) suggested that some species of parasitoids are even dependent on host material. Insects used as an addition need to be easily culturable to reduce extra production costs. Insect components vary from whole-body tissue extracts to small volumes of host hemolymph (De Clercq, 2004; Riddick, 2009).

The hemolymph of lepidopterous pupae, such as *Manduca sexta* L. (Bratti & Nettles, 1988), *G. mellonella* (Dindo et al., 1997), *Spodoptera frugiperda* (J.E. Smith) (Ferkovich et al., 2000), *Antheraea* spp. (Lü et al., 2013; Tan et al., 2013; Nguyen et al., 2014) etc. can be used in artificial diets for parasitoids and predators. This is applied to larval parasitoids such as *Palexorista laxa* (Curran) (Bratti & Nettles, 1988), *Brachymeria intermedia* (Nees) (Dindo et al., 1997), *Diapetimorpha introita* (Cresson) (Ferkovich et al., 1999 & 2000), or *E. larvarum* (Dindo et al., 1999), as well as to oophagous parasitoids, such as *Trichogramma* spp. (Grenier, 1994; Lü et al., 2013) and to predators such as *Orius sauteri* (Poppius) (Tan et al., 2013) and *Amblyseius swirskii* Athias-Henriot (Nguyen et al., 2014). Mostly hemolymph of silkworm species is used, which is cheap and easily obtainable, but also hemolymph of other easily reared insects can be used (Grenier & De Clercq, 2003).

An alternative for natural insect components is the use of insect cells produced in vitro. This reduces the labor and problems related to the production of natural insect components (e.g., melanization) (Grenier & De Clercq, 2003; De Clercq, 2004). Lynn & Ferkovich (2004) developed a diet for *Orius insidiosus* (Say) supplemented with a cell line from *E. kuehniella* embryos. Ferkovich & Shapiro (2007) later developed a diet based on an embryonic cell line derived from *P. interpunctella* eggs. Adding these cells to the diet significantly improved fecundity and survival rates of *O. insidiosus* adults. This approach has also been tested on several parasitoids, such as *Lysiphlebus fabarum* (Marshall) with a *Ceratitis capitata* (Wiedeman) cell line (Rotundo et al., 1988), *Edovum puttleri* Grissell on several cell lines (Hu et al., 1999), etc.

1.4.4. Presentation

An important aspect in the acceptance of an artificial diet in mass rearing of insects is its presentation. Factors influencing optimal presentation are phagostimulants, texture, state of the ingredients, and methods of containment (Cohen & Staten, 1993; Grenier et al., 1994; Cohen,

2004). For predators with chewing mouthparts the artificial diet can be provided ad libitum (Bonte & Samih, 2010). In the case of predators with piercing-sucking mouthparts , artificial diets are mostly encapsulated in Parafilm® domes, using an encapsulation device (Greany & Carpenter, 1998). Encasement reduces desiccation and spoilage, and may serve as a barrier against microbial attack. The Parafilm® coating should provide a firm surface that allows the predator to penetrate the dome using its mouthparts and feed on the diet. However, one should pay attention on the amount of stretching of the Parafilm®. Above that, the effect of membrane chemicals on predator development is not well understood. The use of other forms of encapsulation, for instance Hydrocapsules® or microcapsules, should be researched (Riddick, 2009; Tan et al., 2013).

Another point of interest is the size of the domes. Ferkovich et al. (2007) studied the impact of the size of the diet domes on egg production of *O. insidiosus.* Oviposition was highest when females were fed 10 μl domes, and lowest when fed 50 μl domes. This was explained by the fact that the digestive enzymes that are injected into a prey were significantly diluted in the larger domes (Ferkovich et al., 2007; Bonte & De Clercq, 2010b).

The format of the diet also has an impact on the feeding habits of the reared predatory insect. *Geocoris punctipes* (Say) fed longer on flattened packets made from stretched Parafilm® than on cylindrical artificial 'larvae' (Cohen & Staten, 1993).

For parasitoids, the medium may be presented as a liquid or gel, or can be supported by absorbent cotton. Maintaining larval respiration is one of the key factors in choosing the presentation of the medium (Dindo & Grenier, 2014). Mellini & Campadelli (1996c) found that for *E. larvarum* the compactness of the medium is of great importance. If it is too soft, the newly hatched larvae sink and eventually die from asphyxia. If it is too dense, the larvae feed difficultly, which delays their development and leads to puparia with a low weight and size.

2. *Orius thripoborus* (Hesse)

2.1. Taxonomy and identification

The classification of *Orius* spp. is shown in Figure 1.

Order: Hemiptera
    Suborder: Heteroptera
        Subgroup: Geocorisae
            Infraorde: Cimicomorpha
                Superfamily: Cimicoidea
                    Family: Anthocoridae
                        Subfamily: Anthocorinae
                            Tribe: Oriini
                                Genus: *Orius* (Wolff)
                                    Subgenus: *Orius*
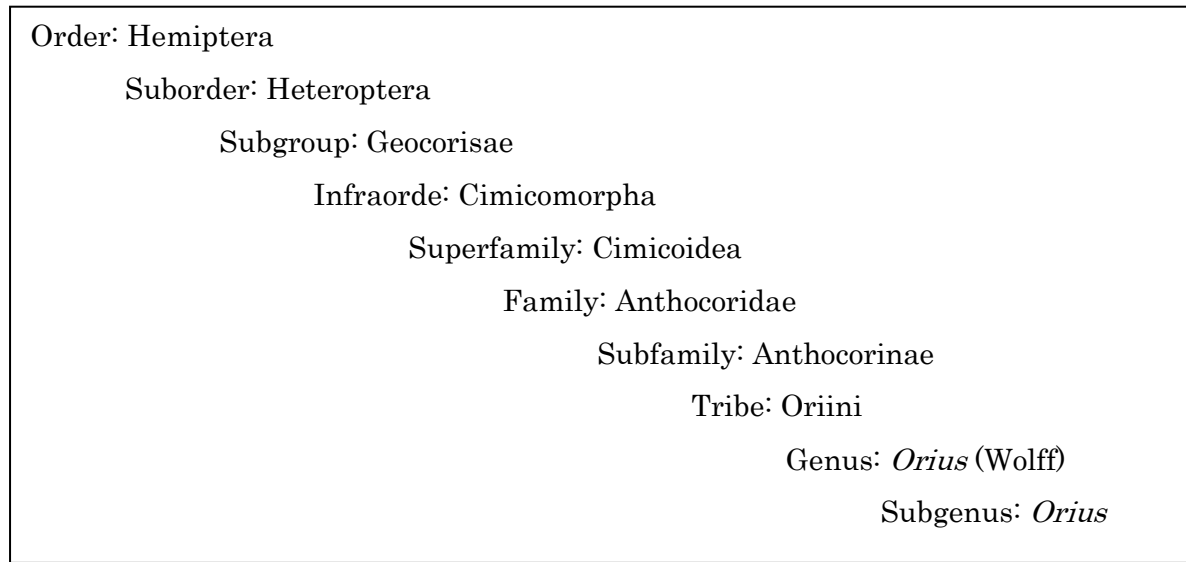
Figure 1: Classification of the subgenus *Orius*

The *Orius* genus belongs to the order of the Hemiptera. This order is characterized by its piercing-sucking mouthparts. The rostrum is formed by the labrum and labium, while the transformed mandibulae and maxillae form a suction tube and salivary duct. Hemiptera have two pairs of wings in the adult stage, however some of them are wingless (Tirry, 2013).

The Hemiptera can be divided into two suborders: the Homoptera and the Heteroptera. *Orius* belongs to the Heteroptera, which are characterized by hemelytra or forewings, of which the anterior part is hardened and the posterior part is membranous. The rostrum is implanted into the front of the head and the wings are held flat on the back of the dorsoventrally flattened body (Tirry, 2013).

Orius belongs to the Anthocoridae family, also called 'flower bugs' or 'minute pirate bugs'. They differ from other heteropterans by their small size (1.4–4.5 mm), three-segmented straight labium, fore wing with clear cuneus, lack of closed cells in the hemelytral membrane, and characteristic structure of the male and female genitalia (Figure 2). Segments six to eight of the male abdomen are very asymmetrical, while the female ovipositor is symmetrical (Schuh & Slater, 1995). They are very similar to the Miridae in terms of external appearance, but are easily recognized by the ocelli on the dorsal surface of the head (Hernández & Stonedahl, 1999).

Flower bugs generally oviposit into plant tissues and are mostly predators, however some consume plant foods as well, or are even entirely phytophagous. They are common to a wide variety of habitats and have functional wings (Lattin, 1999).
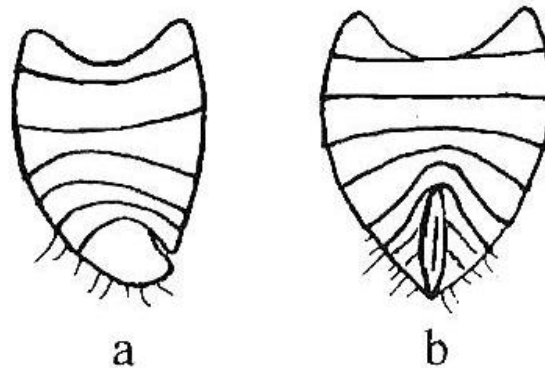
Figure 2: Ventral view of the abdomen of a male (a) and female (b) *Orius* sp. (Maes, 2009)

The genus *Orius* Wolff consists of over 70 species distributed throughout the world (Péricart, 1972). There are many ways of identifying this genus, with the coloring of the wings being one of them. However, the most important (and also the most time consuming) way of characterizing *Orius* species is by comparing the male and female genitalia (Malais & Ravensberg, 2003).

Orius thripoborus can be distinguished from other *Orius* species by its smaller size, dark brown to black cuneus, narrow peritreme of the metathoracic scent efferent system, and the structure of the male and female genitalia. Male genitalia are distinguished by a strongly curved flagellum (a) (Figure 3) while the female copulatory tube opens ventrally on the intersegmental membrane. Female genitalia are short and smooth and are composed of two parts: the basal segment and the apical tube. For *O. thripoborus* females, the basal segment is long and curved (c), and the apical tube is quite short (d) (Figure 4) (Hernández & Stonedahl, 1999).

Orius thripoborus can be found mainly in the eastern part of South Africa (Hernández & Stonedahl, 1999), but has also been observed in Saint Helena (Carayon, 1976) and Kenya (Van den Berg & Cock, 1995).
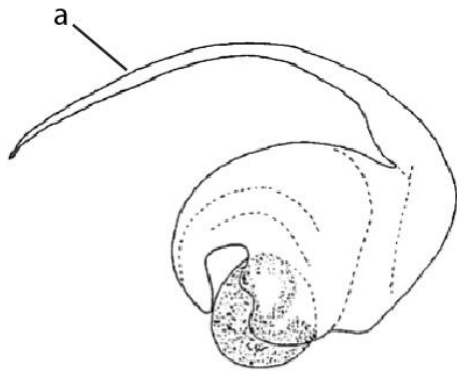
Figure 3: Male genitalia of *O. thripoborus* (Hernández & Stonedahl, 1999)
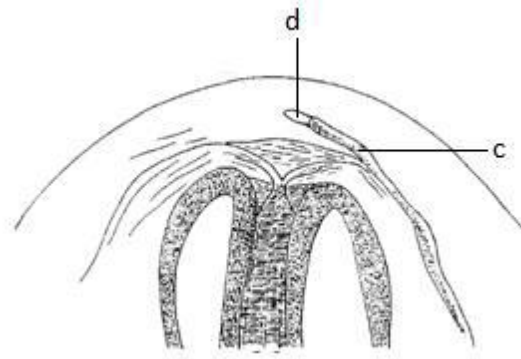
Figure 4: Female genitalia of *O. thripoborus* (Hernández & Stonedahl, 1999)

2.2.   Life cycle of Orius spp.

Orius species have seven stages of development: an egg stage, five nymphal stages, and an adult stage (Malais & Ravensberg, 2003) (Figure 5).
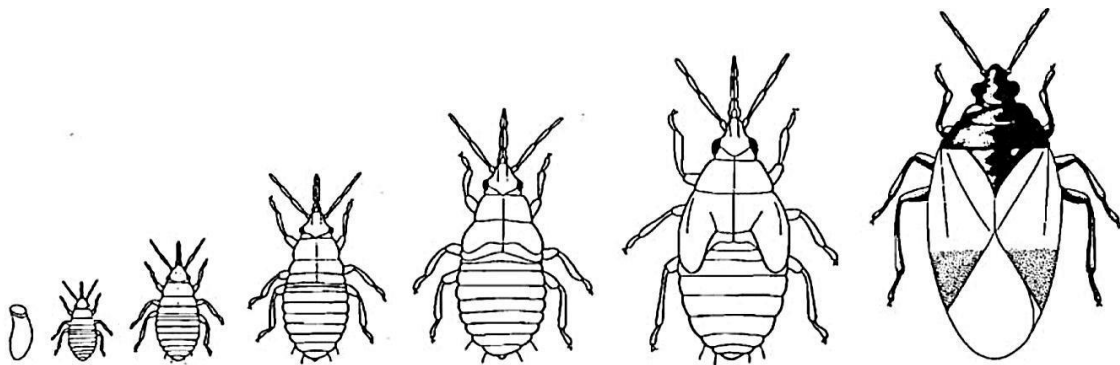
Figure 5: 7 stages in the life cycle of *Orius* sp. (Malais & Ravensberg, 2003)

2.2.1.   Egg

A newly laid egg has a length of ca. 0.4 mm and a width of ca. 0.13 mm. It initially has no color, but later gets a milky white color. Eggs are embedded in the plant tissue, mostly separately, sometimes in small groups by a female adult using her ovipositor. Oviposition sites can differ between species: eggs are often oviposited in the leafstalk or in the lower side of the midrib, but can also be laid in flowerparts. The eggs are hardly visible because they are positioned at the same level as the plant tissue; only the operculum is visible (Malais & Ravensberg, 2003). Eggs hatch after 4-5 days (Applied Bio-nomics, 2013) (Figure 6).

Figure 6 : Eggs of *O. thripoborus* in bean pod tissue (Van De Walle, 2014)

2.2.2. Nymph

Orius spp. are hemimetabolous, which means that nymphs resemble adults but lack functional wings or reproductive organs, and gradually change after each molt (Tirry, 2013). From the very moment the nymphs hatch, the characteristic red ocelli are clearly visible. The color of the nymphs evolves from colorless to yellow a few hours after hatching. In the later nymphal stages the color varies from completely yellow to completely brown, depending on which stage and species is observed (Malais & Ravensberg, 2003). Nymphs of *O. thripoborus* retain their yellow color during all nymphal stages but have additional orange spots in the later stages (Figure 7). Wing development begins in the second stage, but the wings are not visible until the final stage (Malais & Ravensberg, 2003). The development through five nymphal stages takes about 2-3 weeks (Applied Bio-nomics, 2013).

Figure 7: Second (left), third (central) and fifth (right) nymphal stage of *0. thripoborus* (Van De Walle, 2014)

2.2.3. Adult

Right after the final molt, the color of the adults is yellow, but this changes after a few hours when they get their characteristic colors: brown-black with light grey or brown areas on the

wings. The coloring of male and female adults is identical. The wings are deployed about one hour after molting (Malais & Ravensberg, 2003). Adults of *O. thripoborus* have a length of 1.5-2 mm, with female adults being slightly larger than male adults (Hernández & Stonedahl, 1999). Adults live for 3-4 weeks. They can move fast (Applied Bio-nomics, 2013) (Figure 8).

Figure 8: A male adult of *Orius thripoborus* (Van De Walle, 2014)

Immediately after molting, mating begins. Females will lay 2-3 eggs each day on average, starting from 2-3 days after mating. Reproduction is strongly dependent on the temperature (see 2.2.4) and feeding ecology (see 2.3). The lifetime amount of eggs oviposited ranges from 50 to 150 (Applied Bio-nomics, 2013; Malais & Ravensberg, 2003). Leon-Beck & Coll (2009) found females of *O. laevigatus* to be monandrous, avoiding further mating after their first copulation. Males on the other hand were found to be polygynous, however their first mating partner usually lays significantly more eggs than the subsequent females.

Most *Orius* species are prone to diapause when day lengths shorten. In the North-West of Europe, the mated females hide in trees and plants and hibernate from September until April. The photoperiod at which females will go into diapause varies within species and region (Malais & Ravensberg, 2013).

2.2.4.   Thermal biology

The ecology of *Orius* spp. is influenced by a variety of environmental factors, with temperature being one of the most important (Jervis & Copland, 1996; Obrycki & Kring, 1998; Cocuzza et al., 1997b). However at various climatic conditions, differences in terms of development time, survival and reproduction between different *Orius* spp. are limited (Malais & Ravensberg, 2003). Bonte et al. (2012a) studied the effect of temperature on development time, survival, adult weight, reproduction and longevity of *O. thripoborus.* Overall, 25°C appeared to be the optimal rearing temperature, in combination with *E. kuehniella* eggs and a green bean pod. This resulted

in fast development (17.4 days for males, 17.0 for females), good nymphal survival (85%), and a high reproduction (100.7 eggs, of which 66% hatched).

2.3.   Feeding ecology

The Anthocoridae family consists of omnivorous insects, surviving on prey as well as on plant material. They are polyphagous predators of small insects, mites and other arthropods. When plant material is used as food no severe plant damage occurs (Malais & Ravensberg, 2003).

All Hemiptera, including *Orius* spp., use extra-oral digestion (EOD), which means that food is chemically pretreated to increase its nutrient quality or accessibility. Several types of EOD are known. Hemiptera are type 1 non-refluxers, i.e. the chemical liquefaction of the prey happens entirely within the prey's body which turns the prey's exoskeleton into an extension of the predators gut, while there's a one-way flow of digestive enzymes (Cohen, 1995).

2.3.1.   Natural prey

Orius spp. are predators of a series of small arthropods such as thrips, aphids, aleyrodids, young lepidopterous larvae and mites. They are fast predators, and localize their prey by scent and touch rather than by sight. Their antennas play an important role in the detection of prey movement. When the prey population is dense, more prey is consumed than nutritionally needed. Factors influencing prey selection include vulnerability, type, density, and mobility (Malais & Ravensberg, 2003).

Despite *Orius* spp. being polyphagous, they show a preference for thrips (Salas-Aguilar & Ehler, 1977; Malais & Ravensberg, 2003). This can be an inherent prey preference or a preference driven by overlapping habitats (Cloutier & Johnson, 1993; Hansen et al., 2003). Baez et al. (2004) found that *0. insidiosus* can be an efficient predator of different life stages and species of thrips, in various environments. When selecting prey from different life stages, larvae will be preferred, because thrips adults are more mobile.

2.3.2. Factitious prey

As explained in part 1.3, *E. kuehniella* eggs have become the standard food for the production of *Orius* spp. (Richards & Schmidt, 1995; van den Meiracker, 1999; Tommasini et al., 2004; Kakimoto et al., 2005; Bueno et al., 2006; Bonte & De Clercq, 2008; Venkatesan et al., 2008). The nutritional value of *E. kuehniella* eggs exceeds that of pollen or other alternative diets (Bonte & De Clercq, 2010a). It is important to determine the minimum amount of diet needed for one individual to complete its development and achieve acceptable survival and reproduction rates to minimize rearing costs. Yano et al. (2001) found that 30 *E. kuehniella* eggs each four days were sufficient to complete nymphal development, and that 20 eggs each four days were needed for the adult stage. Lower amounts of diet affected survival rate, development time and body weight of both males and females.

As prices of *E. kuehniella* eggs are high (see 1.3), alternatives are being searched. A possible alternative factitious food are cysts of the brine shrimp *A. franciscana*. Arijs & De Clercq (2001a) found that feeding *O. laevigatus* on decapsulated cysts was a viable alternative for *E. kuehniella* eggs, in terms of development, adult weight and reproduction. However, De Clercq et al. (2005b) found that rearing *O. laevigatus* for three consecutive generations on *Artemia* cysts resulted in an increased development time and decreased fecundity. Therefore *Artemia* cysts cannot be used as a sole food in long-term mass rearing of *O. laevigatus*, but they are currently used only in part of the life cycle or in a mixture with lepidopteran eggs (Bonte & De Clercq, 2008; De Clercq et al., 2014).

Another potential factitious food are eggs of the medfly *C. capitata*. *Ceratitis capitata* eggs fed to *O. thripoborus* led to similar development time, body weight and reproductive rates compared to *E. kuehniella* eggs. The percentage of couples laying eggs was even higher. However, nymphal survival and egg hatch rate were lower. Prices of medfly eggs are slightly lower than those of *E. kuehniella* eggs, but increased demand can further lower prices, making this an economically viable alternative (Van De Walle, 2014).

2.3.3. Artificial diets

Bonte & De Clercq (2010a) studied the effect of an artificial diet based on the chemical composition of *E. kuehniella* eggs on the development of *O. laevigatus*. It was found that *E. kuehniella* eggs were nutritionally superior to the artificial diet. A possible explanation was that the studied population had been reared on *E. kuehniella* eggs for multiple generations, and adaptation had occurred to that factitious food.

Arijs & De Clercq (2004) compared several artificial diets based on meat and liver with *E. kuehniella* eggs for the rearing of *O. laevigatus*. Nymphal development was slightly slower on an artificial diet than on *E. kuehniella* eggs (15.0-15.9 days compared to 14.3 days), and nymphal survival was lower as well (68-93% compared to 96%). Fecundity of females fed the liver-based artificial diet was equal or slighty lower than of those fed *E. kuehniella*, and oviposition rate and egg hatch were equal among all diets.

Venkatesan et al. (2008) tested a variety of diets containing natural or artificial food on *Orius tantillus* (Motschulsky)*. It was found that in the absence of host eggs, *O. tantillus* could be efficiently reared on an artificial diet in combination with maize pollen and green bean pods. Bonte & De Clercq (2008) studied the developmental and reproductive fitness of *O. laevigatus* when fed six different diets: two factitious (*E. kuehniella* eggs and *Artemia* cysts) and four artificial foods. Adults fed the factitious foods performed better than those fed an artificial diet. Among the artificial diets, a diet based on liver and ground beef resulted in better developmental and overall fitness than egg yolk based meridic diets.

2.3.4. *Cannibalism*

When prey is scarce, *Orius* spp. need to maintain their population using various strategies. One of these strategies is cannibalism (Dong & Polis, 1992; Malias & Ravensberg, 2003).

To be able to prevent cannibalism, several measures can be taken. Shelter materials can be placed into the cage, such as paper (Chambers et al., 1993; Arijs & De Clercq, 2001a), wax paper (Bonte & De Clercq, 2010a), mesh sheets (Shimizu & Kawasaki, 2001), rice grains (Ito & Nakata, 1998) and wheat grains (Ito, 2007). Nymphal population density should be kept low to prevent mortality caused by cannibalism, competition for food and space, and susceptibility to pathogens (Bonte & De Clercq, 2010a). No correlation was observed between the nutritional value of the presented food and the rate of cannibalism (Leon-Beck & Coll, 2007).

2.4. Plant materials

2.4.1. *Oviposition and living substrate*

An optimal oviposition substrate is important for the success of mass rearing systems. *Orius* females oviposit their eggs in the available plant tissue on the surface level. They will first select the plant that has the thinnest external tissue. When the plant has been chosen they will start

searching for spots with low trichome densities and epidermis thickness. This is mainly to provide the progeny with easily accessible plant material as food source (Lundgren & Fergen, 2006; Lundgren et al., 2009).

In most *Orius* mass rearing systems green bean (*Phaseolus vulgaris* L.) is used as an oviposition medium. However, this is not an optimal substrate because of its perishability, limited seasonal availability, and the risk of contaminating the colony with pathogens or pesticide residues (Castañe & Zalom, 1994; Murai et al., 2001). Other plant material such as small rooted sharp pepper plants, cotton seedlings, geranium stems, or broad bean seeds can also be used (Chyzik et al., 1995; Cocuzza et al., 1997a; Nagai, 1997; Vacante et al., 1997; Murai et al., 2001). Most plant materials have the disadvantage of requiring extra space and labor and thus creating extra costs. Broad bean seeds are herein an exception, being available throughout the year and not requiring much space or equipment for growing (Murai et al., 2001).

To overcome these disadvantages, artificial oviposition substrates could provide valuable alternatives for plant material. Castañe & Zalom (1994) proved that a medium made from carrageen salt of potassium chloride covered with paraffin wax can be used as an alternative. However, plant material also has the function of providing natural shelter to the insects. Taking away hiding spots could lead to greater stress and overall reduced fitness of the insect (Bonte & De Clercq, 2010b). This problem can be solved by placing shelter materials in the cages, as has been explained in 2.3.4.

2.4.2. Moisture source

Orius spp. are facultative phytophagous predators. *Orius thripoborus* nymphs and adults have been observed probing into plant tissue, presumably to take up the moisture (Askari & Stern, 1972; Vacante et al., 1997). When feeding on the plant tissue, *O. insidiosus* was found to feed primarily on xylem sap and mesophyll tissue, and rarely on the phloem. This leads to the conclusion that facultative phytophagy provides the insect primarily with water, but it can also offer some dilute nutrients (see 2.4.3) (Armer et al., 1998).

Bean pods are often used as a moisture source. Bonte et al. (2012b) compared nymphal survival of *O. thripoborus* and *O. naivashae* fed *E. kuehniella* eggs when provided a bean pod or a Parafilm® water dome. Those provided with a bean pod had a 30% higher nymphal survival than those provided a water dome.

Pollen can be offered as a nutrient and moisture source, since pollen are able to take up water from a humid environment into their interior due to capillary effects (Diehl, 2001). Most *Orius* spp. can even survive on pollen alone, however the resulting development time and fecundity are very poor (Salas-Aguilar & Ehler, 1977; Kiman & Yeargan, 1985; Bonte & De Clercq, 2010a; Bonte et al., 2012b).

2.4.3. Extra nutrients

When the nutritional value of an (artificial) diet is not optimal, plant material can be used as a food supplement. This can be seen as an adaptive strategy to sustain the population while prey numbers are low (Cocuzza et al., 1997a). Plant material can offer additional sugars, starches and amino acids (Armer et al., 1998).

Access to a bean pod has been found to have a positive effect on development of *O. laevigatus* (Bonte & De Clercq, 2010a). Cocuzza et al. (1997a) studied the effect of adding pollen to a diet of *E. kuehniella* eggs or feeding on pollen alone for *Orius albidipennis* (Reuter) and for *O. laevigatus* in terms of reproduction. Results were best for the combined diet (fecundity: 152.3 and 187.9 eggs for *O. albidipennis* and *O. laevigatus*, respectively), while feeding on pollen only was suboptimal (fecundity: 38.3 and 79.2 eggs, respectively). *Orius laevigatus* showed less variety in fecundity at different feeding regimes, which makes it a better candidate for biological control programs,   however   other   factors   need   to   be   considered   as   well   (see   2.5).

2.5.   Use as biological control agents

In many agricultural and horticultural crops *Orius* spp. are important natural enemies of a variety of pest species such as aleyrodids, aphids, lepidopterous larvae, thrips and mites (Hernández & Stonedahl, 1999), but they have mainly gained attention for their capability of controlling the western flower thrips, *F. occidentalis* (Cocuzza et al., 1997a) and the onion thrips, *T. tabaci*. These are economically important pests that are difficult to control with pesticides. Thrips species damage the crop by sucking out plant cells, leading to empty air-filled cells with a silver shining. This causes deformations, discoloration, and cosmetic damage to fruit, flowers and leaves (Biobest, 2015). Many thrips species can also be a vector of viruses, e.g., *F. occidentalis* for the tomato spotted wilt virus (Broadbent et al., 1987).

There are many advantages of using *Orius* species as biological control agents (Applied Bionomics, 2013; Biobest, 2015). *Orius* species are polyphagous and are able to feed on plant

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material, which enables their preventive use in pollen producing crops. These predators feed on all life stages of thrips and often kill more prey than needed to survive. *Orius* species are good flyers and locate prey efficiently. They have a small size, allowing them to prey on thrips between touching fruit (Dennill, 1992). *Orius* species can easily be combined with other biological control agents. A disadvantage of using *Orius* species as biological control agents is that most species are prone to diapause when day length shortens, so supplemental lighting may be needed when used in protected cultivation. Cannibalism is a recurring problem between adults or between adults and nymphs. During shipping this can be prevented by providing hiding places in the containers, e.g., by adding buckwheat hulls or other materials (Applied Bionomics, 2013). The shelf life of these containers is quite low. *Orius* species are also affected by several chemical pesticides, such as teflubenzuron and imidacloprid (Biobest, 2015).

Orius spp. can be used preventively at a 5000-10000 *Orius*/ha density in pollen-bearing crops such as sweet pepper, strawberries, egg plants,... In non-pollen producing crops such as cucumber, *Orius majusculus* (Reuter) can be used as this species can feed on a larger variety of prey and plant juice (Applied Bio-nomics, 2013; Biobest, 2015). *Orius* spp. are often used in combination with predatory mites (Malais & Ravensberg, 2003). They can also be released curatively at 5-10 *Orius*/m² near local pest outbreaks (Biobest, 2015).

3. *Exorista larvarum* L.

3.1. Taxonomy and identification

The classification of the genus *Exorista* Meigen is shown in Figure 9 (McAlpine, 1987; Tree of Life Web Project, 2007)

```
Order: Diptera
        Suborder: Brachycera
                Infraorder: Muscomorpha (= Cyclorrhapha)
                        Section: Schizophora
                                Subsection: Calyptratae
                                        Superfamily: Oestroidea
                                                Family: Tachinidae
                                                        Subfamily: Exoristinae
                                                                Tribe: Exoristini
                                                                        Genus: *Exorista* Meigen
```

Figure 9: Classification of the genus *Exorista* Meigen

The order of Diptera, or (true) flies, contains more than 100 families. They can be distinguished from other orders of insects by a pair of flight wings on the mesothorax and a pair of halters, derived from the hind wings, on the metathorax. Diptera have a holometabolous life cycle, during which the larvae have no true legs (Wiegmann & Yeates, 2007; Tirry, 2013).

The Diptera can be divided into two suborders, the Nematocera and the Brachycera. The latter can be identified most easily by reduced antenna with fewer than eight flagellomeres. The larvae of Brachycera have mandibles composed of two distinct parts (Wiegmann & Yeates, 2007; Tirry, 2013).

The Muscomorpha can be separated from other Brachycera by the larvae, which have reduced head capsules, and the pupae, which are formed inside the exoskeleton of the final larval instar, forming a puparium (Tirry, 2013).

The classification of the different Muscomorpha can be done by observing the adult emerging from the puparium. Schizophora use a ptilinum (an inflatable membranous sac) during adult emergence, while the Aschiza do not. A further identification within the Schizophora can be made by the presence of calypters, lobes covering the halters. These calypters are possessed by the Calyptratae but not by the Acalyptratae (Tirry, 2013).

The genus *Exorista* belongs to the family of the Tachinidae. Species of this family are known to appear in a wide range of sizes, colors and degrees of bristling, but most are larger and more bristly than a house fly. Adult tachinids have a well-developed postscutellum, which is a cushion-like bulge below the rear part of the scutellum. First instar larvae have a hook-like labrum which is fused with the cephalopharyngeal skeleton (Van Emden, 1954; McAlpine, 1987; O'Hara, 2008).

Four families can be distinguished within the Tachinidae: Phasiinae, Dexiinae, Tachininae and Exoristinae. All of them are parasitoids. Exoristinae is taxonomically the most difficult group. Members of this group have homogeneous appearances, often being colored grayish black and being fairly bristly. At this moment the phylogenetic relationships between the different Tachinidae are unclear, but more research on the male and female genitalia and immature stages could provide clearer characteristics for phylogenetic analysis (O'Hara, 2008).

To distinguish the genus *Exorista* Meigen from other Exoristinae, one should look at the frontal bristles, which descend to the level of the facial ridge (Figure 10).
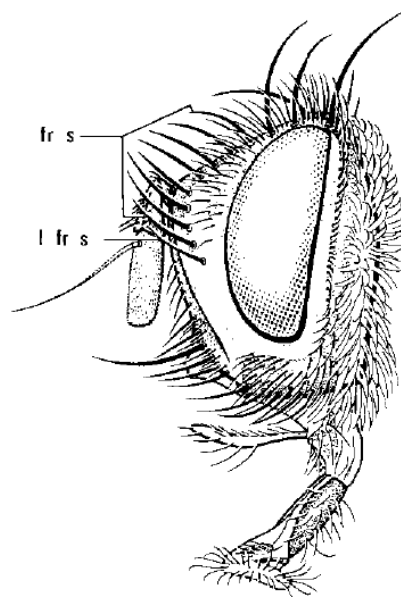
36 Exorista larvarum ♂

Figure 10: Lateral view of the head of *E. larvarum* (fr s = frontal bristle, l fr s = lower frontal bristle) (McAlpine, 1987)

Exorista larvarum has a wide geographical range, with presence in Europe, Asia, Africa, and North America, where it was introduced in the beginning of the 20th century (Herting, 1960).

3.2.   Life cycle of *E. larvarum*

The complete life cycle of *E. larvarum* consists of an egg stage, three larval stages, a pupal stage and an adult stage (Hafez, 1953). Observations at the laboratories of DipSA (*Alma Mater Studiorum* Università di Bologna, Italy) showed that at 27°C, the total development from egg to adult takes on average 16 days (Marchetti, 2006).

3.2.1. Egg

E. larvarum eggs are deposited directly by the female using an extensible ovipositor. *E. larvarum* is an oviparous tachinid, meaning that the egg is undeveloped when oviposited and the first instar can only be seen after a few days (O'Hara, 2008). The macrotype eggs have a length of ca. 0.6 mm and a width of ca. 0.3 mm. Eggs are ovoid and white directly after deposition: as development proceeds they turn pale yellow (Hafez, 1953).

Oviposited eggs can get lost due to the hosts rubbing against each other or against surfaces, or when a host larva molts. Therefore parasitization is most likely to be succesful when the host larva has reached maturity (Mellini et al., 1993a; Mellini & Campadelli, 1996a; Hafez, 1953). The eggs do not provide long term protection for the first instar. Tachinidae do not hibernate in the egg stage or as free-living first instar (Mellini, 1991; O'Hara, 2008).

At a temperature of 26-27°C, *E. larvarum* eggs hatch after about three days. The larva leaves the egg at a set breaking line and penetrates the host's integument with its pointed hook-like labrum, using saliva to soften the integument (Hafez, 1953).

3.2.2. Larval instars

E. larvarum, like all Tachinidae, has three larval instars (Hafez, 1953). The first instar is ca. 0.7 mm long and ca. 0.25 mm wide immediately after hatching, but doubles in size and width during this stadium. The larva is pyriform, colorless and transparent. Its body consists of a head and 11 body segments, all covered with dense rows of small spines (Hafez, 1953). As soon as the larva penetrates the host, a primary respiratory funnel begins to form. This is a wound-induced immune response in which host hemocytes form a sclerotized sheath around the hind part of the larval body (Figure 11)(Hafez, 1953; Michalkova et al., 2009). This can be seen as a dark spot on the integument of the host (Figure 12) (Hafez, 1953; Marchetti, 2006). By using the host immune response to its favor through forming primary respiratory funnels, the larva breathes atmospheric air during its entire development and is able to grow fast (Mellini, 1991; Valigurovà et al., 2014).The first instar larva feeds on host hemolymph, avoiding damage to vital organs. It lives more like a parasite than a parasitoid (O'Hara, 2008).
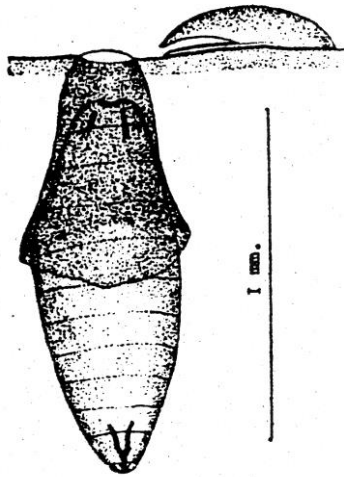
Figure 11: *E. larvarum* **first instar larva inside an integumental primary respiratory funnel (Hafez, 1953)**

Figure 12: Hatched eggs and penetration holes of the larvae of *E. larvarum* **(Photo Archive of the Entomology area of DipSA)**

When the larva reaches its second instar, the single hook it used to cut open the egg is replaced by two hooks. These are clearly visible and can be used to identify the second instar. The larva remains anchored to the respiratory funnel and does not move in the host body. It still feeds on body fluids and adipose tissue cells, without attacking vital organs. Its size increases to a length of ca. 3 mm and a width of ca. 1.5 mm (Hafez, 1953).

The third and last instar leaves the respiratory funnel and moves freely in the host. In this stage the larva uses larger amounts of food and grows rapidly. The host now quickly dies. The larva keeps feeding until it is fully developed or until the food supply is exhausted (Marchetti, 2006; O'Hara, 2008). The third instar has a length of ca. 8 mm and a width of ca. 3 mm. It can be distinguished by the posterior spiracles of the respiratory system being clearly visible on the surface of the last body segment (Hafez, 1953).

At 27°C, the total development from first instar larva to the formation of a puparium takes on average 6 days (Marchetti, 2006). *Exorista larvarum* is a gregarious parasitoid, i.e. more than one larva can complete development in a single host (Michalkova et al., 2009).

3.2.3. Pupa

Pupation takes place in a puparium, the hardened skin of the third larval instar. The pupa is of the exarate type, having free appendages. The barrel-shaped puparium has the same segmentation and distribution of spines as the third instar, and the anterior and posterior

spiracles of the third instar remain visible. The color changes from pale yellow right after pupation to dark red. The puparium has a length of ca. 8 mm and a width of ca. 4 mm, however these dimensions can vary depending on various factors (Figure 13). It was found that male puparia are slightly bigger (0.1-0.5 mm larger and 0.1-0.3 mm wider) than female puparia, and that an increasing number of parasitoid puparia per host decreases the puparium size (Hafez, 1953; O'Hara, 2008). Pupation mostly takes place outside the host's body, but it is possible that the larva stays inside the body to pupate (Hafez, 1953).

At 27°C, the time needed for an adult to emerge from the puparium is approximately 8 days (Marchetti, 2006). Adults emerge by inflating the ptilinum, a membranous balloon-like sac located behind the face, which forces the anterior top of the puparium to open along a particular line of weakness (O'Hara, 2008). Females take a few days more to emerge than males (Hafez, 1953).

Figure 13: Puparia of *E. larvarum* (photo author)

3.2.4.   Adult

Male and female adults can be easily distinguished: the pretarsi of males are clearly forked, while this bifurcation is much less evident in females (Hafez, 1953).

Mating begins immediately after emerging (Marchetti, 2006) (Figure 14). The female extends her ovipositor, after which the male bends his terminalia ventrally and inserts his aedeagus into the female genital atrium. Mating usually happens in the morning and takes 1-5 hours. According to Hafez (1953), males and females can mate more than once, with the same or with several partners.

Figure 14: Two adults of *E. larvarum* mating (Michalkova et al., 2009)

The length of the preoviposition period depends upon the temperature and season, varying from two to three days in summer to six days in winter. It can even be reduced to one day in laboratory conditions at 31°C (Hafez, 1953, Dindo et al., 2007).

To be able to locate a potential host, *E. larvarum* females rely on host-induced plant volatiles. Visual cues are of lesser importance (Depalo et al, 2012). For *Exorista japonica* Townsend it was found that host size, color, texture and movement may influence oviposition behavior (Ichiki et al., 2011). Oviposition begins when the female approaches the host, and after some time staying still near it, the female slowly extends its ovipositor between its legs until the tip reaches the host's integument. Then an egg is suddenly stuck to the host's body. They are attached to the host's body surface with a glue-like substance, in such a way that the ventral side of the eggs fits perfectly with the convexity of the host's surface. The majority of the eggs are deposited on the lateral and dorsal side of the host (Hafez, 1953; Mellini et al., 1993a). Before the next deposition the female usually rests for a few minutes. When the female is presented several stages of host larvae, it will choose to oviposit on advanced larval instars. Super-parasitism is very common (Hafez, 1953).

Female adults lay about 7 eggs each day for a period of 20-25 days (Hafez, 1953). Under laboratory conditions, it was however found that the number of eggs laid per day can be much higher for young females. Most eggs are laid during the first ten days after the oviposition onset (Dindo et al., 1999). When a suitable host remains absent for a few days, the females will keep the eggs in the vagina piriformis and will eventually oviposit on other objects, for instance dead bodies of other *E. larvarum* (Mellini & Campadelli, 1996a). Fecundity is dependent upon larval development of the females. The more individuals the female larva had to share the host with, the less food it could obtain and the less eggs it will lay as an adult. Hafez (1953) found that the number of eggs laid decreased from 243 to 152 when the female had to share its host with one

other individual, and to 104 eggs when shared with two other individuals. Parthenogenesis does not occur in Tachinidae (Hafez, 1953).

Longevity of adults depends upon available food, temperature and sex. Females survive longer than males, with a difference going up to 20 days in the field in winter or 9 days in the laboratory. In the culture maintained in the laboratories of Entomology at DipSA, the female longevity was 21 days at 26°C, 60% RH and 16:8 L:D photoperiod (Dindo et al., 1999). Increased temperatures decrease longevity of males and females. The sex ratio in a population is 1:1 (Hafez, 1953).

Exorista larvarum does not kill or paralyze its host when first entering it. Therefore it has been classified as a koinobiont, as all tachinids (Askew & Shaw, 1986). However, *E. larvarum* and other tachinid species do not have synchronized developments with their hosts. These parasitoids grow quickly and kill their host quite rapidly after attack, which means they behave as zoonecrophages for a large part of their larval development. This would classify them as idiobionts. Dindo (2011) therefore suggests not using the koinobiont/idiobont classification for Tachinidae, but rather to classify them based on the presence of synchronized development with the host.

3.3. Ecology

Exorista larvarum is a polyphagous larval parasitoid. It has a wide range of potential natural hosts. These hosts may however not be easily rearable. Therefore factitious hosts (e.g., *G. mellonella*) may be considered for *in vivo* rearing. *Exorista larvarum* is also very suitable for *in vitro* rearing due to several reasons: it is a polyphagous species, its larvae are gregarious, its development is not synchronized with the host, and its larvae build primary respiratory funnels and thus grow rapidly (Mellini & Campadelli, 1996a; Dindo et al., 1999).

3.3.1. Natural hosts

Natural hosts of *E. larvarum* are mostly lepidopterans. Hafez (1953) and Herting (1960) reported more than 45 species as natural hosts many years ago. Since then, host records have been scarce and are often incomplete (Kara & Tschorsnig, 2003; Cerretti & Tschorsnig, 2010). *Exorista larvarum* has a wide geographical range and is polyphagous. Therefore, each natural habitat of *E. larvarum* houses its own range of natural hosts. Cerretti & Tschorsnig (2010) and Kara & Tschorsnig (2003) catalogued all natural hosts for the Tachinidae of respectively Italy

and Turkey. Within the wide range of possible hosts for *E. larvarum*, some are known to be pest species, the most important ones being *L. dispar*, *Hyphantria cunea* (Drury) (Kara & Tschorsnig, 2003; Cerretti & Tschorsnig, 2010), and *Dendrolimus pini* L. (Csoka et al., 1989). *Spodoptera littoralis* has been recorded as a natural host of *E. larvarum* in Egypt (Hafez et al., 1976).

3.3.2. Factitious hosts

As explained above (see 1.3), the greater wax moth *G. mellonella* is a safe and easily available alternative to natural hosts, providing most of the nutritional requirements needed for full development (Campadelli, 1988). *E. larvarum* has been reared on *G. mellonella* for many generations in the laboratory of Entomology of the University of Bologna. Mellini & Campadelli (1996b) found a puparium yield of 21% out of 169 eggs oviposited on 46 host larvae, and a mean puparial weight of 33.28 mg for 100 puparia obtained from a container with parasitized larvae. In both occasions superparasitism occurred which influenced the results. Adult emergence reached 87% for 113 puparia obtained from another rearing container.

Depalo et al. (2010) compared *G. mellonella* with *S. littoralis*, a natural host of *E. larvarum*, in terms of acceptance and larval development. For both species the time to obtain four to six eggs per larva was similar, so they are equally accepted by *E. larvarum* females. Parasitized larvae of both species were significantly influenced by the parasitization, yielding higher mortality than in control larvae. However, only 1.3% of larvae parasitizing *S. littoralis* formed a puparium versus 75% of larvae parasitizing *G. mellonella*. This may be due to the fact that *E. larvarum* had been reared on *G. mellonella* for many generations and had problems adapting to a different host.

A key factor in successful *in vivo* rearing on natural or factitious hosts is the parasitization success. This depends on several factors, such as the number of eggs oviposited per host, and host age at parasitization (Dindo & Grenier, 2014). Mellini & Campadelli (1996a) found that results are optimal when four to six eggs are deposited on a last instar (mature) *G. mellonella* larva. However, development can also be completed in younger host larvae, except when they are too small (Baronio et al., 2002).

3.3.3. Artificial media

When *in vitro* rearing a dipteran parasitoid, one should pay attention to several biological aspects of the insect. The first is the host range: *Exorista larvarum* is polyphagous. The second is the oviposition strategy. This has an influence on the way the eggs have to be collected in order

to be placed on the artificial medium. *Exorista larvarum* females oviposit directly on the host integument, implying that the same direct strategy should be used on the artificial medium. The third biological aspect is the interaction between the host and the parasitoid. *Exorista larvarum* larvae use the host's immune response to their advantage by forming primary respiratory funnels. This means that they need to stay in contact with air from the first instar, eliminating the possible use of liquid media (Dindo & Grenier, 2014).

Mellini et al. (1993b) developed the first two (similar) artificial media for *E. larvarum*. These media were composed of bovine serum, extract or homogenate of *G. mellonella* pupae, and some additives. Adult yields of 36% were obtained. In following studies efforts were made to delete host components, since the maintenance of the lepidopterous insect colony requires labor. Bratti & Coulibaly (1995) reared *E. larvarum* on four tissue culture media-based media (TMM-FH, SCHNEIDER'S, EX-CELL 400 and SF-900) and found that there was no difference in adult yield and puparium weight between the different media, with adult yield going up to 55%. When *G. mellonella* pupal extract was added to one of these media, adult yield and puparium weight were higher than on media without extract. Mellini & Campadelli (1996c) used a basic medium of yeast, egg yolk (both replacing *G. mellonella* larval homogenate), saccharose, and sterile water (replacing bovine serum) and made several attempts to make the medium even cheaper. One of the main findings was that skimmed milk can be used to partially replace yeast, leading to adult yields of 43-44%, which was similar to the results on *G. mellonella*. Dindo et al. (1999) developed two media based on Gerber veal homogenate (a food intended for human babies), combined with yeast extract, egg yolk and wheat germ (1) or saccharose (2), both devoid of insect components. Adult yields of resp. 29 and 33% were obtained. Longevity and fecundity of the females on medium 1 were similar to that of adults obtained from puparia formed in *G. mellonella* larvae. Puparial weights (of both sexes) were higher and development time was longer on the artificial medium. Dindo et al. (2010) further simplified the standard artificial medium containing skimmed milk, yeast extract, egg yolk, sucrose and gentamicin by deleting sucrose. Results obtained with the basic medium were similar to the results with the simplified medium in terms of development. In general, *E. larvarum* shows a great adaptability to a variety of artificial media, proving once more its great potential for *in vitro* rearing (Mellini & Campadelli, 1996c; Dindo et al., 1999).

Dindo et al. (2006) compared *E. larvarum* reared on the factitious host *G. mellonella*, on an artificial skimmed milk medium and on a veal homogenate medium. A higher puparial yield and average puparial weight was obtained on both insect-free media, but no difference was found in longevity and parasitization rate. In another experiment it was found that *E. larvarum* females reared on a milk medium oviposited fewer eggs than females reared on *G. mellonella*, despite

both having similar puparial weights. This led to the conclusion that puparial weight alone cannot be seen as a reliable quality parameter.

In general, agar is used as a physical support of the artificial medium, but this is quite expensive. Absorbent cotton can be used as a cheaper alternative, resulting in similar puparial yields and adult emergence as with agar. Puparial weight was highest (66-69 mg for males, 59-60 mg for females) when 15 mg cotton was used to absorb 0.4 ml medium in multiwell plates, each well having a diameter of 17 mm (Dindo et al., 2003).

Dindo et al. (2007) tested the possibility of rearing *E. larvarum* from eggs that are not laid on an insect host. Eggs oviposited on a plastic sheet and reared *in vitro* showed no significantly different hatching rate, puparial weight and adult yield as compared with eggs removed from *G. mellonella* larvae for one generation. Marchetti et al. (2008) however found that the quality of the second generation of *E. larvarum* in terms of hatching rate, percentage of puparia and percentage of adults was decreased, especially when the insect was reared in vitro for two generations starting from eggs laid on plastic. Instead, when the eggs were collected from hosts and then transferred to the diet, the quality loss in the second in vitro generation was less dramatic. In both studies the number of eggs laid on the plastic sheet was significantly lower than those laid on host larvae.

In an attempt to compare *in vivo* and *in vitro* reared *E. larvarum* adults in terms of effectiveness, Dindo et al. (2002) reared *E. larvarum* on an artificial egg yolk and skimmed milk-based medium or on *G. mellonella* larvae and tested their effectiveness against the natural host *L. dispar*, both in the laboratory and in the field. No significant difference was found between *in vivo* and *in vitro* reared adults in the laboratory. In the field *in vivo* reared females laid significantly more eggs on *L. dispar* larvae than *in vitro* reared females (9.8 and 0.5 eggs, respectively), but nevertheless the percentage of larvae from which *E. larvarum* puparia were obtained was the same (17.5%, both for *in vitro* and *in vivo* reared adults).

Superparasitism is very common in *E. larvarum*. However, when mass producing the insect, excessive superparasitism should be avoided to optimize production since the decrease in vital space per individual can lead to lower adult size and higher mortality (Mellini & Campadelli, 1996a). Vital space is also an important factor for the rearing on artificial media. Baronio et al. (2002) studied the effect of vital space on the number and size of puparia. When reared in a 0.38 cm^3 well, only 7% of the individuals developed a puparium and none of the adults emerged, while when reared in a 2.27 cm^3 well with the same amount of food presented, 83% developed a puparium and 92% of adults emerged. Puparial weight remained equal.

3.3.4. Adult maintenance

The food source of the adult parasitoid is generally considered as less important, because it is assumed that any sugar-rich substrate may be sufficient (Wäckers, 2003). *Exorista larvarum* adults can be provided with a sugary or honey-water solution absorbed in cotton balls. Sugar cubes can also be provided. Water is provided by moistened cotton (Dindo et al., 1999, 2010).

3.4. Use as biological control agents

Exorista larvarum is a polyphagous parasitoid, and many of its natural hosts are known to be pest species. It can be reared easily both *in vivo* and *in vitro.* Therefore it has been considered as a potential biological control agent for many of these pest species. Research aimed at improving in vitro rearing techniques, making the use of *E. larvarum* more convenient and efficient, is thus justified (Grenier, 2009; Dindo & Grenier, 2014).

At the laboratory of Entomology of the University of Bologna there has been extensive research on the mass rearing of *E. larvarum.* These studies show promising results for augmentative releases of *E. larvarum* to control populations of noctuid pest species, such as *P. saucia*, *Pseudaletia unipuncta* (Haworth), *Xestia c-nigrum* L. (Simões et al., 2004), *S. littoralis* (Depalo et al., 2010) and *Mythimna unipuncta* (Haworth) (Depalo et al., 2012).

In cork oak forests in Sardinia, *E. larvarum* is an important biological control agent of several lepidopteran defoliators such as *L. dispar*, *Malacosoma neustria* L. (Delrio et al., 1983) and *Tortrix viridana* L. (Delrio et al., 1988). *Exorista larvarum* can cause mortality of up to 50% on *L. dispar* (Luciano & Prota, 1984).

Exorista larvarum can be considered the second most important antagonist species of *L. dispar* according to Herting (1960). *Lymantria dispar* is an important pest species in Europe, Africa and North America. It is a defoliator of more than 300 species of woody plants, making them more susceptible to secondary pests and pathogens (CUES University of Minnesota, 2015). From 1970 to 2010 80.4 million acres were defoliated in the US (US Forest Service, 2015). Many efforts have been made to introduce natural enemies (parasitoids, predators and pathogens), but most failed to establish (Hoy, 1976). The use of *E. larvarum* as a biological control agent of *L. dispar* has been limited to sporadic interventions in North America, where it became established (Sabrosky & Raerdon, 1976; Kenis & Lopez Vaamonde, 1998). *Exorista larvarum* females prefer to oviposit on

female larvae of *L. dispar* rather than on male larvae, giving *E. larvarum* an extra asset in its use as a biological control agent (Babaei et al., 2009).

Finally the availability of efficient rearing techniques for *E. larvarum* (both *in vitro* and *in vivo*) may also be helpful in studies concerning pesticide effects on non-target species, e.g., the effect of conventional and transgenic *Bacillus thuringiensis galleriae (Btg)* toxin on *E. larvarum* (Marchetti *et al.*, 2009; 2012). Aspects concerning the rearing of this tachinid is therefore important for biological control from different points of view.

PART II: MATERIALS & METHODS

1. Experiments with *Orius thripoborus*

1.1. Laboratory culture

The culture of *O. thripoborus* at the Laboratory of Agrozoology, Ghent University, was started with nymphs and adults obtained in August and September 2008 in Mpumalanga (26°S, 30°E) and Kwazulu-Natal (29°S, 31°E), two provinces in South Africa. They were collected in and nearby sugarcane fields (*Saccharum officinarum*), particularly in the vicinity of the flower borders. This stock colony was cultured in cylindrical Plexiglas® containers, 4 cm high and with a diameter of 9 cm, comprising six ventilation holes (diameter 1 cm) each covered with meshed nylon gauze (80 μm). On the upper side there is a seventh hole, which holds a rubber plug. Food was being provided through the latter hole. On the bottom of the container a smaller aperture can be found which fits the stem of a sharp pepper plant (*Capsicum annuum* L. cultivar 'Creta', long red). The cylindrical ring and two disks closing the container were put together by semitransparent sticky tape (Scotch 3M, Magic Tape, St. Paul, MN, VSA). All remaining gaps between the plant and the container were filled up with Pritt Buddies (N.V. Henkel, Brussel, Belgium) preventing the insects from escaping.

Ephestia kuehniella eggs were used as food for *O. thripoborus* nymphs and adults. These eggs were supplied by Koppert B.V. (Berkel en Rodenrijs, Netherlands) and were stored at -18°C. Before being used as food, the eggs were sieved and thawed. In this way the eggs could be kept in a refrigerator at 4°C for a few days without significantly losing quality.

When constructing the container, *E. kuehniella* eggs were added using the tip of a spatula. Dried bee pollen (Weyns N.V., Beveren, Belgium) were also added to improve development and reproduction (Bonte et al., 2011). A 4- to 5-leaved sharp pepper plant (4-5 weeks old) was provided as an oviposition substrate, moisture source and shelter for the mobile stages. Before adding the plant to the container the cotyledons were removed. Lastly a wrinkled piece of wax

paper was also placed in the container. This was done to provide extra shelter so as to reduce cannibalism (Bonte & De Clercq, 2010) (Figure 15).

Figure 15: A container used in the rearing of *O. thripoborus* (Van De Walle, 2014)

The containers were placed in a gutter filled with water in such a way that the roots of the sharp pepper plant could take up the water, without allowing water to infiltrate into the container. This could otherwise lead to mold infections and drowning of the insects. The gutters were placed in a climatic cabinet at a temperature of 25 ± 1 °C, a relative humidity of 65 ± 5%, and a photoperiod of 16:8 h (L:D).

Maintenance of the containers was being done every Monday, Wednesday and Friday. On these days, adults were transferred to a fresh container allowing them to oviposit for 2 or 3 days on the sharp pepper seedlings. On the next day of maintenance, the surviving adults were moved to a new container and their number was complemented to 80 adults, using 4- to 5-day-old adults. Remaining food was also removed and, as a result, the rearing unit only contained a sharp pepper plant with a high number of oviposited eggs which were to hatch during the following days. *O. thripoborus* stayed in the same container for their entire nymphal life, until they had reached the early adult stage. In this way the continuous culture of *O. thripoborus* was assured.

All containers were provided with *E. kuehniella* eggs adhered to one or more pieces of household paper. These pieces of paper were made by pulling apart the two layers of one sheet of household paper, and moistening these separate sheets. The sheets were then partially dried on a second piece of household paper. Sieved and thawed *E. kuehniella* eggs were dispersed over the sheets, and after complete drying the sheets were cut into smaller pieces of ± 1cm² and transferred into the rearing containers. Every Friday, all paper pieces with *E. kuehniella* eggs were replaced by new ones to avoid mold infection. Every Monday also the wax paper was changed.

The transfer of adults from container to container was done by using an aspirator (Figure 16). This aspirator consists of a mouthpiece, a piece of PVC-tube and a pipette tip, covered by meshed

nylon gauze (80 µm) at the widest end. By sucking on the side of the mouthpiece, the insects were dragged into the pipette tip against the nylon gauze. By gently blowing in the aspirator and tapping the side of the pipette tip the insects would then fall out again.

Figure 16: An aspirator used in the handling of *O. thripoborus* (Van De Walle, 2014)

1.2.   Diets

In this thesis a factitious food (*E. kuehniella* eggs) and an artificial diet with or without insect components were compared in terms of development and reproduction of *O. thripoborus*. The tested diets are described in the following section.

1.2.1.   *Ephestia kuehniella eggs*

Ephestia kuehniella eggs are considered to be an excellent factitious food for *Orius thripoborus*, and are used in the laboratory culture as described above. *Ephestia kuehniella* eggs were therefore used as a control diet. The eggs were being provided to the insects on household paper in the same way as was done in the stock culture.

1.2.2.   *Egg yolk diet*

The goal of this thesis was to test whether adding insect hemolymph to an artificial diet would affect the quality parameters of *O. thripoborus*. As the basic artificial diet, the egg yolk diet developed by Arijs & De Clercq (2002) was chosen.

This artificial diet consisted of ±30 g egg yolk, 53.9 g water, 3 g casein, 2.5 g casein enzymatic hydrolysate, 2 g soy enzymatic hydrolysate, 3 g lactalbumin, 1 g dextrose, 0.5 g Wesson's salt, 0.06 g vitamin mix, 1 g groundnut oil, 3 g soy oil and one drop of vitamin E. The powdered

vitamin mix consisted of 0.15 g nicotinic acid, 0.029 g vitamin B2, 0.00289 g vitamin B1, 0.00877 g vitamin B6, 0.073 g Ca-pantothenate, 0.00592 g folic acid, 0.001 g biotin and 0.32 g vitamin C. The vitamin E was not included in the vitamin mix because it needed to be added to a hydrophobic solution. The composition of this vitamin mix was derived from beef liver, but without vitamins A, D and K, since insects do not need these vitamins.

Fresh hen's egg yolk was mixed with the water in a glass jar and then heated and continuously mixed on a magnetic stirrer heating plate until the solution began boiling. A watch-glass was placed on top of the beaker containing the solution to prevent water from escaping. When the solution started coagulating, it was cooled down by holding the beaker under running water for about 1-2 minutes until it reached a temperature below 40°C. Then the casein, casein enzymatic hydrolysate, soy enzymatic hydrolysate, lactalbumin and glucose were weighed and added to the solution. After mixing, the Wesson's salt and vitamin mix were added and mixed again. Then the groundnut oil and soy oil were pipetted into the solution, and a drop of vitamin E was added to the oil. The diet was then thoroughly mixed with a blender until a homogenous solution was obtained.

The diet was provided to the insects in a hemispherical Parafilm® dome (70 µl), obtained with an encapsulation device (Analytical Research Systems, Inc., www.ars-fla.com). By stretching the Parafilm® before encapsulation, proper stylet penetration by early stages of the predatory bug was assured. The domes were sealed with transparent Scotch 3M Packaging Super Tape (3M, www.3m.com). The domes could be stored in a refrigerator at 4°C for a few days.

1.2.3. *Egg yolk diet with hemolymph of* Hermetia illucens *L.*

Hemolymph of prepupae of the black soldier fly, *H. illucens*, was added to the artificial egg yolk diet described in 1.2.2. The hemolymph was obtained from flies reared at the Laboratory of Agrozoology, Ghent University. A colony of black soldier flies was initiated with specimens supplied by Millibeter BVBA (Antwerp, Belgium). Larvae were reared in stainless steel containers (34 x 25 x 20 cm) with vented hoods on solid organic waste derived from a fruit and vegetable fermentation installation. Every week substrate was added until the *H. illucens* larvae reached the prepupal stage. The prepupae were then placed in potting soil for two days before collecting the hemolymph, to reduce the risk of impurities during extraction.

The actual collection of the hemolymph started by collecting *H. illucens* prepupae (± 5 days old) and washing them with tap water to remove dirt. The prepupae were then placed in a beaker filled with water at a temperature of 60°C for 10 minutes to avoid melanization of the

hemolymph. After this period the prepupae were surface sterilized by washing them with 75% ethanol. The hemolymph was collected by cutting one end of the prepupa open and squeezing the hemolymph out of the prepupa and into an Eppendorf 1.5 ml tube. From 20-25 *H. illucens* prepupae ca. 1 ml hemolymph could be obtained. The hemolymph could then be stored in a freezer at -18°C. This method of collecting *H. illucens* has been developed by Nguyen et al. (2015).

When hemolymph was to be added to the artificial egg yolk diet, a sufficient amount of hemolymph was thawed and centrifuged at 8000 rpm for 10 minutes at a temperature of 4°C, to separate the hemolymph from extracted tissue. The basic artificial diet was weighed, and then hemolymph was added until the amount of hemolymph accounted for 20% of the total weight. After thorough mixing of the diet, Parafilm ® domes were obtained as described in 1.2.2..

1.2.4. Egg yolk diet with hemolymph of Antheraea pernyi *Guérin-Méneville*

In another treatment, the hemolymph of the Chinese oak silkworm *A. pernyi* was used in an attempt to enrich the artificial egg yolk diet. This hemolymph was obtained from a culture at the Guangdong Entomological Institute, China. The process of collecting the hemolymph of *A. pernyi* was the same as for *H. illucens* and is described by Nguyen et al. (2013). First, the pupae were immersed in a water bath at 60°C for 10 minutes to avoid melanization of the hemolymph. Then the pupae were surface sterilized by washing them with 75% ethanol. The hemolymph was collected by squeezing the pupae under sterile conditions. The hemolymph was then lyophilized and stored in a freezer at -18°C. Upon using the hemolymph, 10 ml of demineralized water needed to be added to the vial and the mixture was thoroughly shaken until all hemolymph was dissolved. The hemolymph could then be added in the same 20/80 (hemolymph/artificial diet) weight ratio to the diet. Parafilm ® domes were obtained as described in 1.2.2..

1.3. Bioassays

1.3.1. General

All experiments were performed in small plastic containers, 2.5 cm high and with a diameter of 4 cm, with a ventilation hole on top covered by meshed nylon gauze (80 μm). Each container was labeled with semitransparent sticky tape (Scotch 3M, Magic Tape, St. Paul, MN, VSA). These

containers were placed in climate cabinets with a temperature of 25 ± 1 °C, a relative humidity of 65 ± 5 % and a photoperiod of 16:8 (L:D) h (Figure 17).

In the first part of the experiment a development test was performed, in which *O. thripoborus* individuals were reared from first instar to adult on each of the tested diets. A newly hatched nymph from the stock culture was carefully transferred to an individual small container by using a small brush. The nymph was then provided with a Parafilm® dome filled with tap water as a moisture source (obtained with an encapsulation device as described above), and with one of the three foods as described in section 1.3.2 (Figure 18). The food and water dome were changed every day during the first five days, and every two days after day 5. Every time food and water were changed survival of the nymph was checked.
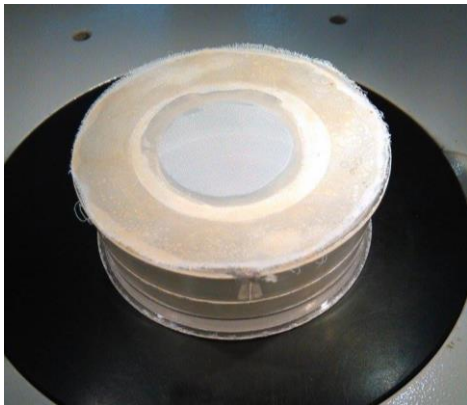

Figure 17: Plastic container used in the experiments with *O. thripoborus* (photo author)
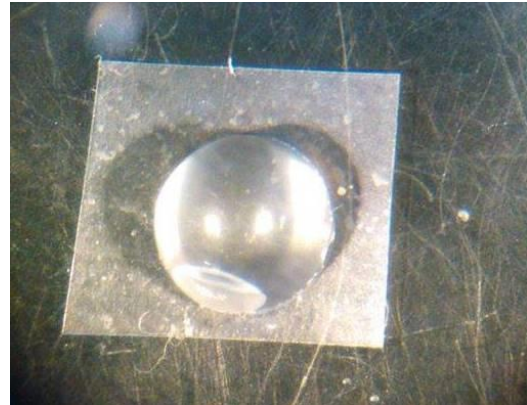

Figure 18: A Parafilm® dome filled with tap water (photo author)

When the adult stage was reached, the predator was weighed on an analytical balance (Sartorius, Goettingen, Germany), sexed by examining the ventral side of the insect through a microscope (see Figure 2), and mated with a partner. Each couple was placed in an individual container containing supplied with a Parafilm® dome filled with tap water, food (the same food as during their nymphal life) and a small piece (ca. 2 cm) of flat green bean pod (*Phaseolus vulgaris*) as an oviposition substrate, but also serving as an extra moisture and nutrient source (Bonte et al., 2012). The water dome, food and bean pod were changed every other day. The green bean pod was checked every day for oviposited eggs, which were mainly laid on the sides and the cut surface of the bean pod. By checking oviposition every day the preoviposition period could be determined. When eggs were observed, their number was counted and the bean pod was placed in a separate plastic cup in order to monitor egg hatch. Once a couple was found ovipositing, it was only checked every two days.

The experiment lasted until both adults had died. When one of the adults died before the first oviposition, it was replaced with a virgin adult from the same treatment group. When a nymph

or both adults were found to be dead, the content of the container was discarded and the container was cleaned for later use.

Parameters recorded in these experiments were: nymphal survival, female and male development time, female and male adult weight, sex ratio, preoviposition period, fecundity, proportion of ovipositing females, egg hatch, and female and male longevity.

1.3.2. Treatments

In total, five treatments were done. In treatment 1, 120 *O. thripoborus* first instars were provided with a Parafilm® dome filled with the basic egg yolk diet. In treatment 2, 120 nymphs were offered a Parafilm® dome filled with artificial diet containing hemolymph of *H. illucens*. Treatment 3 had the same approach as treatment B, but in this case the diet was enriched with *A. pernyi* hemolymph.

Further, several treatments involving *E. kuehniella* eggs were set up. In treatment 4, 60 *O. thripoborus* nymphs were offered *E. kuehniella* eggs during the first six days of their life, approximately from the first up to the third instar. From the fourth instar on (day 7), a Parafilm® dome filled with artificial diet containing *A. pernyi* hemolymph of was provided. In treatment 5, 60 nymphs were offered *E. kuehniella* eggs throughout their life cycle. The latter treatment was considered to be the control treatment.

2. Experiments with *Exorista larvarum*

2.1. Laboratory cultures

2.1.1. Exorista larvarum

The culture of *E. larvarum* at the laboratories of Entomology of DipSA (Dipartimento di Scienze Agrarie, *Alma Mater Studiorum* Università di Bologna, Italy) was started in 1992. One hundred individuals that had emerged from larvae and pupae of the fall webworm *H. cunea* were collected in the Italian province of Forli-Cesena (44° 13′ 21″ N, 12° 2′ 27″ E) by Amadou K. Coulibaly. In 2004 the culture was renewed by Luca Sighinolfi from puparia obtained on *H. cunea* in the Italian province of Modena (44° 10′ 49″ N, 10° 38′ 54″ E). The adults of this culture

were maintained in transparent Plexiglas cages (40 x 30 x 30 cm), with 50-70 individuals per cage (Figure 19). These cages had ventilation holes (diameter ± 15 cm) covered with a fine metal mesh on the sides, and a door at the top used to feed the insects and to insert/remove the host larvae to be exposed to parasitoid females for parasitization. The cages were placed in climate chambers at a temperature of 25-26°C, a relative humidity (RH) of 70-80% and a photoperiod of 16:8 (L:D).

The food of the *E. larvarum* adults consisted of sugar cubes (± 6-8 per cage) and a solution of honey mixed with water (20% honey), provided in absorbent cotton balls (3-4 per cage), both presented on plastic Petri dishes. Water was provided in plastic cups (150-200 ml) with a cover containing an aperture. This aperture was filled with a piece of absorbent cotton, which remained moist by keeping contact with the distilled water in the cup. The sugar cubes and water cup remained in the cage for the entire life of one generation of *E. larvarum* adults. The cotton balls soaked in honey/water solution were changed every Monday, Wednesday and Friday.

In order to create a new cage, every Wednesday the adults were allowed to parasitize larvae of the greater wax moth, *G. mellonella*. After removing the entire content of the cages (cotton balls, sugar cubes and water cups), about 80 *G. mellonella* mature larvae were placed inside the cages. This number was used to provide each female with approximately three larvae to oviposit on. It is important that the host larvae would not be molting soon, because when this would happen the parasitoid eggs could be exuviated together with the old integument 1-3 days after oviposition (Mellini et al., 1993). For this reason, mature larvae (far from pupation) were used. After approximately one hour the larvae were placed inside a Plexiglas container (24 x 17 x 9 cm) with a ventilation hole covered with a fine metal mesh. Each host larva should at least have 4 eggs oviposited on its surface. According to Mellini & Campadelli (1996a) this is the optimal number of eggs for the production of this tachinid. The cages of the *E. larvarum* adults were then cleaned to remove remaining silk and feces of the *G. mellonella* larvae, after which the content of the cages was placed back. About one week after parasitization the first puparia of the tachinid could be detected. These were then placed in a new cage with sugarcubes, awaiting the emergence of the adults. About three days after adult emergence mating began. In this way the continuous culture of *E. larvarum* was assured.
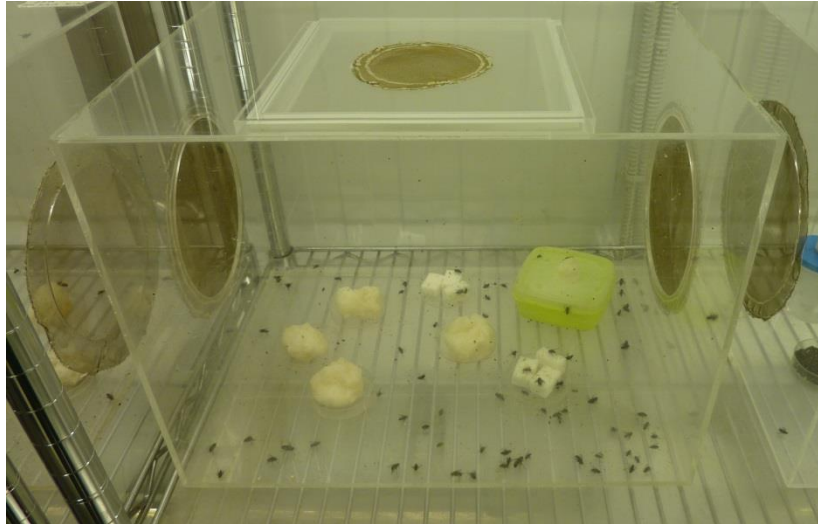
Figure 19: Rearing cage for *E. larvarum*, containing cotton balls soaked in honey/water solution, sugarcubes and a water cup (photo author)

2.1.2. *Galleria mellonella*

The laboratories of Entomology of DipSA held their own culture of *G. mellonella*. Adults were kept in Plexiglas containers (24 x 17 x 9 cm) of which the cover had been modified with a central circular hole (ca. 6 cm diameter). This hole was covered by disks of tissue paper on which the female adults could oviposit their eggs. Emerged adults mated and after one hour females began laying eggs on the edge of the tissue paper. Each female lays about 1,500 eggs during her life, of which about 90% hatches (Mellini & Coulibaly, 1991). The adults were stored in a climatic cabinet at a temperature of 30 ± 1 °C, a relative humidity of 60-70%, and constant darkness.

About twice a week a new container for *G. mellonella* larvae was prepared. This started with cleaning a Plexiglas container (24 x 17 x 9 cm) with sodium hypochlorite (10%), to avoid infection of the culture by a virus or bacterium. Then the tissue paper disks of the adult containers were removed and replaced, and one part of a collected disk (with ca. 2 cm of *G. mellonella* eggs) was cut and placed inside the new container, together with a small amount of artificial diet. The container was then labeled (serial number and date) and stored in the same climatic cabinet as for the adults.. The rest of the tissue paper disks containing eggs were placed in a refrigerator (for maximum 10 days) and served as a backup in case the culture would need to be restored. The larvae of *G. mellonella* reach maturity after 25-30 days (Figure 20). The pupae were then used to set up a new container of adults.

The artificial diet used to rear the *G. mellonella* larvae was developed by Campadelli (1973), based on a diet developed at the Institute of Entomology of the Czech Academy of Sciences (Sehnal, 1966). It contains 2000 g white wheat flour, 1000 g whole wheat flour, 1000 g whole corn flour, 1000 g milk powder, 500 g brewer's yeast (dry powder), 1000 g pure glycerin, 900 g

beeswax and 2000 g honey. All ingredients were placed in a stove at 100°C for sterilization. After about 2-3 hours the ingredients were mixed. First the different types of flour were mixed, and then sequentially the glycerin, honey and beeswax were added and mixed. When a homogenous mixture was obtained, the diet was cut into smaller portions (5 x 5 x 5 cm). The diet was then stored at room temperature for 2-3 days to allow it to harden, and chopped into thin curls using a grating machine. These curls were stored in a storage box (30 l) in the climate chamber where the *E. larvarum* culture was maintained. Every Monday, Wednesday and Friday all *G. mellonella* containers were supplied with extra diet, forming a uniform layer of about 2 cm.

Every mass rearing system is prone to epidemics caused by a virus or other micro-organism (Mellini & Coulibaly, 1991). *Galleria mellonella* larvae that are affected by a virus or bacterium are easily recognized by their flabby and black body after death. When such a larva was found, the entire container was immediately isolated and placed in a freezer to avoid contamination of the other containers. Preventive measures were taken by immersing the containers for at least 24 hours in a bath containing sodium hypochlorite solution (10%) followed by a thorough washing at high temperature in a dishwasher before reuse.

Figure 20: Plexiglas container holding 1 month old *G. mellonella* mature larvae (photo author)

2.2. Media

2.2.1. *Egg yolk and skimmed milk medium*

The artificial medium developed by Mellini & Campadelli (1996c) and described by Farneti et al. (1998) was used in this thesis. The ingredients needed for one 24-well plate are 9.63 ml skimmed milk, 0.25 g sucrose, 3.31 ml distilled water, 0.88 g yeast extract (Sigma Chemical Co., USA, cod. Y-1000), 1.75 ml egg yolk and 0.75 ml Gentamicin (Sigma Chemical Co., USA).

The milk and sucrose were poured in a 50 ml beaker, and the yeast was added to the distilled water in a 25 ml beaker. All materials needed for preparation of the medium, including these two beakers, were then covered with tin foil and placed in an autoclave at 120°C for 10 minutes to assure sterility. A chicken egg was separately surface-sterilized with 60% ethanol and washed with distilled water. The further preparation of the medium took place under a fume hood.

The egg was broken and separated in egg yolk and egg white. The egg yolk was kept in a Petri dish. With a sterile syringe (Terumo Europe N.V., Leuven, Belgium) the correct amount of egg yolk was added to the milk/sucrose solution and mixed with a sterile glass rod. Then a 10% gentamicin solution was prepared by taking up 0.1 ml of gentamicin with a syringe and adding it up to 1 ml with distilled water. The correct amount of gentamicin was added to the medium, and after adding the yeast solution the medium was mixed with a glass rod. This medium was always prepared fresh and was not stored for later use.

2.2.2. Egg yolk and skimmed milk medium with hemolymph

Similarly to the experiments on *O. thripoborus*, the artificial medium of *E. larvarum* was supplemented with hemolymph of *H. illucens* or *A. pernyi* (Figure 21). To be able to transport the hemolymph from Ghent to Bologna without losing quality by chemical or microbiological degradation, the hemolymph of *H. illucens* was also lyophilized. The same procedure of adding the hemolymph to the medium was used as that described for *O. thripoborus*. The hemolymph was added in a 20/80 (hemolymph/medium) weight ratio after preparing the basic medium and mixed thoroughly.

Figure 21: Vials containing lyophilized hemolymph of *H. illucens* (left) and *A. pernyi* (right) (photo author)

2.3. Bioassays

2.3.1. General

The rearing of *E. larvarum* on each artificial medium was performed in 24-well plastic rearing plates (Falcon, Corning inc. NY, USA), each well having a diameter of 1.6 cm. A piece of cotton (15 ± 0.5 mg) was placed in each well as a supporting substrate for the medium (Dindo et al., 2003). Subsequently, 0.4 ml of medium was pipetted into each well, after which the medium-soaked cotton was flattened using a sterile glass rod to allow a homogenous filling of the well. The multiwell plate was then sealed with Parafilm®, covered with tin foil, labeled, and placed in a refrigerator at 5°C (Figure 22).

Figure 22: 24-well plate containing flattened pieces of cotton soaked with artificial medium (photo author)

After one day the plate was taken out of the refrigerator in order to place the *E. larvarum* eggs on the medium. First, all material needed for manipulation of the tachinid eggs was placed in an autoclave for 10 minutes at 120°C. Meanwhile, 6-7 larvae of *G. mellonella* were put inside one of the adult rearing cages of *E. larvarum*, until enough eggs were obtained (minimum 24 per plate). A 60% alcohol solution was prepared and poured in two separate 25 ml beakers. A *G. mellonella* larva was placed inside the first beaker to sedate it. With a sterilized forceps the larva was moved to a beaker with distilled water to wash it, and with a second forceps it was placed on a piece of household paper to dry. The larva was then stretched on a paraffin plate using two pins. The eggs oviposited on its surface were removed by scraping over the caterpillar's integument with two small spatulas, and placing the eggs in a watch glass filled with water. When all eggs were removed the larva was discarded. This operation was repeated for each *G. mellonella* larva until sufficient eggs were collected. The eggs were then washed by accurately removing the water in the watch glass with a pipette and filling it with 60% alcohol solution from the second beaker that was prepared earlier. The alcohol solution was removed with a second pipette and the watch glass was filled with distilled water to wash the alcohol away. This process was then

repeated, and finally a bit of water was left in the watch glass together with the collected *E. larvarum* eggs. The 24-well plates were then opened to begin with the deposition of the eggs on the artificial medium. An egg was placed on top of a big spatula and gently moved towards the medium-soaked cotton plugs. This process was repeated for every well. The plate was then again sealed with Parafilm® and covered in tin foil, and placed in a climatic cabinet at a temperature of 26°C, relative humidity of ±70% and complete darkness.

During the test, the plate was checked every day under a microscope to be able to observe hatched eggs. The development of each hatched larva was monitored. When an *E. larvarum* larva reached the puparial stage, the puparium was removed from the well, washed with distilled water, weighed on an analytical scale (Mettler AE 160, Metalchim S.N.C., Bologna, Italy), and placed in a test tube sealed with a piece of cotton to prevent the emerged adult from escaping. The remaining medium was removed from the well. The puparia were stored in the same climate chamber as that of the stock culture of *E. larvarum*. When all puparia had been removed from a plate or the remaining larvae had passed eventually died, the plate was discarded.

When an adult of the tachinid emerged from a puparium, it was sexed and placed inside a small Plexiglas cage (20 x 20 x 20 cm) together with 2 sugar cubes, a cotton ball soaked in honey/water solution, and a water cup. Emerged females on the medium were paired with a male from the laboratory culture regardless of its age. Emerged males on the medium were paired with a female from the laboratory culture that had emerged in the same period as the male (within ca. 48 h). To prevent the latter females from having previously mated in the stock culture cages, a number of puparia from the laboratory culture were isolated in advance by placing them in a test tube plugged by a piece of cotton. When two females of the same replicate emerged within 48 hours, they could be placed in the same cage, and paired with two males from the stock culture.

A fecundity test was performed for each pair. Three days after pairing the first parasitization took place. The cages were emptied and three mature *G. mellonella* larvae of approximately 1 month old were placed inside each cage for one hour. The females could use this time to lay eggs on the host larvae. After one hour, the cage was cleaned to remove remaining silk and feces. Then, the host larvae were taken from the cage and the eggs deposited on the larvae were counted. The larvae were then placed in a small plastic cup (6 cm diameter) with small ventilation holes in the cover and stored in the climate chamber. This process of parasitization was repeated almost every day for a period of 10 days starting from the first parasitization, or until the female died. The cups containing the parasitized larvae were checked about one month after parasitization had taken place. Puparia and emerged adults of *E. larvarum* were counted.

Parameters recorded in these experiments were: percentage of hatched eggs, percentage of puparia formed (calculated on the original egg number), percentage of adults emerged (calculated on the number of puparia), female and male puparial weight, sex ratio, female and male development time from egg to puparium and puparium to adult, female and male total development time, preoviposition period, oviposition in 10 days (starting from the beginning of oviposition), percentage of eggs which produced a puparium and proportion of ovipositing females.

2.3.2. Treatments

Three treatments were tested. 48 individuals of *E. larvarum* were tested per treatment. In treatment 1 the basic artificial medium without added hemolymph was tested. This was considered as the control medium. In treatment 2 the artificial medium enriched with hemolymph of *A. pernyi* was used, and in treatment 3 *H. illucens* hemolymph was added to the medium.

3. Data analysis

The data were analyzed using the statistical program IBM SPSS Statistics 21 (IBM Corp., 2012). The significance level was set to 0.05 for all statistical procedures.

Continuous data (e.g., puparium weight and adult weight) were first tested for normality using a Kolmogorov-Smirnov test. When means were found to be normally distributed, a one-way ANOVA (analysis of variance) was performed since the effect of only one factor (treatment) had to be reviewed. A Levene test indicated whether the variances of means were homoscedastic or not. In case of homoscedasticity the means were separated using a Tukey test to search for significant differences between means. If the Levene test indicated heteroscedasticity of variances, a Tamhane test was used instead.

If data were not normally distributed, a non-parametric Kruskal-Wallis H test was used to indicate significant differences between means. If differences occurred, means were compared pairwise with a Mann-Whitney U test.

Countable data (e.g., development time, preoviposition period, lifetime oviposition, oviposition in ten days and longevity) were analyzed using a generalized linear model, with a Poisson

distribution if applicable (means ≈ variances) or a negative binomial distribution in case of overdispersion (means < variances), as determined by the deviance and Pearson goodness- of-fit statistics. If the variances were smaller than the means, or if the goodness-of-fit criteria did not support a generalized linear model, a non-parametric model, as explained above, was applied to indicate differences between means.

Parameters expressed as percentages (e.g., egg hatch, nymphal survival, puparium formation, adult emergence, proportion of ovipositing females and eggs which produced a puparium) were treated as binary data and compared by using a logistic regression. This generalized linear model makes use of a probit (log odds) link and a binomial error function. For each pairwise test, a linear regression coefficient was calculated, and it was tested whether the coefficient was significantly different from zero. For one parameter expressed as percentages (proportion of ovipositing *E. larvarum* females (FOM)) a logistic regression could not be used as two data sets were completely identical. Therefore the means were separated and compared pairwise using a Chi-square test. Considering the low number of cases (<50), the Yates correction for continuity was applied.

The sex ratios of the different treatments were not compared to each other but versus an equal male:female distribution (1:1 ratio), by using a Chi-square test.

PART III: RESULTS & DISCUSSION

1. *Orius thripoborus*

1.1. Survival and development

In this thesis, five different foods were tested on *O. thripoborus*, corresponding to five treatments. In the first treatment, a basic egg yolk diet (BE) was offered. This diet will further be called diet 1. The second and third treatment comprised the BE diet enriched with hemolymph of *H. illucens* (diet 2) or *A. pernyi* (diet 3). In the fourth treatment *E. kuehniella* eggs were offered to the first and second instar, and, from third instar on, BE enriched with *A. pernyi* hemolymph was offered. This combined diet will further be called diet 4. In the fifth and last treatment *E. kuehniella* eggs were presented throughout the entire life cycle (diet 5). Developmental parameters of *O. thripoborus* on these five different foods are shown in Table 1.

Nymphal survival (χ^2 = 46.301; df = 4; P < 0.001) was significantly lowest on diet 1, with only 20 out of 119 nymphs reaching adulthood. The highest nymphal survival rates were found on diets 4 and 5. Although the survival rate on diet 5 was 11% higher than on diet 4, the difference was not significant. No difference was found in survival rate between both hemolymph enriched diets (Table 1).

Developmental times for both females and males differed on nearly every diet (χ^2 = 60.593; df = 4; P < 0.001 and χ^2 = 53.152; df = 4; P < 0.001 for females and males, respectively). Only diets 2 and 3 resulted in similar development times for both sexes. Nymphs offered a combined diet (diet 4) developed faster than nymphs offered solely an artificial diet (diet 1, 2 or 3). The control diet (diet 5) yielded the shortest development times (Table 1).

Body weights ranged from 0.22 to 0.36 mg for female adults (F = 38.102; P < 0.001; df = 4, 72) and from 0.20 to 0.30 mg for males (F = 35.016; P < 0.001; df = 4, 71), and were only higher for adults fed *E. kuehniella* eggs (Table 1).

For all treatments, no significant deviation from a 1:1 sex ratio was observed (Table 1).

1.2.   Reproduction and longevity

Reproduction and longevity parameters of *O. thripoborus* on the five different diets are shown in Table 2.

No significant differences were found between the different diets for mean preoviposition period (χ^2 = 3.906; df = 4; P = 0.419) and proportion of ovipositing females (χ^2 = 2.034; df = 4; P = 0.729). These results could be caused by the low number of couples that could be tested, resulting in high variances. Preoviposition periods ranged between 4.7 (diet 3) and 7.0 days (diet 1). The proportion of ovipositing females ranged between 33.3% (diet 2) and 60.0% (diet 3) (Table 2).

Due to the low number of couples monitored, variances for lifetime oviposition were also quite high (χ^2 = 14.155; df = 4; P = 0.007). However, it was clear that females reared on the control diet (diet 5) oviposited the highest number of eggs. Diet 4 resulted in the lowest number of oviposited eggs, although the difference in lifetime oviposition when *O. thripoborus* was offered other artificial diets was not significant (Table 2).

The percentage of eggs hatched (χ^2 = 14.886; df = 4; P = 0.005) was, surprisingly, highest on diet 2. This is the only reproductive parameter for which a significant difference was found between *O. thripoborus* fed either of the two hemolymph enriched diets (diets 2 and 3). The lowest percentage of eggs hatched on diet 4, but the difference was only significant compared to diets 2 and 3 (Table 2).

Females provided with the *E. kuehniella* eggs (diet 5) lived longest, although this result was only significantly different from that on the combined diet (diet 4)(χ^2 = 9.541; df = 4; P = 0.049). No significant difference in longevity was found between females solely offered the three artificial diets (diets 1, 2 or 3). Female life span on diet 3 was only significantly different from that on diet 4. Male longevity (χ^2 = 24.061; df = 4; P < 0.001) on diet 5 was significantly higher than on all other diets. Male longevity on the other diets was not significantly different and values ranged between 8.54 days (diet 4) and 10.00 days (diet 1) (Table 2).

1.3.   Discussion

As was expected by analyzing the literature, the control treatment offering *E. kuehniella* eggs to *O. thripoborus* (diet 5) showed the best results, both in terms of development and reproduction

(Cocuzza et al., 1997; Ferkovich and Shapiro, 2004; Bonte and De Clercq, 2008; Bonte et al., 2012b; De Clercq et al., 2014). Compared with the other diets, *E. kuehniella* eggs resulted in the highest nymphal survival, fastest development and heaviest adults (Table 1). In terms of reproduction, the highest lifetime oviposition and male and female life spans were obtained on *E. kuehniella* eggs (Table 2). Bonte et al. (2012b) reared *O. thripoborus* nymphs on *E. kuehniella* eggs and a Parafilm® dome as a moisture source. The nymphs originated from the same laboratory culture as that being used in this thesis research, and were reared at the same conditions (25 ± 1 °C; RH 65 ± 5% ; 16:8 h (L:D)). This resulted in a nymphal survival of 64%. In the same study, nymphal survival on *E. kuehniella* eggs and a bean pod as a moisture source was 95%. In our research, no experiment was set up with an alternative moisture source, but it can be concluded that moisture source has a large influence on nymphal survival of this anthocorid. Other parameters observed in the research by Bonte et al. (2012b) included development time (15.6 and 16.3 days for females and males, respectively) and adult weight (0.41 and 0.31 mg for females and males, respectively). The different values obtained in this study compared with those observed by Bonte et al. (2012b) could be explained by fluctuations in both the quality of *E. kuehniella* eggs supplied by the commercial distributor as in the genetic variability of the *O. thripoborus* stock culture used in both studies.

The aim of this thesis was to study the effect on developmental and reproduction parameters of *O. thripoborus* when the artificial egg yolk diet developed by Arijs & De Clercq (2002) was enriched with hemolymph of *H. illucens* or *A. pernyi*. This enrichment should yield better results than the basic egg yolk diet, since the insect is expected to profit from specific growth factors and feeding stimulants usually found in the natural prey (De Clercq, 2004; Riddick, 2009). The developmental results in Table 1 support this hypothesis: both hemolymph enriched artificial diets (diets 2 and 3) resulted in higher nympal survival rates and lower male and female development times than the basic egg yolk diet (diet 1). In terms of reproduction (Table 2), the results are less clear since little significant differences were found. However, it seems that the hemolymph enriched diets tended to influence lifetime oviposition in a positive way, and, if more couples could have been formed, statistical analysis may have yielded less uncertainty. Overall, it can be concluded that a hemolymph enriched artificial diet supported development and reproduction of *O. thripoborus* in a better way than a basic artificial diet.

The results in Table 1 and Table 2 show that there was no effect of hemolymph type on the overall performance of *O. thripoborus*. Only egg hatch was differently affected by both hemolymph enriched diets, but these percentages could be influenced by molds infecting the bean pods in which the eggs were oviposited. When mold was observed, the number of eggs that hatched decreased markedly. To compensate for this, a correction factor was applied. The

number of eggs hatched on the normal bean pods was used to make an assumption of the number of eggs that would hatch on the moldy bean pod. Mold infected ± 15% of the bean pods, mostly for diets 3, 4 and 5.

None of the artificial diets (diets 1, 2 or 3) alone could match the results obtained on *E. kuehniella* eggs (diet 5). For all tested parameters, diet 5 yielded significantly better or equal results compared to diets 1, 2 and 3. Only in terms of egg hatch the results obtained on diet 2 were significantly better than those observed on diet 5, but as explained above, these numbers could have been influenced by moldy bean pods.

Nguyen et al. (2013) studied the effect of enriching a honey and egg yolk-based artificial diet with *A. pernyi* hemolymph on the predatory mite *A. swirskii*. Results showed that the enriched artificial diet led to faster female development, longer oviposition periods and higher oviposition rates of *A. swirskii* compared to the basic artificial diet. These results are similar to the ones found in this thesis. However, Nguyen et al. (2013) found that the enriched artificial diet supported development and reproduction of *A. swirskii* to the same extent as *Carpoglyphus lactis* L., a factitious prey which is routinely used in the mass rearing of the phytoseiid. In our research none of the tested artificial diets could match the results of the commonly used factitious prey *E. kuehniella*. Nguyen et al. (2013) noted that more research is warranted since only one generation of *A. swirskii* was observed. Nutritional imbalances within the diet could be revealed in subsequent generations (De Clercq et al., 2005a).

Diet 4, the combined diet, was used to investigate whether providing the first two instars with *E. kuehniella* eggs would contribute to better survival chances for these vulnerable stages and allow them to more easily deal with artificial food in the subsequent stages. This combined diet could link the good development and quality of *O. thripoborus* on a diet of *E. kuehniella* eggs with the lower price of an artificial diet. In terms of development, diet 4 yielded significantly better results compared with diets 2 and 3, but could not match the results of diet 5 (*E. kuehniella* eggs). However, reproduction of *O. thripoborus* reared on the combined diet was not better than when fed diets 2 or 3. Even more, lifetime oviposition on diet 4 was the lowest of all treatments. These results could possibly be explained by the inability of the *O. thripoborus* individuals to switch to the artificial diet in Parafilm® domes after having been fed as young nymphs on moth eggs. A notable high number of nymphs was found to have died some days after the new diet was provided. This lack of adaptability of the nymphs could have been the reason for poor reproduction and quality of the resulting adults.

Interestingly, for all diets packaged into the Parafilm® domes (i.e., all artificial diets except for diet 5) it was often observed that the domes leaked. This was detrimental for the *O. thripoborus*

nymphs and adults since they could drown in the liquid diet oozing out of the domes. After some time the remaining diet desiccated impeding the predators to feed from it. Furthermore, the leakages prevented the insects from effectively piercing through the Parafilm® surface using their stylet. This ultimately led to starvation of the predators in a number of cases. The quality of the currently used batch of Parafilm® was questioned. Therefore a sixth experiment was set up with 60 first instars of *O. thripoborus*, using a new batch of Parafilm® to create domes filled with artificial diet containing *A. pernyi* hemolymph. The water domes remained being created with Parafilm® from the 'old' batch. This experiment, however, was ended prematurely because too much of the tested nymphs again died.

These results lead to three conclusions: (1) Enriching an artificial diet with hemolymph improved the quality of the egg yolk diet, but development and reproduction of *O. thripoborus* reared on an enriched artificial diet could still not match that of *O. thripoborus* reared on *E. kuehniella* eggs. (2) A combination of *E. kuehniella* eggs offered to the first two instars and an enriched artificial diet offered from the third instar on into the adult life led to faster development and higher nymphal survival than the hemolymph enriched artificial diet alone, but overall did not result in better reproduction and longevity. (3) The type of hemolymph did not influence the quality of the artificial diet for *O. thripoborus*. Both hemolymph enriched artificial diets led to similar development and reproduction of this anthocorid.

Table 1: Developmental parameters for *O. thripoborus* on five different diets

Nr.	Treatment	Nymphal survival (%)	Development time (days) ♀	Development time (days) ♂	Adult weight (mg) ♀	Adult weight (mg) ♂	Sex ratio (♂/♀)
1	BE	(n=119) 16.8 ± 3.4 c	(n=12) 20.08 ± 0.67 d	(n=8) 20.88 ± 0.69 d	(n=12) 0.23 ± 0.01 b	(n=8) 0.21 ± 0.01 b	(n=20) 1 : 1.5*
2	BE + hemolymph *H. illucens* (20 w/w%)	(n=117) 29.9± 4.3 b	(n=15) 17.07 ± 0.42 c	(n=20) 17.90 ± 0.38 c	(n=14) 0.24 ± 0.01 b	(n=18) 0.22 ± 0.01 b	(n=35) 1 : 0.75*
3	BE + hemolymph *A. pernyi* (20 w/w%)	(n=117) 28.2 ± 4.2 b	(n=17) 18.00 ± 0.47 c	(n=16) 17.81 ± 0.54 c	(n=17) 0.24 ± 0.01 b	(n=14) 0.20 ± 0.01 b	(n=33) 1 : 1.06*
4	*E. kuehniella* eggs (N1-N2) and BE + hemolymph *A. pernyi* (20 w/w%) (N3-death)	(n=60) 51.7 ± 6.5 a	(n=17) 15.65 ± 0.50 b	(n=20) 14.71 ± 0.29 b	(n=17) 0.22 ± 0.01 b	(n=20) 0.20 ± 0.01 b	(n=31) 1 : 1.21*
5	*E. kuehniella* eggs	(n=59) 62.7 ± 6.3 a	(n=17) 12.76 ± 0.18 a	(n=14) 13.30 ± 0.15 a	(n=17) 0.36 ± 0.01 a	(n=16) 0.30 ± 0.01 a	(n=37) 1 : 0.85*

Mean ± standard error of mean; values in the same column followed by the same letter are not significantly different (P > 0.05): binary probit test (% nymphal survival); Kruskal-Wallis ANOVA with Mann-Whitney U post-hoc test (female and male development time); or one-way ANOVA with Tukey post-hoc test: (female and male adult weight).

n = number of replicates

BE = basic egg yolk diet

* none of the values differs significantly from a 1:1 ratio; χ^2 and P values were 0.800, 0.371; 0.714, 0.398; 0.030, 0.862; 0.290, 0.590; and 0.243, 0.622, respectively (Chi-square test, df = 1).

Table 2: Reproduction parameters and longevities for *O. thripoborus* on five different diets

Nr.	Treatment	Preoviposition period (days)	Lifetime oviposition (eggs/♀)	Ovipositing females (%)	Egg hatch (%)	Longevity (days) ♀	♂
1	BE	(n=5) 7.00 ± 1.30 a	(n=5) 15.2 ± 6.03 b	(n=9) 55.56 ± 17.6 a	(n=76) 53.9 ± 5.8 bc	(n=12) 15.83 ± 2.95 ab	(n=7) 10.00 ± 3.21 b
2	BE + hemolymph *H. illucens* (20 w/w%)	(n=4) 6.25 ± 0.85 a	(n=4) 18.25 ± 2.93 ab	(n=12) 33.33 ± 14.2 a	(n=73) 74.0 ± 5.2 a	(n=14) 12.36 ± 1.95 ab	(n=18) 9.56 ± 1.21 b
3	BE + hemolymph *A. pernyi* (20 w/w%)	(n=9) 4.67 ± 0.33 a	(n=9) 19.67 ± 5.37 b	(n=15) 60.00 ± 13.1 a	(n=177) 57.1 ± 3.7 b	(n=16) 18.59 ± 2.23 a	(n=15) 9.60 ± 1.71 b
4	*E. kuehniella* eggs (N1-N2) and BE + hemolymph *A. pernyi* (20 w/w%) (N3-death)	(n=7) 6.43 ± 0.30 a	(n=7) 7.29 ± 3.90 b	(n=14) 50.00 ± 13.9 a	(n=51) 41.2 ± 7.0 c	(n=17) 8.53 ± 1.20 b	(n=18) 8.54 ± 0.95 b
5	*E. kuehniella* eggs	(n=8) 6.38 ± 1.02 a	(n=8) 53.13 ± 19.10 a	(n=16) 50.00 ± 12.9 a	(n=425) 54.1 ± 2.4 bc	(n=17) 24.06 ± 2.14 a	(n=13) 20.78 ± 2.91 a

Mean ± standard error of mean; values in the same column followed by the same letter are not significantly different (P > 0.05): generalized linear model with Poisson distribution (preoviposition period); generalized linear model with negative binomial distribution (lifetime oviposition; female and male longevity); or binary probit test (% ovipositing females; % egg hatch).
n = number of replicates
BE = basic egg yolk diet

2. *Exorista larvarum*

2.1. Survival and development

Exorista larvarum individuals were reared from egg to adult on three different media, corresponding to three different treatments. In the first treatment, a basic egg yolk and skimmed milk medium (BEM)(medium 1) was used to rear *E. larvarum* from egg to pupa. The second and third medium were based on the same BEM, but were enriched with hemolymph. The same two types of hemolymph as those utilized in the experiments on *O. thripoborus* were now also employed for *E. larvarum*. Medium 2 consisted of BEM enriched with hemolymph of *H. illucens*. For medium 3 the BEM had been enriched with hemolymph of *A. pernyi*.

As an adult, every individual was offered sugar cubes, cotton balls soaked in honey-water solution and a water cup, regardless of the treatment. Developmental parameters of *E. larvarum* on the three different media are shown in Table 3.

In previous studies, the egg hatch rate and adult emergence rate of *E. larvarum* were not influenced by the medium, even when compared with rearing *in vivo* (Dindo et al., 2003; Dindo et al., 2006). The results in Table 3 show that this trend was be confirmed in this thesis research. None of the three tested media led to a significant difference in terms of egg hatch rate (χ^2 = 0.905; df = 2; P = 0.636) or adult emergence (χ^2 = 2.169; df = 2; P = 0.338). The medium influenced the formation of puparia (Table 3), since less puparia were produced on medium 1 compared to media 2 and 3. However, the differences were not significant (χ^2 = 2.981; df = 2; P = 0.225). This was possibly caused by the rather low number of replicates, leading to high variances.

Analysis of puparium weight led to different conclusions for males and females. For females, medium 3 yielded significantly lower female puparium weights than medium 1 (F = 5.2299; df = 2,16; P = 0.018). Puparium weights on medium 2 did not differ significantly from those obtained on either media 1 or 3. For male puparium weights, no significant difference was found among the three media (F = 5.2299; df = 2,16; P = 0.018), however variances were high and in absolute figures medium 3 again yielded the lowest weights. On none of the media a significant difference was found between the observed *E. larvarum* sex ratio and a 1:1 sex ratio.

Development times are shown in Table 4. The fact that the sum of the number of cases of males and females is not equal to the number of cases of the general development time from egg to

pupa is caused by individuals that developed a puparium, but did not emerge as an adult. Therefore these pupae could not be sexed, since sex can only be detected in adult flies. The development time of these unsexed individuals was however taken into account for the general development time from egg to pupa.

Results for the development time from egg to pupa were similar for females and males (Table 4). For both sexes, development of *E. larvarum* on medium 2 was significantly faster than that on medium 1 (χ^2 = 7.524; df = 2; P = 0.023 and χ^2 = 6.359; df = 2; P = 0.042 for females and males, respectively). In general, development time from egg to pupa on both hemolymph enriched media (media 2 and 3) was faster than on the basic egg yolk and skimmed milk medium (medium 1) (χ^2 = 16.9667; df = 2; P = 0.0002). It is clear that both types of hemolymph fastened larval development. Development times from pupa to adult were not significantly different among *E. larvarum* individuals reared on the three media (χ^2 = 1.197; df = 2; P = 0.550, χ^2 = 0.224; df = 2; P = 0.894 and χ^2 = 1.010; df = 2; P = 0.774 for female, male and general development time, respectively). Differences in the total development time (from egg to adult) were similar to the differences in development time from egg to pupa (χ^2 = 9.467; df = 2; P = 0.009, χ^2 = 8.640; df = 2; P = 0.013 and χ^2 = 15.2212; df = 2; P = 0.0005 for female, male and general development time, respectively).

2.2. Reproduction

For this set of parameters a separation was made between females (FOM) and males (MOM) obtained from the medium. Newly emerged females obtained from the medium were paired with a male from the laboratory culture, regardless of age. Males obtained from the medium were paired with a virgin female from the laboratory culture that had emerged in the same period as the male (within ca. 48 h.). Reproduction values of the latter were based on reproduction of the female with which the male was paired. Results are shown in Table 5.

Due to time constraints, the number of eggs oviposited per female was only observed for a ten day period, starting from the beginning of oviposition. This is the time interval during which most eggs are oviposited by *E. larvarum* (Dindo et al., 1999) and other entomophagous insects (Sighinolfi et al., 2008; 2013).

Because only one female was obtained from medium 3, the data referred to this female were not taken into account for the statistical analysis of most reproduction parameters. Therefore only females obtained on medium 1 and 2 were compared to each other (Table 5). This was an indication of the poor quality of the adults obtained on medium 3. Preoviposition period and

oviposition in ten days were similar for females obtained on media 1 and 2 (χ^2 = 1.725; df = 1; P = 0.189 and χ^2 = 0.015; df = 1; P = 0.904, respectively). Puparial yield was significantly higher for females obtained on medium 2 (χ^2 = 31.401; df = 1; P < 0.001). This may indicate that the hemolymph of *H. illucens* provided to the larvae had a positive influence on the quality of the females.

With regard to the reproduction parameters of the females paired with males obtained on the medium, none of the parameters showed a significant difference between either one of the three media (χ^2 = 2.844; df = 2; P = 0.241, χ^2 = 1.049; df = 2; P = 0.592, χ^2 = 3.387; df = 2; P = 0.184 and χ^2 = 0.342; df = 1; P = 0.843 for preoviposition period, oviposition in 10 days, percentage of eggs which produced puparia and percentage of ovipositing females, respectively) (Table 5). However, variances were high, and although the differences were not significant, 10-day oviposition and puparial yield were highest on medium 2, suggesting again the higher quality of adults reared on this medium.

2.3.   Discussion

In the laboratories of Entomology of DipSA (Dipartimento di Scienze Agrarie, Alma Mater Studiorum Università di Bologna, Italy) *G. mellonella* larvae are used as a factitious host for the development of *E. larvarum* to assure a continuous culture. This factitious host provides most of the nutritional requirements needed for full development (Campadelli, 1988). In this thesis no experiment was set up in which the development and reproduction of *E. larvarum* on *G. mellonella* was tested. Dindo et al. (2006) however studied the biological and biochemical differences between *in vitro* and *in vivo* reared *E. larvarum* on a similar laboratory culture and at the same conditions as those being maintained during this thesis research. The basic egg yolk and skimmed milk medium was supported by agar. Dindo et al. (2003) studied the effect of using cotton as a support for the medium instead. The most important results from both studies will be discussed.

The percentage of puparia formed on *G. mellonella* larvae is generally lower than on an artificial medium, because often too many eggs are oviposited on one larva to provide enough space for the development of each of those eggs (Dindo et al., 2006). However, the percentage of puparia formed on medium 1 in this thesis research was even lower than the percentage found by Dindo et al. (2006) on *G. mellonella* larvae (22.4% and 30.2%, respectively). Comparing medium 1 with the cotton-supported medium by Dindo et al. (2003) the percentage of puparia formed on medium 1 was almost three times as low (22.4 % and 61.1%, respectively). The percentage of

puparia formed on media 2 and 3 was higher than on medium 1, but still almost twice as low as on the cotton-supported medium by Dindo et al. (2003). These differences could be explained by the genetic variability of the parasitoid laboratory culture and/or unknown environmental factors. A third explanation could be mold infecting the wells. *Exorista larvarum* larvae are quite resistant to mold but fungal contamination of the medium could still lead to a lower number of eggs hatched and puparia formed (Dindo et al., 2003). Mold had infected 1 well out of 49 containing medium 1, and 13 wells out of 49 containing medium 2. None of the 47 wells containing medium 3 was infected.

Female and male puparium weight of *E. larvarum* reared on the three media tested in this thesis research was lower than that observed on the cotton-supported medium by Dindo et al. (2003). However, the latter medium did result in slower development than any of the three media tested here. The fast developmental times of *E. larvarum* on media 2 and 3 even matched those obtained by Dindo et al. (2006) on *G. mellonella* larvae. The results for reproduction parameters were promising as well. The average percentages of puparium-forming eggs oviposited by adults reared on medium 2 were 30.59% (FOM) and 27.67% (MOM) (Table 5). These are higher percentages than those observed on the agar-supported medium (21.9%) or on *G. mellonella* larvae (25.9%) by Dindo et al. (2006).

The aim of this thesis part was to enrich the artificial egg yolk and skimmed milk medium by developed by Mellini & Campadelli (1996c) and described by Farneti et al. (1998) with hemolymph of *H. illucens* or *A. pernyi*, and study the effect on developmental and reproduction parameters of *E. larvarum* reared on these enriched media. The first artificial media for *E. larvarum* developed by Mellini et al. (1993b) and, later, Dindo et al. (1999), were enriched with *G. mellonella* hemolymph. Because the maintenance of the lepidopterous insect colony requires labor, subsequent efforts were aimed at deleting host material from *E. larvarum* media (see literature study). However, since rearing on insect material-free media may, in the long run, result in lower parasitoid production and quality (Mellini et al., 1996), new research has now aimed towards enriching artificial media with insect components.

In the present work, puparium formation was higher and development time faster for *E. larvarum* larvae reared on both hemolymph enriched artificial media (media 2 and 3) compared to medium 1, although differences were not always significant. The type of hemolymph did not affect developmental parameters of *E. larvarum*, as there were no significant differences between larvae reared on media 2 and 3 (Table 3 and Table 4).

The reproduction parameters of *E. larvarum* adults reared on media 2 and 3 did not corroborate the hypothesis that both types of hemolymph delivered similar results. Only 1 out of 5 females

obtained from medium 3 oviposited, and although the number of cases was too low to perform statistical analysis, it was clear that the integration with *A. pernyi* hemolymph negatively affected the quality of the *in vitro*-reared females. Females obtained from medium 2 performed far better. Compared to females obtained from medium 1, preoviposition period and oviposition in ten days were not significantly different, but the percentage of eggs which produced a puparium was significantly higher for females obtained from medium 2. For males obtained from the medium (MOM's), none of the reproduction parameters resulted in a significant difference between the three tested media (Table 5).

These results lead to three conclusions: (1) There was a clear difference between the two types of hemolymph. Enriching the standard artificial medium of *E. larvarum* (BEM) with hemolymph of *H. illucens* led to far better results than enriching it with hemolymph of *A. pernyi*, especially in terms of reproduction.The latter medium even led to poorer results than the basic artificial medium. This could mean that different types of hemolymph contain different types of nutrients. Specific components essential for *E. larvarum* development may not be present in *A. pernyi* hemolymph. (2) Enriching the basic artificial medium with *H. illucens* hemolymph seemingly improved the quality of the medium for the tachinid. It led to faster developmental times and a higher yield of second generation puparia, despite a high number of wells infected with mold. (3) The quality of the *E. larvarum* male obtained on the medium had a low (or no) influence on the reproduction parameters of the female with which it was paired, for at least one generation.

Table 3: Developmental parameters for *E. larvarum* on three different media

Nr.	Medium	Egg hatch (%)	Puparium formation (%)	Adult emergence (%)	Puparium weight (mg) ♀	Puparium weight (mg) ♂	Sex ratio (♂/♀)
1	BEM	(n=49) 67.3 ± 6.8 a	(n=49) 22.4 ± 6.0 a	(n=11) 90.9 ± 9.1 a	(n=6) 46.78 ± 3.42 a	(n=4) 45.65 ± 6.89 a	(n=10) 1 : 1.5 a*
2	BEM + hemolymph *H. illucens* (20 w/w%)	(n=49) 61.2 ± 7.0 a	(n=49) 36.7 ± 7.0 a	(n=18) 72.2 ± 10.9 a	(n=6) 44.88 ± 2.76 ab	(n=7) 50.80 ± 5.77 a	(n=13) 1 : 0.88 a*
3	BEM + hemolymph *A. pernyi* (20 w/w%)	(n=47) 70.2 ± 6.7 a	(n=47) 36.2 ± 7.1 a	(n=17) 88.2 ± 8.1 a	(n=7) 35.37 ± 2.06 b	(n=8) 38.96 ± 2.64 a	(n=15) 1 : 0.86 a*

Mean ± standard error of mean; values in the same column followed by the same letter are not significantly different (P > 0.05): binary probit test (% egg hatch; puparium formation; adult emergence); one-way ANOVA with Tukey post-hoc test (female and male adult weight); Chi-square test (sex ratio).

n = number of replicates

BEM = basic egg yolk and skimmed milk medium

* none of the values differs significantly from a 1:1 ratio; χ^2 and P values were 0.400, 0.527; 0.067, 0.796 and 0.077, 0.782, respectively (Chi-square test, df = 1).

Table 4: Development times for *E. larvarum* on three different media

Nr.	Medium	Developmental time (days)								
		from egg to pupa			from pupa to adult			Total (from egg to adult)		
		♀	♂	general	♀	♂	general	♀	♂	general
1	BEM	(n=6) 13.00 ± 0.26 a	(n=4) 13.00 ± 1.22 a	(n=11) 13.00 ± 0.43 a	(n=6) 8.67 ± 0.33 a	(n=4) 8.75 ± 0.48 a	(n=10) 8.70 ± 0.26 a	(n=6) 21.67 ± 0.33 a	(n=4) 21.75 ± 0.85 a	(n=10) 21.70 ± 0.37 a
2	BEM + hemolymph *H. illucens* (20 w/w%)	(n=6) 10.00 ± 0.00 b	(n=7) 9.43± 0.36 b	(n=17) 9.79 ± 0.15 b	(n=6) 9.00± 0.65 a	(n=7) 9.00± 0.38 a	(n=13) 9.00± 0.25 a	(n=6) 19.00 ± 0.37 b	(n=7) 18.43 ± 0.30 b	(n=13) 18.69 ± 0.24 b
3	BEM + hemolymph *A. pernyi* (20 w/w%)	(n=7) 9.57 ± 1.38 ab	(n=8) 10.75 ± 0.75 ab	(n=18) 9.82 ± 0.70 b	(n=7) 9.00 ± 0.37 a	(n=8) 8.88 ± 0.23 a	(n=15) 8.93 ± 0.32 a	(n=7) 18.57 ± 1.27 b	(n=8) 19.63 ± 0.56 ab	(n=15) 19.13 ± 0.65 b

Mean ± standard error of mean; values in the same column followed by the same letter are not significantly different (P > 0.05): Kruskal-Wallis test (female, male and general development time pupa to adult); Kruskal-Wallis ANOVA with Mann-Whitney U post-hoc test (female, male and general development time egg to pupa; female, male and general total development time)
n = number of replicates
BEM = basic egg yolk and skimmed milk medium

Table 5: Reproduction parameters for *E. larvarum* on three different media

Nr.	Medium	Preoviposition period (days)		Oviposition in 10 days (eggs/♀)		Eggs which produced puparia (%)		Ovipositing females (%)	
		FOM	MOM	FOM	MOM	FOM	MOM	FOM	MOM
1	BE	(n=6) 4.67 ± 0.49 a	(n=3) 4.67 ± 0.88 a	(n=6) 54.83 ± 17.80 a	(n=3) 59.33 ± 33.07 a	(n=329) 12.77 ± 1.84 b	(n=178) 21.92 ± 3.11 a	(n=6)# 100.0 ± 0.0 a	(n=3) 100.0 ± 0.0 a
2	BE + hemolymph *H. illucens* (20 w/w%)	(n=6) 5.67 ± 0.61 a	(n=4) 6.25 ± 0.48 a	(n=6) 58.83 ± 7.28 a	(n=4) 91.25 ± 16.37 a	(n=353) 30.59 ± 2.46 a	(n=365) 27.67 ± 2.35 a	(n=6)# 100.0 ± 0.0 a	(n=6) 66.7 ± 21.2 a
3	BE + hemolymph *A. pernyi* (20 w/w%)	(n=1)* 3.00 ± 0.00	(n=3) 7.00 ± 1.15 a	(n=1)* 8.00 ± 0.00	(n=3) 41.33 ± 5.78 a	(n=8)* 25.00 ± 16.37	(n=124) 20.97 ± 3.67 a	(n=5) 20.00 ± 20.0 b	(n=6) 50.0 ± 22.4 a

Mean ± standard error of mean; values in the same column followed by the same letter are not significantly different (P > 0.05): Kruskal-Wallis test (preoviposition period FOM and MOM); generalized linear model with negative binomial distribution (oviposition in 10 days FOM and MOM); binary probit (eggs which produced puparia FOM and MOM; ovipositing females MOM); Chi-squared test (ovipositing females FOM)

n = number of replicates

BEM = basic egg yolk and skimmed milk medium

FOM = female obtained from the medium

MOM = male obtained from the medium

* The number of replicates was too low to be considered in the statistical analysis.

 # Medium 1 and 2 yielded the exact same results. Therefore a binary probit could not be used. A Chi-squared test was used instead to test the significance of the differences between the three diets pairwise.

SUMMARY AND FUTURE PERSPECTIVES

Artificial diets/media can offer an alternative for natural or factitious hosts/prey in the mass rearing of beneficial insects. Many artificial diets have been developed in the past few decades, both for predators and parasitoids, mainly devoid of insect components. However, such insect components can provide the reared insect with specific growth factors or feeding stimulants typically found in the natural host. This thesis research aimed at improving a basic artificial diet for the anthocorid predator *O. thripoborus* and a basic artificial medium for the tachinid parasitoid *E. larvarum* by enriching it with insect hemolymph. Two types of hemolymph were tested: hemolymph of the black soldier fly *H. illucens* and hemolymph of the Chinese oak silkworm *A. pernyi*.

For both insects, adding hemolymph of *H. illucens* to the basic artificial diet proved to be beneficial. The enriched artificial diet led to faster development and higher numbers of individuals reaching adulthood for both *O. thripoborus* and *E. larvarum* compared to the basic artificial diet. Results for reproduction parameters were less clear but promising as well. If there had been more time to do research using a greater number of replicates, results may have been more decisive.

The effect of adding *A. pernyi* hemolymph to their basic artificial diet differed strongly between *O. thripoborus* and *E. larvarum*. For *O. thripoborus* , the diet enriched with *A. pernyi* hemolymph resulted in similar values for developmental and reproduction parameters compared to the *H. illucens* enriched artificial diet. Both types of hemolymph influenced the development and quality of the anthocorid in a positive way as compared to the basic artificial diet. In contrast for *E. larvarum*, the medium enriched with hemolymph of *A. pernyi* led to similar development but far worse reproduction compared to the *H. illucens* enriched artificial medium. For some parameters results on the medium enriched with *A. pernyi* hemolymph were even worse than for *E. larvarum* reared on the basic artificial medium. This leads to the conclusion that the type of hemolymph added to the artificial diet influences development and quality of *E. larvarum*.

Another point of interest in this thesis was whether the enriched artificial diet could replace the factitious prey or host currently used in the rearing of *O. thripoborus* and *E. larvarum* (*E. kuehniella* eggs and *G. mellonella* larvae, respectively) while maintaining a high quality of the natural enemy. This question again led to different answers. For *O. thripoborus* the answer seems to be negative. Development as well as reproduction values of *O. thripoborus* reared on both hemolymph enriched artificial diets could not match the results of *O. thripoborus* reared on

E. kuehniella eggs. For *E. larvarum* no simultaneous treatment was set up using *G. mellonella* larvae, but previous research by Dindo et al. (2006) could be used for comparison of the results. *Exorista larvarum* reared on the artificial medium enriched with *H. illucens* hemolymph developed equally fast as on *G. mellonella* larvae as observed by Dindo et al. (2006). Puparia were heavier and more eggs oviposited by adults reared on the medium formed a puparium compared to rearing on *G. mellonella* larvae. Thus, results are promising, but to be able to draw a clear and unequivocal conclusion, further research should be done.

In the experiments with *O. thripoborus* Parafilm® domes were used. Parafilm® slows down bacterial growth and allows better feeding by the predatory bug on the diet. However, in our research, many problems occurred with leaking Parafilm® domes. Future research should be aimed towards optimizing the presentation of the artificial diet for this predator. A possible approach could be to provide the *O. thripoborus* individuals an enriched artificial diet as food and a piece of bean pod as a moisture and extra nutrient source. The diet could be provided by placing a droplet on the bean pod, keeping it moist for some days. This may lead to higher survival rates and shorter development times (Bonte et al., 2012b), but would also solve the Parafilm® problem since the leaking domes would be eliminated from the experiments. Another possibility to solve the Parafilm® problem is to use other forms of encapsulation, for instance Hydrocapsules® or microcapsules (Riddick, 2009; Tan et al., 2013).

Other research could be aimed towards optimizing the content of the artificial diet for *O. thripoborus*. Numerous artificial diets have been developed for predators, for *Orius* species in particular, but none of them has been able to match the results of *O. thripoborus* reared on eggs of lepidopterans. In this thesis research an egg yolk based artificial diet was used, but further research could study the effects of adding hemolymph to other developed artificial diets.

The hemolymph of various insects has been used in previous research on many different predators and parasitoids. In this thesis, hemolymph of *H. illucens* and *A. pernyi* was tested. *Hermetia illucens* has great potential for industrial-scale production due to several beneficial traits (e.g., short development cycle, high conversion rate, ability to develop on a variety of food sources,…)(Nguyen et al., 2013). *Antheraea pernyi* used to be available at relatively low prices throughout China, but in recent years they have become less easily available and prices have increased (Lü et al., 2013). Different types of hemolymph which are cheaper and more easily obtainable could be tested in future research. The components of the pupal hemolymph which are responsible for increased performance should be identified, especially for *E. larvarum* since for this parasitoid a clear difference between the two tested types of hemolymph was observed.

Further research may also study the potential of trehalose to replace insect hemolymph entirely, as has been done before for *Trichogramma* species (Lü et al., 2013).

This thesis research only observed *O. thripoborus* and *E. larvarum* reared on an artificial diet for one generation. Future research should monitor the performance of both species reared on a hemolymph enriched artificial diet for multiple generations. The quality of the reared insects could decrease over subsequent generations. For example, for *E. larvarum* no difference was found in terms of reproduction for the females paired with males obtained on the different artificial media. So the quality of the male has no influence on reproduction parameters in the first generation. But it is not unlikely that after subsequent generations a loss of quality in the males will ultimately affect reproduction.

Finally, future research may also focus on the effectiveness of *O. thripoborus* and *E. larvarum* as a biological control agent after being reared on an artificial diet for subsequent generations (Grenier and De Clercq, 2003). Dindo et al. (2002) compared *in vitro* and *in vivo* reared *E. larvarum* in terms of their effectiveness against *L. dispar*, showing no significant difference in the percentage of *L. dispar* larvae from which *E. larvarum* puparia were obtained. Bonte & De Clercq (2010c) observed that final instar nymphs and female adults of *O. laevigatus* reared on artificial diet or pollen killed as many second instars of *F. occidentalis* as their peers reared on *E. kuehniella* eggs, despite a lower body weight. Results of both studies were promising, but effectiveness of the predator and parasitoid reared on an artificial diet were only observed for one generation. This type of research should be extended to observing subsequent generations of *Orius* and *Exorista* species reared on an artificial diet.

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