CHARACTERIZATION OF ANTIGENIC AND MORPHOLOGICAL PROPERTIES OF *GASTEROPHILUS INTESTINALIS* LARVAE

Thèse de Doctorat

présentée à la

Faculté des Sciences, Institut de Biologie
de l'Université de Neuchâtel

par

Liselore Roelfstra

Biologiste diplômée de l'Université de Neuchâtel

Jury

Prof. Kurt Pfister, Directeur de thèse (Munich)
Prof. Bruno Betschart (Neuchâtel)
Prof. Peter-Allan Diehl (Neuchâtel)
Dr. Jean-François Graf (Neuchâtel)
Dr. Petr Kopacek (Ceske Budejovice)
Dr. Pierre Brocard (Grandson)

Neuchâtel, le 21 août 2009

Université de Neuchâtel

FACULTE DES SCIENCES
Secrétariat-Décanat de la faculté
- Rue Emile-Argand 11
- CP 158
- CH-2009 Neuchâtel

IMPRIMATUR POUR LA THESE

Characterization of antigenic and morphological properties of *Gasterophilus intestinalis* larvae

Liselore ROELFSTRA

UNIVERSITE DE NEUCHATEL

FACULTE DES SCIENCES

La Faculté des sciences de l'Université de Neuchâtel,
sur le rapport des membres du jury

MM. K. Pfister (Université de Neuchâtel/LMU, München D, directeur de thèse),
B. Betschart (co-directeur de thèse), P.-A. Diehl, J.-F. Graf,
P. Brocard (Grandson CH) et P. Kopàcek (Ceské Budejovice, Czech Republic)

autorise l'impression de la présente thèse.

Neuchâtel, le 8 septembre 2009

Le doyen :
F. Kessler

UNIVERSITE DE NEUCHATEL
FACULTE DES SCIENCES
Secrétariat - décanat de la faculté
Rue Emile-Argand 11 - CP 158
CH-2009 Neuchâtel

Felix Kessler

Table of contents

1 Introduction

Myiasis is the infestation of live humans and vertebrate animals with the larvae of diptera, which at least for a certain period, feed on host's dead or living tissues (Zumpt 1965). Animal and human infestations by dipteran larvae occur worldwide predominantly caused by species of the Calliphoridae, Sarcophagidae and Oestridae families. Some myiasis-causing species are considered as major pests, being responsible for important economic losses to the livestock industry, particularly in developing countries (Otranto et al. 2006; Stevens et al. 2006).

The extent of the disease induced by myiasis-causing larvae and the host immune response depend on the fly species involved (Otranto 2001). The myiases caused by Calliphoridae (blowflies) and Sarcophagidae (fleshflies) are of short duration (4-7 days), engendered by both obligate and/or facultative parasites, and generally cause acute skin diseases. Such infestations offer few opportunities for an immune response and lead to the animal's death without treatment (Sandeman 1996). In contrast, the Oestridae (botfly larvae) are all obligatory parasites that remain in the host body for a long duration (few weeks to months) at specific locations, according to the species. The botfly larvae, according to the period they spend in the host body, interact with the host immune system and stimulate cellular and humoral responsiveness (Sandeman 1996; Otranto 2001). The host immune response to myiasis-causing larvae is related to animal health and to the larval biology, thus depend on various parameters including the site of parasitism, the nature of the antigens or the host immune defense mechanisms (Hall and Wall 1995). During the past decade, considerable attention was given to the biology, immunology, epidemiology and control of myiasis-causing species. Most investigations focused on hypodermosis (*Hypoderma bovis* and *Hypoderma lineatum*), oestrosis (*Oestrus ovis*) and on fly strike in sheep (*Lucilia cuprina* and *Lucilia sericata*) (Baron and Colwell 1991), but there are still many aspects and species about which little is known (Hall and Wall 1995). If this can be understood for myiases in wildlife, it is surprising for gasterophilosis which can cause severe damages to equids and consequently to the horse industry.

Indeed, the infestation of horses by *Gasterophilus* larvae is highly damaging resulting in major economic and welfare problems. The migration of the larvae in the host's mouth can lead to serious tissue damages and consequently to bacterial infections. The larvae attached in the host stomach cause inflammation and ulceration. The general health can be affected as infested equids may be depressed, and show reduced ability to feed and loose weight. The key problem is that while adult flies are visible around the host and eggs can be recognized on the horse's hairs, the presence of the larvae inside the host body is not detectable. Despite the negative impact of these parasites on their host, appropriate research on them has been neglected. A single immunological approach has been reported on the development of antibodies against *Gasterophilus* spp larvae in horses and donkeys (Escartin-Pena and Bautista-Garfias 1993) however the specificity of the immune reactions could not be established. There is therefore an urgent need to study this host-parasite interaction in more detail.

The present study was realized to elaborate some characteristics of *Gasterophilus intestinalis* larvae and to understand the interaction of the larvae with horses, by using different approaches:

(i) A proteomic analysis to characterize second and third instar larvae proteins of *G. intestinalis*, and to analyze the immune reaction of horses and immunized mice against larval antigens
(ii) A morphological study of the midgut and salivary glands of second and third instar larvae of *G. intestinalis* by light, scanning and electron microscopy
(iii) A preliminary chromatographic study to analyze the protein profile of third instar larvae and to identify the antigenic properties of the collected fractions
(iv) An analysis by ELISA tests of the antigenic properties of several *G. intestinalis* larval extracts when exposed to horse sera and immunized mice sera
(v) A preliminary analysis of antigenic properties of *G. intestinalis* first instar larvae using ELISA tests and Western blots

As only limited research focused on *Gasterophilus* spp, the present approach aims to enhance the understanding of this host-parasite interaction but also to demonstrate its complexity. This study reveals the necessity to identify a reliable diagnostic tool to allow the detection and treatment of infested animals.

2 Literature review

2.1 Generalities

Biology is a branch of the natural sciences which studies living organisms. Most biological sciences are specialized disciplines and grouped by the type of organism being studied. Entomology is the scientific study of Insects (Arthropoda). Insects account more than 2/3rds of all known organisms (1.3 millions described species), dating back some 400 millions years (Chapman 2005), and having many interactions with all forms of life on earth. Entomology is rooted in nearly all human cultures and has involved throughout history an enormous list of personalities, including famous figures such as Charles Darwin or Karl Ritter von Frisch (1973 Nobel Prize). Often considered as a science preserved in dusty files, entomology entered modern popular culture via the forensic inquiries of recent American cult series. Medical and veterinary entomology more specifically study the Insects at the origin of pathologies in man and animals. Insects can be considered as vectors when they transmit pathogens or can be themselves pathogenic. Some diseases are caused by the larvae of Insects, belonging specifically to the Order of Diptera, and the consequence of these infestations are known under the term of Myiasis. Myiasis has a great economic impact as it is responsible for significant losses to the livestock industry worldwide (Baron and Colwell 1991).

2.2 Definition and classification of myiasis

The first term that refers to animal diseases caused by larvae of insects was scholechiasis, proposed by Kirby and Spence (1818). A few years later, Hope (1840) proposed the term myiasis (Greek *myia* = fly) to differentiate these diseases caused only by fly larvae. The most used definition was proposed by Zumpt (1965) who defined myiasis as “the infestation of live human and vertebrate animals with dipterous larvae, which, at least for a certain period, feed on the host’s dead or living tissues, liquid body substances, or ingested food”.

The different forms of myiasis have been classified in two ways:

1. Anatomically: according to the anatomical site of infestation on the host (Zumpt 1965):

- Dermal/Subdermal: including as well species which infest wounds, called traumatic myiasis (*Cochliomyia* spp.; *Chrysomyia* spp.; *Wohlfahrtia* spp; *Lucilia* spp.), then species that undergo a systematic migration and develop in boil-like swellings, called furuncular myiasis (*Dermatobia* spp.; *Hypoderma* spp.).
- Nasopharyngeal: the larvae are deposited in nostrils or mouth and invade sinuses and pharyngeal cavities. (*Oestrus* spp.; *Pharyngobolus* spp.; *Rhinoestrus* spp.).
- Intestinal: eggs or hatched larvae are ingested by the host and larval development occurs in the gastrointestinal tract (*Gasterophilus* spp.).
- Urinogenital: these are facultative infestations by larvae of the genera *Musca* or *Fannia* that occur in case of bad environmental and corporal hygiene conditions.
- Sanguinivorous: this term designates blood sucking maggots that otherwise do not invade the body (*Auchmeromyia* spp).

Oestrids do not adopt a sanguinivorous strategy, nor do they invade the urinogenital tract.

2. Parasitologically: according to their level of dependence on the host (Patton 1922; Zumpt 1965). Three main groups of myiasis producing species can be distinguished:

- Obligatory parasites: larvae must develop on live hosts to complete the lifecycle but imagos are free-living and generally have a very brief life. Some species are very specific in the selection of their host (*Oestrus* spp.; *Hypoderma* spp.; *Gasterophilus* spp.). Other species can infest animals or humans indistinctly (*Cochliomyia hominivorax; Chrysomyia bezianna; Dermatobia hominis*; *Wohlfahrtia magnifica*).

- Facultative parasites: larvae live free, on dead organic matter or under certain conditions they can infest live organisms particularly in presence of a wound. They can be divided in two groups, the primary species which are able to initiate myiasis (= facultative 1) and the secondary species which occur after obligate or primary species have initiated it (= facultative 2) (Table 2).

Zumpt (1965) described a fourth group, the accidental myiasis or pseudomyiasis, which occur when fly eggs or larvae are inadvertently swallowed.

2.3 Taxonomy

The order Diptera (true flies) is among the more species-rich group on earth. Their estimation made by Groombridge reported 125’000 species (Groombridge 1992), but the real number of existent fly species must be at least twice more important (Yeates and Wiegmann 1999). A huge variety of different ecological and biological behaviours exist among this order, including numerous groups of medical and veterinary interest.

The Calyptratae is one of the major and most diversified dipteran group (McAlpine 1989; Yeates and Wiegmann 1999). According to McAlpine’s classification (1989), the most widely accepted, the Calyptratae are divided into three superfamilies, Hippoboscoidea, Muscoidea and Oestroidea. The three major families of myiasis-producing flies; Oestridae, Calliphoridae (blowflies), Sarcophagidae (fleshflies), belong to the superfamily Oestroidea (Shewell 1987b).

The development of molecular techniques in the last 20 years has provided great insights in the clarification of the systematics and population genetics. A huge amount of sequence data and associated phylogenetic analysis of insects are now widely available (Otranto and Stevens 2006), but it has been demonstrated that molecular investigations of higher Dipterans phylogeny face two major problems: a limited number of species and families represented by DNA sequences, and a lack of reliable phylogenic information covering all taxonomic levels within the Calyptratae (Nirmala et al. 2001). Nowadays, it is accepted that the family Oestridae includes 25 genera and 151 species, ranked in four subfamilies: Cuterebrinae, Gasterophilinae, Hypodermatinae and Oestrinae (Wood 1987; Hall and Wall 1995; Pape 2001) (Table 1).

Table 1. Summary of the classification of the order Diptera (McAlpine 1989)

Class	Order	Suborder	Division	Section	Superfamily	Family	Subfamily	Species
Insect	Diptera	Nematocera	Tipulomorpha		Tipuloidea	Tipulidae		*Tipula sp.*
			Culicomorpha		Culicoidea	Simuliidae		*Simulium damnosum*
		Brachycera	Orthorrhapha		Tabanoidea	Tabanidae		*Chrysops niger*
					Asiloidea	Asilidae		*Laphria sp.*
			Cyclorrhapha	Acalyptratae	Tephritoidea	Tephritidae		*Anastrepha fraterculus*
					Drosophiloidea	Drosophilidae		*Drosophila melanogaster*
				Calyptratae	Hippoboscoidea	Glossinidae		*Glossina palpalis*
						Hippoboscidae		*Melanophagus ovinus*
						Nycteribiidae		*Basilia sp.*
						Streblidae		*Brachytarsina sp.*
					Oestroidea	Calliphoridae		*Lucilia cuprina*
						Sarcophagidae		*Wohlfahrtia magnifica*
						Tachinidae		*Nemoraea pellucida*
						Rhinophoridae		*Rhinomorinia sarcophagina*
						Mystacinobiidae		*Mystacinobia zealandica*
						Oestridae	Oestrinae	*Oestrus ovis*
							Gasterophilinae	*Gasterophilus intestinalis*
							Hypodermatinae	*Hypoderma lineatum*
							Cuterebrinae	*Dermatobia hominis*
					Muscoidea	Muscidae		*Musca sp.*

2.4 Presentation of the major myiasis-causing species

This chapter will focus on the three major families of myiasis-producing flies: the Calliphoridae (blowflies), the Sarcophagidae (fleshflies) and the Oestridae.
Cuterebrinae, *Oestrinae* and *Hypodermatinae* subfamilies will be discussed in the same section while *Gasterophilinae* will be discussed in detail separately.

2.4.1 Calliphoridae and Sarcophagidae

The Calliphoridae have a worldwide distribution. More than 1000 species classified in 150 genera are described. More than 80% of the species and 60% of the genera are restricted to the Old World (Shewell 1987b). The Sarcophagidae family is almost worldwide with over 2000 described species in nearly 400 genera, most of them occurring in tropical to warm temperate areas (Shewell 1987a).
Most Calliphoridae females are oviparous and oviposit on fresh or cooked meat, fish or dairy products as well as on carcasses. Some species may vector causal agents of bacterial infections after contact with excrements or decaying bodies (Shewell 1987b). These flies are also valuable tools in forensic science, since they are usually the first insects to come in contact with dead bodies (Anderson 2000).
Sarcophagidae are without any exception viviparous or ovoviviparous (Shewell 1987a). This family includes a variety of different specialists ranging from inhabitants of pitcher plants to bat coprophages, crab saprophages, wasp nest inquilines, and insect parasitoids (Pape et al. 2006). Even if they do not have the same notoriety as pests as have some Calliphoridae, some species can be associated with the transmission of diseases, for instance by the ingestion of contaminated food (Shewell 1987a). The larviposit on dead bodies gives them an important role on the crime scene.

At least 80 species in these two families have been recorded as causing myiasis and include both facultative and obligate parasites (Zumpt 1965). Only a few calliphorids and sarcophagids species are of veterinary and economic importance (Table 2). The most damage is caused by the three obligate screwworms, provoking wound myiasis, i.e. *C. hominivorax*, *C. bezziana*, *W. magnifica*, and by two species of facultative blowflies, i.e. *L. sericata* and *L.*

cuprina, responsible of the sheep strike (Hall and Wall 1995). The life duration of the larval stages tends to be shorter than that of the adult stages (Colwell et al. 2006a).

Table 2. Summary of Calliphoridae and Sarcophagidae species of veterinary importance, with their degree and main site of parasitism (facultative 1 = primary species)

Family	Species	Degree of parasitism	Site of parasitism
Calliphoridae	*Cochliomyia hominivorax*	Obligate	Dermal, wound
	Chrysomyia bezziana	Obligate	Dermal, wound
	Auchmeromyia spp	Obligate	Sanguinivorous
	Cordylobia anthropophaga	Obligate	Dermal, furuncular
	Lucilia sericata	Facultative 1	Dermal, wound
	Lucilia cuprina	Facultative 1	Dermal, wound
Sarcophagidae	*Wohlfahrtia magnifica*	Obligate	Dermal, wound
	Wohlfahrtia vigil	Obligate	Dermal, furuncular

2.4.1.1 *Cochliomyia hominivorax* and *Chrysomyia bezziana*

The New World screwworm (*C. hominivorax*) and the Old World screwworm (*C. bezziana*) are both obligate parasites of mammals and are unable to develop in carrion (Zumpt 1965). Broadly, a similar life cycle can be associated to most Calliphoridae screwworms. The females deposit their eggs on the edge of fresh wounds where the larvae hatch within 24 hours. They pass through three instars and feed on wound fluids and live tissue (Hall and Wall 1995). After a pupal stage, the new adults emerge to mate and locate a new host. Adult females mate only once during their lifetime, this behaviour is an important factor in biological control. The wound can enlarge because of multiple infestations and can lead to serious tissue damage (Humphrey et al. 1980). Without an effective treatment, the animal will die.

2.4.1.2 *Lucilia cuprina*

The Australian sheep blowfly (*L. cuprina*) is responsible for initiating most blowfly strikes. Under normal conditions, these flies lay their eggs in faeces or in carcasses. In facultative myiasis, the adults are attracted by stimuli, usually an odour, coming from a wound, skin lesion, bacterial contamination or hair soaked with urine or faeces (Hall and Wall 1995). The eggs are laid on the fleece; they will hatch within 24 hours depending of the moisture conditions, producing larvae (maggots) that stay at the same site and ingest nutrients from wounds, exudates or soiled surfaces. This condition is known as fly strike (or strike). The second larval stage uses its mouth hooks to damage the skin and obtain nutrients. Maggots can tunnel through the thinned epidermis. Strikes can spread rapidly and attract more blow flies. Mild strike can cause a fast lost of condition and in case of a bad strike the animals can die from shock, intoxication or secondary infection (Merck 2005).

2.4.1.3 *Wohlfahrtia magnifica* and *Wohlfahrtia vigil*

W. magnifica is an obligate parasite of warm-blooded vertebrates that can cause rapid and severe cutaneous myiasis in man and most livestock (Zumpt 1965). Larvae are deposited directly at sites of wounding or besides body orifices on the host, where they feed and mature in 5-7 days before leaving the site for pupation (Zumpt 1965). *W. vigil* deposits its larvae usually on unbroken skin, mostly in young animals, causing a furuncular myiasis (Hall and Wall 1995). In some cases the penetration can go deeper, until the coelomic cavity. The larval development in host tissues can be accompanied by secondary bacterial infections and thereby produce intense irritation and inflammation (Merck 2005).

2.4.1.4 Atypical myiasis-causing species

The genera *Auchmeromyia* (on mammals) (Colwell et al. 2006a) and *Protocalliphora* (on nesting birds) (Shewell 1987b) are considered as atypical myiasis-causing species. They have blood-sucking larvae that are obligate, haematophagous ectoparasites (sanguinivorous myiasis).

2.4.2 Oestridae

2.4.2.1 Biology

All oestrids are obligate parasites during their larval stage. They tend to be highly host-specific with a strong preference for herbivores (Schaëfer 1979). The only reported species able to parasitize carnivores and herbivores is *D. hominis* (Colwell et al. 2006a). The host association can be linked to the taxonomic classification of the fly (Colwell et al. 2006a) (Table 3).

Their nutritional requirements for basic metabolism, survival, dispersal and reproduction have to be ingested during the obligatory parasitic larval stage (Hall and Wall 1995). The adult oestrids function essentially as a mean for reproduction and transfer of the population to a new host, therefore the adult stage is proportionally very short when compared to the duration of the larval stage (Hall 1995). The reproductive organs of the males are fully developed at eclosion and they are able to inseminate the females shortly after emerging (Anderson 2006a). When females eclose, they already bear the mature eggs or previously accumulated enough nutrients to finish oogenesis (Anderson 2006a).

The host location by adult females is a critical phase in the life cycle. Unlike most myiasis-causing calliphorids and sarcophagids generally attracted by stimuli associated to host trauma (i.e. wounds, necrosis), oestrids are attracted to normal healthy hosts probably by both olfactory and visual stimuli (Hall 1995). Usually, the Hypodermatinae, Gasterophilinae and Oestrinae adopt the “on-the host” oviposition or larviposition strategy, except *Gasterophilus pecorum* that lays its eggs on grass. The Cuterebrinae generally adopt an “off-the host” strategy, laying their eggs at sites frequented by the hosts.

2.4.2.2 Taxonomy of the Oestridae

Table 3. The four oestrid subfamilies, their host group and the anatomical site of infestation (Colwell et al. 2006a), (*From Zumpt, 1965)

Family	Subfamily	Genus	Host groups	Site of myiasis*
Oestridae	Oestrinae	*Cephenemyia*	Cervidae	Nasopharyngeal
		Cephalopina	Cameline	
		Gedoelstia	Antelopes	
		Kirkioestrus	Antelopes	
		Oestrus	Ovine, caprine	
		Pharyngobolus	Elephant	
		Pharyngomyia	Cervidae, zebras, pigs, giraffe, hippopotamus, springbuck, sheep	
		Rhinoestrus	Equine	
		Tracheomyia	Kangaroos	
	Gasterophilinae	*Cobboldia*	Elephant	Intestinal
		Gasterophilus	Equine	
		Gyrostigma	Rhinoceros	
	Hypodermatinae	*Hypoderma*	Bovine	Dermal/Subdermal
		Oestroderma	Pikas	
		Oestromyia	Mice, marmots, pikas	
		Pallasiomyia	Saiga antelope	
		Pavlovskiata	Goitered antelope	
		Portschinskia	Mice, pikas	
		Przhevalskiana	Caprine, gazelles	
		Strobiloestrus	Bovidae, Kobus species	
	Cuterebrinae	*Cuterebra*	Rodents, lagomorphs, howler monkey	Dermal/Subdermal
		Dermatobia	Non-specific, larger mammals, birds	

	Cuterebrinae (probably)§	*Neocuterebra*	African elephant
		Ruttenia	African elephant

§The classification of the two monospecific genera *Neocuterebra* and *Ruttenia* is still under discussion. As it is not a major concern in this work and in order to make it as clear as possible, they will not be placed into the Gasterophilinae subfamily as suggested by Zumpt(1965). Wood (1987) noted some morphological similarities between the species of *Ruttenia*, *Neocuterebra* and *Cuterebra* and suggested a possible phylogenic relationship. Although Pape (2001) grouped these two genera with the stomach parasites, their affiliation to any group remains to be clarified; therefore they will remain in this classification as belonging to the Cuterebrinae subfamily.

2.4.2.3 Morphology of adult Oestridae

Each Oestridae subfamily is quite heterogeneous, and shared derived character states for each of the subfamilies are not obvious. Some major characteristics of the Oestridae family will be described (Zumpt 1965; Wood 1987; Wood 2006).

The adult oestrids vary considerably in size, from 8 to 35 mm in length. The head of most oestrids is as wide as the thorax. The enormously widened ventral part of the head is a result either of an enlarged genal region and correspondingly narrowed face (most species) (Fig.1A,B) or conversely, in most Hypodermatinae, of a greatly widened face and correspondingly reduced genal region (Fig.2). The frontal vitta (Fig.1A), conspicuous in all calyptrate because of the presence of a strip of longitudinally wrinkled and dark cuticle, is readily recognizable in all oestrids. The lunule in calyptrate is usually shiny and bare, and this is true of most oestrids as well (Fig.1A). However some Hypodermatinae have small hairs arising on the lunule (Fig.2). The ocellar triangle is present in all oestrids (Fig.1A), and three ocelli are present in most species. One of the most noticeable aspect of most oestrids is the relatively minute antennae (Fig.1A,B, 2). Indeed each antenna is sunken into a deep pit (Fig.1A, 2), so that only the arista is visible in profile (Fig.1B). Even in frontal view little but the first flagellomere and arista are visible (Fig.1B). The parafacial of the majority of oestrids is haired, sometimes densely and quite narrow as for *Hypoderma* (Fig.2), or it may be broad and sparsely haired as for *Gasterophilus* and *Cuterebra* (Fig.1A).

The mouthparts of the Oestridae are usually present even though in reduced or rudimentary form, and are always undersized relative to the rest of the head. If most of the species do not feed, some may imbibe fluids (*Cephenemyia*, *Cuterebra*).

The Oestrids have a pruinose thorax that is also extensively pilose compared to the other muscoids flies. The quality of the vestiture of pile associated to the coloration is probably governed, in many oestrids, by selection for mimicry which might be defensive or protective. The postpronotum, scutum and scutellum are almost entirely covered with pile which might be recumbent (Fig.1B) or erected according to the species: e.g. the scutellum of *Gasterophilus* and *Hypoderma* are covered with erect pile, similar to that of the scutum, which complements their bee-like appearance (Fig. 2,7). If most of the *Hypoderma* and *Cuterebra* species resemble bumblebees or carpenter bees; many species of the Oestrinae genera (*Oestrus, Rhinoestrus)*, with their black, brown gold and white coloration resemble bird droppings, which can be more associated to a cryptic coloration strategy.

Some oestrid species that seem to mimic bumblebees or other bees have rather stout hairy abdomens, e.g. *G. intestinalis* (Fig.7), while the species that seem to have a cryptic mimicry strategy, e.g. *Oestrus*, *Rhinoestrus*, have characteristic mottled patterns of white, gold and brown or black pruinosity. As is typical of all calyptrate, the first abdominal tergite is usually greatly reduced and fused to tergite 2 to form syntergite 1+2. The tergites 3-5 are usually about the same length, each narrowing progressively to the apex (Fig.1B). Each of the 6 first tergites bears a spiracle on each side.

The legs of most oestrids are of moderate size and covered with appressed hairs. In *Cuterebra*, the legs are rather short and stout, and the tarsomeres are broad and dorsoventrally flattened (Fig.1B).

The wings of most oestrids are hyaline, with a vague pattern of darker colour in some species of *Gasterophilus* (Fig.7). The wings of *Cobboldia elephantis*, *Cobboldia loxodontis*, of all *Gyrostigma* and of many, perhaps all, species of *Cuterebra* are dark brown (Fig.1B). The calypters, both upper and lower, are present in all oestrids but might be reduced as in *G. intestinalis*.

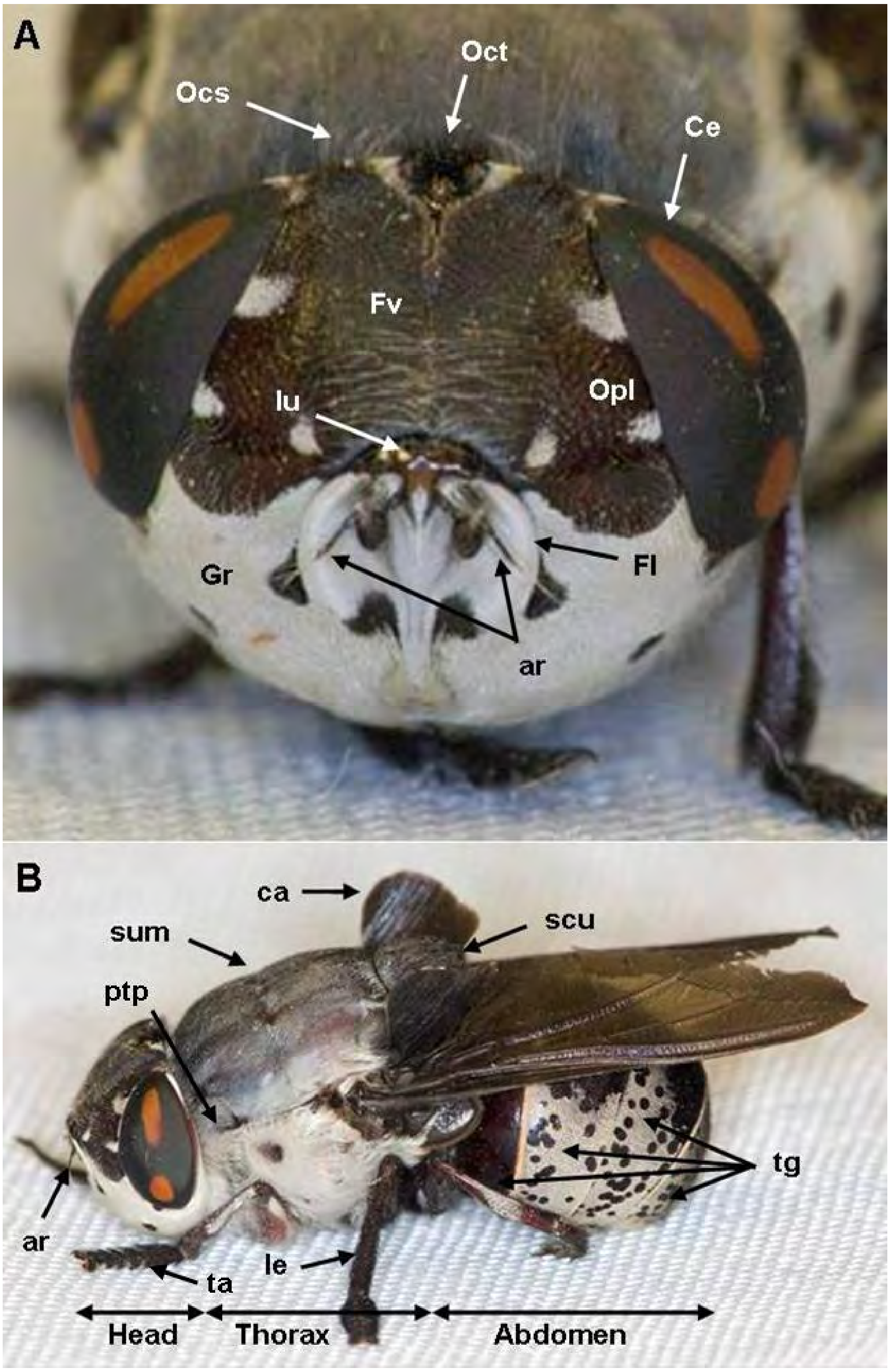


Fig.1. Pictures of *C. buccata* by Tam Stuart. (A) Head in frontal view. Arista (ar), compound eye (Ce), ocellar seta (Ocs), ocellar triangle (Oct), first flagellomere (Fl), frontal vitta (Fv), genal region (Gr), lunule (lu), orbital plate (Opl). (B) Body in lateral view showing the 3 major regions: head, thorax and abdomen. Calypters (ca), legs (le), postpronotum (ptp), scutellum (scu), scutum (sum), tarsomeres (ta) tergites (tg). © Tam Stuart, all rights reserved.

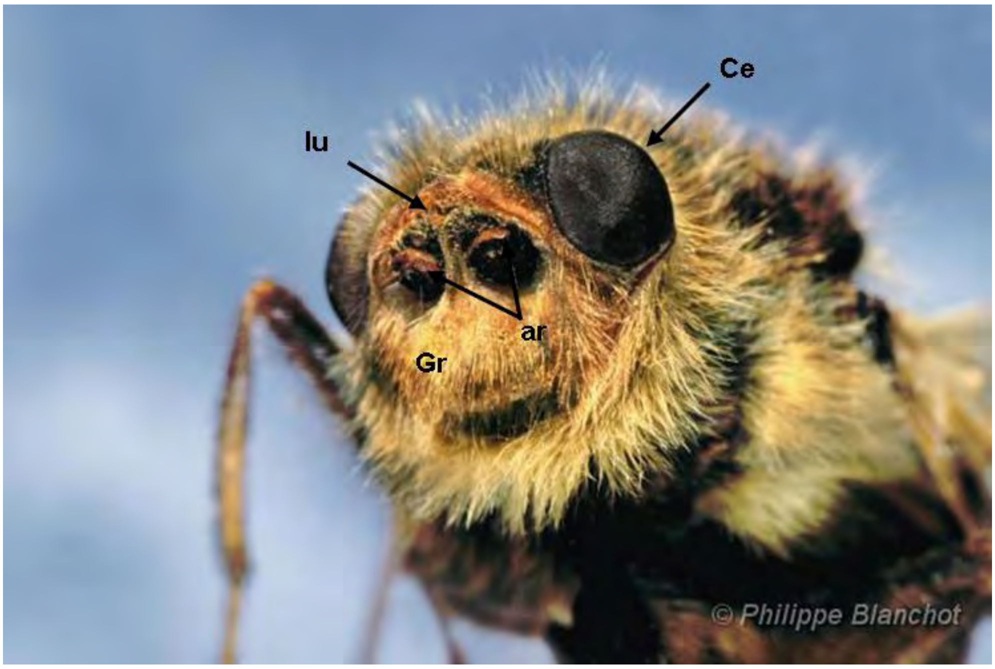


Fig.2. Picture of *H. bovis* by Philippe Blanchot. Arista (ar), compound eye (Ce), genal region (Gr), lunule (lu). ©Philippe Blanchot, all rights reserved.

2.4.2.4 Host-parasite interaction

Although botflies have been considered as less pathogenic than other myiasis-causing flies, (e.g. *L. cuprina*, *C. hominivorax*, *W. magnifica*) they can cause significant morbidity, have an impact on animal growth and general health (Otranto 2001). Quite an amount of research work on the host immune response focused on myiasis-causing species that are of economic importance (e.g. *O. ovis*, *H. bovis*, *H. lineatum*, *D. hominis*), whereas there remains a gap with regard to species of less significant impact (e.g. *Gasterophilus* spp).

The host-parasite interactions vary in degree and intensity according to the species of oestrid involved. During the migration and maturation phases inside a host's body, the larvae can cause different trauma to the host's tissue; e.g. induced by hook and spines or by proteolytic activity of excretory and secretory products (Dorchies et al. 2006). According to the damage produced by a larval infestation, the host immune system can be greatly stimulated and nonspecific (inflammatory reaction) as well as specific immune responses can be described (Otranto 2001). In order to understand the immunological response of the host, it is of high priority to determine the origin and the nature of the antigenic substances produced by the larvae during the different phases of their life cycle inside the host (Baron and Colwell 1991).

The immunological interaction of *O. ovis*, *Hypoderma* spp. and *Gasterophilus* spp. with their respective hosts will be further discussed in the corresponding section.

2.4.3 Cuterebrinae

The Cuterebrid bot flies are insect parasites of wild mammals. The 83 known species are separated in six genera restricted to the New World (Catts 1982). 80% of the species belong to the *Cuterebra*, but the most important Cuterebrid in economic terms, belongs to the *Dermatobia* genus, and is called *D. hominis* (Hall and Wall 1995). This section will concentrate on the two genera *Cuterebra* and *Dermatobia*.

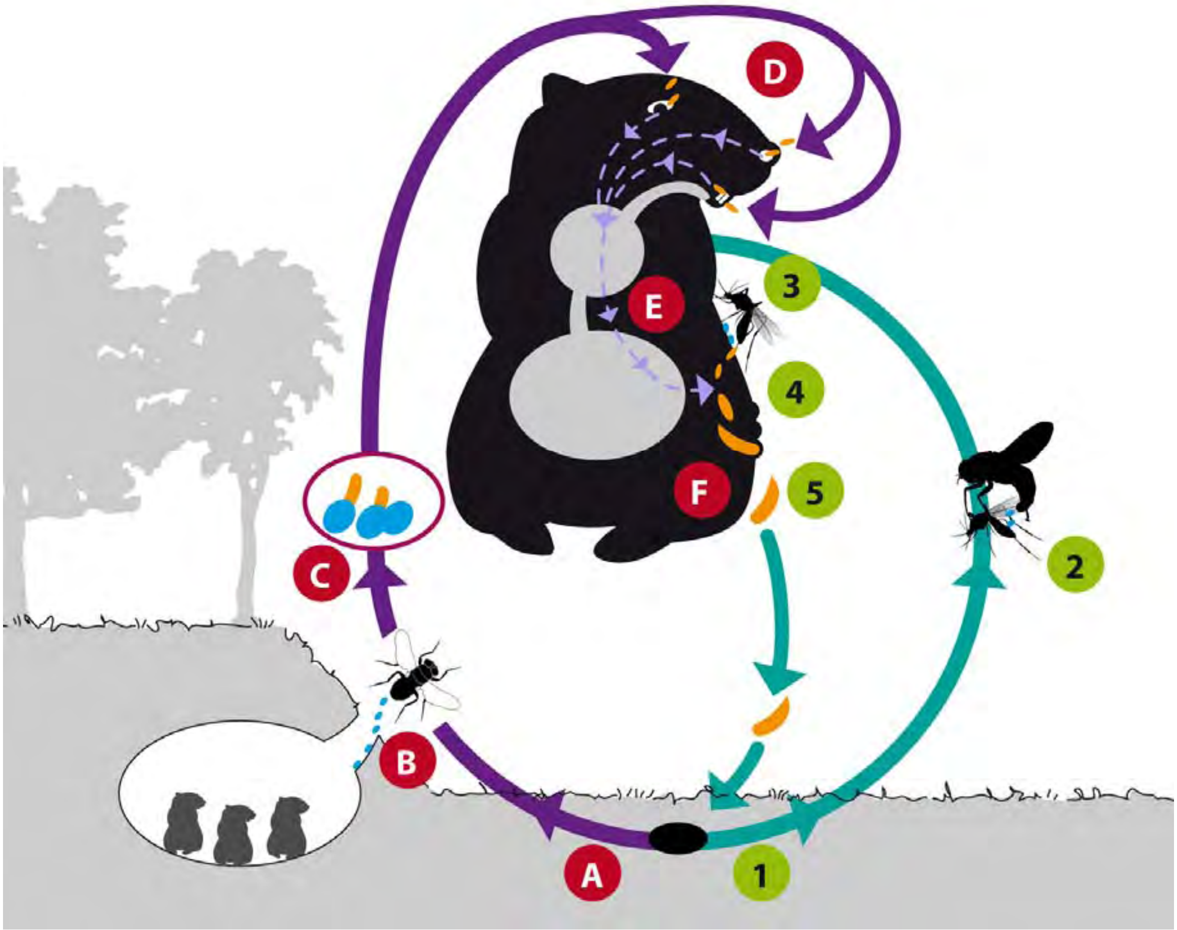


Fig.3. Life cycle of *Dermatobia* (1-5) and *Cuterebra* (A-F): (1) the adults emerge, they mate and they capture blood-sucking arthropods to lay eggs on their body using a glue-like substance (2). Bot fly larvae develop within the egg and when the vector takes a blood meal on mammalian or avian host, they penetrate host's tissue (3). The larvae feed for 5-10 weeks in a subdermal cavity (4). Mature larvae drop to the ground and pupate in the environment (5). Life cycle of *Cuterebra*. (A) the adults emerge, they mate and lay their eggs in the vicinity of the host (B) the eggs hatch by external stimulation associated to the host presence

(C) and the newly hatched larvae enter host body via body openings (D).After a short migration, the larvae complete their development at subdermal site where they form a warble (E). Mature larvae drop to the ground and pupate in the environment. (F). ©S. Zanou, all rights reserved.

2.4.3.1 Genus *Cuterebra* (Clark, 1815)

The different *Cuterebra* species have much stricter host specificities than *D. hominis* and cause myiasis in rodents, lagomorphs, marsupials and occasionally humans in the New World (Baird et al. 1989).

2.4.3.1.1 Life cycle of *Cuterebra*

After eclosion female Cuterebrid need several days to complete the egg development and to become receptive to mating (Fig.3 (A)) (Colwell 2006a). Immediately after copulation, females begin oviposition and eggs are deposited at close proximity of the host, as at the entrances of burrows (Fig.3 (B)). After 4-10 days, the eggs are fully embryonated and they can hatch in response to increased environmental temperature and elevation of CO_2 associated with the presence of a potential host (Fig.3 (C)) (Catts 1982). The newly hatched larvae search for the host and enter the body through appropriate site. Most species invade the host through moist body openings (e.g. mouth, nasal openings, eyes) (Fig.3 (D)) or skin lesions. First-instars *Cuterebra* spp. undertake a short internal migration (4-6 days) through the abdominal cavity (Fig.3 (E)) and reappear at the final subdermal site (Lello 2006) where they open a breathing hole (warble pore) in the host skin (Catts 1982). Larvae at the subdermal site will be surrounded very quickly by a host granuloma, forming a characteristic warble where larvae will complete their development (Fig.3 (F)) (Cogley 1991). The location of those granulomatous cysts seem to be species-specific (Colwell et al. 2006b). Larvae require a period of 20-40 days to complete their development and exit the host body through the breathing hole. Pupation occurs under surface litter or deep in the soil (Fig.3 (A)) and its duration is variable, depending on the *Cuterebra* species and environmental factors. A pupal winter diapause is often recorded in northern latitudes (Catts 1982; Colwell et al. 2006b).

2.4.3.1.2 Pathogenesis

At average intensities *Cuterebra* spp. infestations produce no significant pathologies or important disease symptoms other than the characteristic cyst (Colwell et al. 2006b). Field studies provide little evidence of strong deleterious impact on host populations, but the negative affect of those infestations on the population dynamics is still under discussion (Slansky 2007). The economic impact of *Cuterebra* myiasis is negligible because of the hosts generally involved (Catts 1982). Accordingly, no treatment is required for the management of wild host populations (Colwell et al. 2006b).

2.4.3.2 Genus *Dermatobia* (Linnaeus, 1781)

Dermatobia is a monospecific genus of the neotropics. *D. hominis* (Linnaeus), also called tropical warble fly, infest a wide variety of mammals, birds and surpasses all other cuterebrids in economic and public health importance because of its impact on livestock production and its zoonotic potential (Catts 1982). Human infestations with larvae of *D. hominis* are so common in Central and South America that most cases are not reported (Anderson 2006b).

2.4.3.2.1 Life cycle of *Dermatobia*

D. hominis have an atypical method to infest their host. Mated females will deposit their eggs on zoophilic or anthropophilic insects that act as “porters” (Fig.3 (2)) (phoretic insects). Most of the porters are mosquitoes or muscoids. The eggs incubate on a porter and need one week to fully embryonate (Catts 1982). They hatch in response to a sudden increase of temperature associated with the proximity of a potential host (Fig.3 (3)). The selection of the final host is down to the porter and explains the catholic range of hosts parasitized by *D. hominis* (Colwell et al. 2006a). The larval penetration occurs close to the point of initial contact (Fig.3 (4)). They can penetrate intact skin and will complete their development by forming a boil-like ulcer in the subcutaneous tissue (Catts 1982; Colwell et al. 2006b). Once in the host they do not migrate and their time of development will depend on the selected host; 40-60 days in cattle and less in small animals. Mature larvae exit through the warble pore (Fig.3 (5)) (or breathing hole) and burrow into upper soil or debris where pupation takes place (Fig.3 (1)). Adults emerge 35-60 days later and after a few hours, mating can occur (Colwell et al. 2006b).

2.4.3.2.2 Pathogenesis

As *Dermatobia* warbles develop at any place on the host‘s body, they are responsible of many annoyances. Some hosts covered with warbles have been described as emaciated and weakened; clustering of warbles can cause deformities that are subject to cause secondary bacterial infections or other parasitic infestations (Catts 1982). The economic impact on domestic livestock in most of Central and South American countries is huge (milk, meat and hide production losses) and is estimated each year to hundred millions of US$ (Catts 1982).

2.4.4 Oestrinae

The Oestrinae subfamily includes nine genera (Zumpt 1965). The different species are usually host specific, including wild or domestic mammals, and their distribution depends of the location of their host. A specificity of this subfamily is that the females of all species larviposit directly on or into the nostrils of their host and larvae develop and migrate in their nasal passages and head sinuses. Two genera will be further developed: *Rhinoestrus* spp. and *Oestrus* spp.

2.4.4.1 Genus *Rhinoestrus* (Brauer, 1886)

The main description of the genus was made by Zumpt (1965). Four *Rhinoestrus* species among the eleven described were reported as affecting equids (Zumpt 1965). The myiases caused by larvae of *Rhinoestrus purpureus* (Brauer) and *Rhinoestrus usbekistanicus* Gan are of veterinary importance since they are responsible of severe respiratory diseases in equids (horses, donkeys and zebras) (Traversa and Otranto 2006). Although *Rhinoestrus* was thought to be confined to African and Asian Countries, recent studies reported their presence in Europe, more specifically in southern Italy (Otranto et al. 2004).

2.4.4.1.1 Life cycle

After mating, *Rhinoestrus* females produce 700-800 larvae which are deposited in batches of 8-40 on the host’s nostrils. According to the climatic conditions, first instars can remain in this stage for a few weeks or a few months. For instance, in warmer parts of Central Asia two annual generations have been observed (adult flies observed from March to mid-June and again in September-October), conversely in higher latitudes first instars seem to undergo hypobiosis and complete moult into second instars at the arrival of warmer spring conditions. As the first instars mature they migrate further into the nasal cavities. The second and third instars are usually found at this same site even if some move into upper pharyngeal areas. The mature third instar larvae exit their host through the nose and pupation occurs under surface litter (Zumpt 1965; Otranto et al. 2004; Colwell et al. 2006b).

2.4.4.1.2 Pathogenesis

Larvae of *Rhinoestrus purpureus* and *Rhinoestrus usbekistanicus* induce irritation and inflammation of the nasal cavities, sinuses and pharynx of equids. Lesions to the upper respiratory tract and lungs have been described (Kaboret et al. 1997). In addition, the olfactory nerves can be injured and hosts can develop nervous symptoms characteristic of encephalomyelitis due to the penetration of the ethmoid bone and soft cerebral membrane (Zumpt 1965; Otranto et al. 2004; Colwell et al. 2006b).

Human infection is usually benign, limited to the first instar and result in a mild ophtalmomyiasis (Colwell et al. 2006b) and conjunctivitis (Peyresblanques 1964).

Scanty documentation reports the presence of equine nasal myiasis, because the consumption of horse meat is not popular in many European areas and horse’s heads are only partially submitted to the veterinary inspection after slaughtering. The detection of larvae is very difficult in live animals by an endoscopic approach and no serological methods allow yet the detection of rhinoestrosis in equids (Traversa and Otranto 2006).

2.4.4.2 Genus *Oestrus* (Linnaeus, 1758)

The cosmopolitan sheep nasal bot fly, *Oestrus ovis* (Linnaeus), is the most important species in this genus in veterinary and economic terms (losses in meat, wool and milk production). The other *Oestrus* species are distributed mainly in Africa and central Asia where they parasitize wild animals (Zumpt 1965). The *O. ovis* larvae provoke the naso-sinusal myiasis of sheep and goats (ovine oestrosis) in all sheep-farming areas of the world (Hall and Wall 1995). The adult flies also affect sheep and goat’s behaviour by their intense larviposition activity, this condition is known as fly strike. The number of generation per year and the regional variations in strike seem to be closely related to local climate (Dorchies et al. 2006).

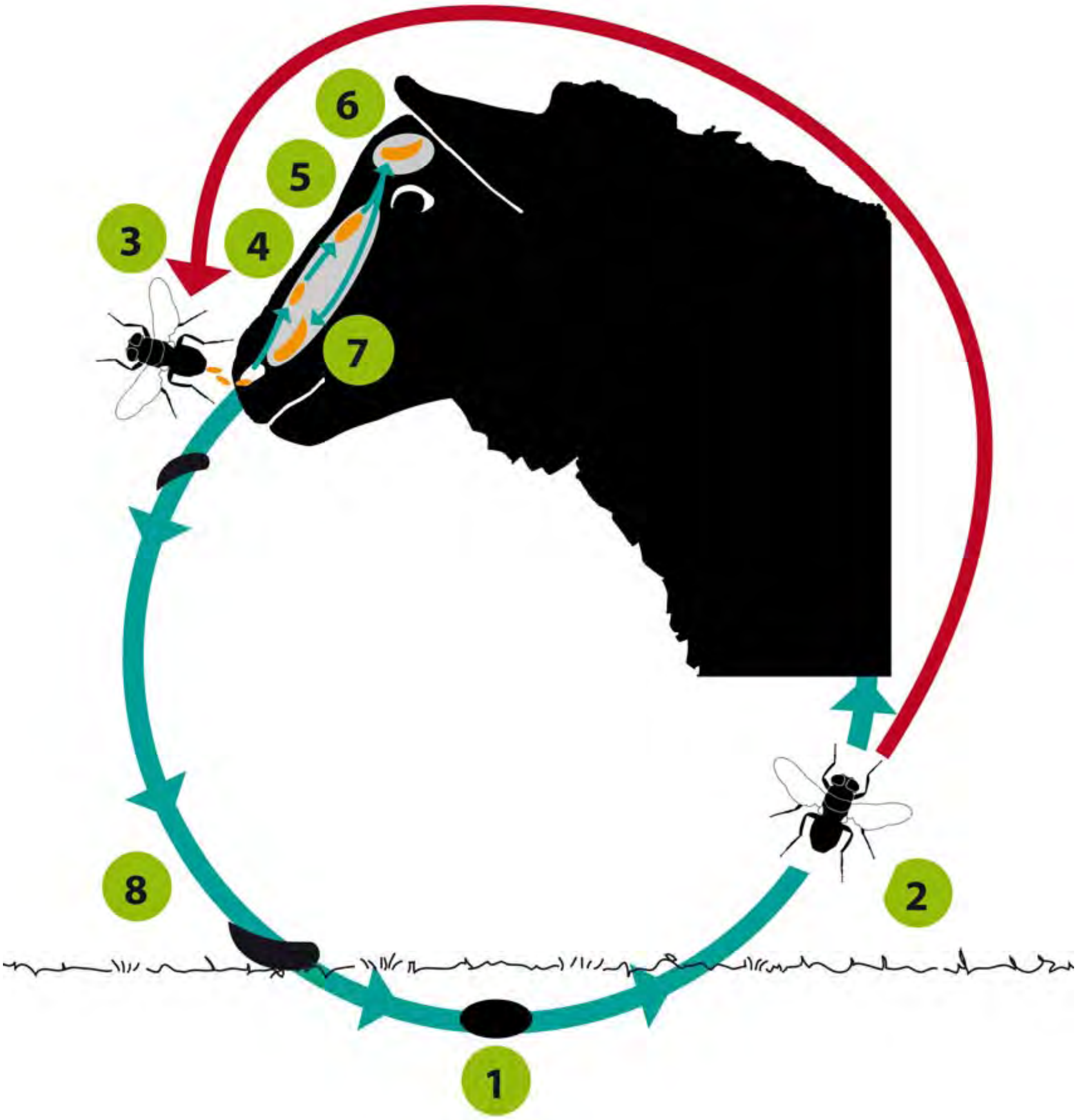


Fig.4. Life cycle of *Oestrus ovis* (1-8): after pupation (1) new adult emerge (2) and after mating first instar larvae are deposited into the host nostrils (3). After migration and maturation phases in the host naso-sinusal cavities (4-7), mature larvae expulsed and pupate underground (8). ©S. Zanou, all rights reserved.

2.4.4.2.1 Life cycle of *O. ovis*

After adult emergence (Fig.4 (2)), the mating takes place and the mated females deposit at each attempt a group of 2-20 first instar larvae into the host's nostrils, up to a total of 500 larvae/female (Fig.4 (3)). These L1 start a quick migration into the nasal cavities (Fig.4 (4)) (nasal septum, turbinates and ethmoid bone) of their host (Angulo-Valadez et al. 2007). At this site the period of development of the larvae can vary widely from several days to several months, depending probably on climatic condition and host immunity (Colwell 2006a). These larvae can undergo hypobiosis, passing the cold season within the host's nostrils, but with optimal conditions the complete larval development occurs in 25-35 days (Colwell et al. 2006b). Once the L1 are in the nasal passages, they moult into the second instar larvae and usually migrate to another site (frontal sinus, horn cavities) where they moult into the third instar larvae (Fig.4 (5,6)). The exit migration of mature larvae via the host's nostrils is often accompanied by intense sneezing behaviour (Fig.4 (7,8)). The pupation takes place underground (Fig.4 (1)) and after 25-30 days in warm weather, or longer in cooler periods a new adult emerge. According to the temperature there will be 2-3 generations per year (Colwell et al. 2006b).

2.4.4.2.2 Pathogenesis

After emergence, the gravid females swarm around the heads of sheep or goats to larviposit. This intense activity disturbs the sheep (goats less sensitive) which get nervous, shake their heads or typically keep their noses inside the fleece of other sheep (Dorchies et al. 2006). The time of grazing is considerably reduced resulting in a loss of the animal's condition. The most damages are due to the larval stages in the nasal cavities. Light infestations can be well tolerated but heavy ones can have serious consequences, including purulent discharges from the nostrils, sometimes haemorrhagic, accompanied by sneezing and head shaking, and breathing difficulties (Hall and Wall 1995; Colwell et al. 2006b). Secondary infections can lead to lung abscesses and interstitial pneumonia (Dorchies et al. 1993). In rare cases, the larvae can penetrate the brain causing a condition known as "false gid" (ataxia, circling and head pressing) (Hall and Wall 1995).

More recent work demonstrated that the pathogenicity associated to *O. ovis* larvae was mainly a consequence of proteolytic activity of excretory/secretory products plus the effect of type I hypersensitivity (Dorchies et al. 2006).

Accidental myiases in humans associated with *O. ovis* are reported all around the world and are very common. Most cases involve people in close contact with sheep or goats. Generally the larvae are deposited in the eyes, rarely into the mouth or nostrils and result in most cases in an acute conjunctivitis (Zumpt 1965).

2.4.4.3 Immunological aspects

Larval antigens

A crucial step for developing sensitive and specific tools to diagnose oestrosis, as well as other myiases, is to identify specific immunogenic antigens and to analyse the induced host immune response against them (Tabouret et al. 2001). Crude larval extracts of the different larval stages (L1, L2 and L3) were initially used in several studies to diagnose *O. ovis* infestations (Bautista-Garfias et al. 1988; Marchenko et al. 1991), but the results were not satisfactory with regard to sensitivity and specificity. The determination of the larval tissue extracts that elicit the strongest host immune response is essential and it was demonstrated on different myiasis models that the content of secretory organs might provide target antigens (Pruett et al. 1988; Innocenti et al. 1995; Casu et al. 1996). Larvae secrete enzymes for extracorporeal pre-digestion and the external protein digestion by proteases is essential for the acquisition of nutrients by parasitic fly larvae (e.g. *Hypoderma lineatum*, *Lucilia cuprina*) (Angulo-Valadez et al. 2007). The products excreted and secreted (ESP) by *O. ovis* larvae (mainly enzymes) are the result of both salivary gland and digestive tube activity (Tabouret et al. 2001). These enzymes degrade host mucosa into smaller units easily ingestible to support larval growth and development (Tabouret et al. 2003a). Proteases (mainly trypsin-like serine proteases) were identified in the ESP and seem to originate from the larval gut (Tabouret et al. 2003a). Recently the proteolytic activity of salivary gland products has been demonstrated for the first time (Angulo-Valadez et al. 2007). It was established that the salivary gland proteins are major immunogens in infested sheep (Innocenti et al. 1995) and important antigens (28 kDa protein complex) were identified in salivary gland secretions (Tabouret et al. 2001).

Host-immune reactions

The understanding of the host immune response has been improved by the identification of reliable antigens. Organ contents (salivary glands, digestive tube contents) or ESP are now currently used for diagnosis and immunization trials in sheep (Tabouret et al. 2001; Alcaide et al. 2005; Sanchez-Andrade et al. 2005; Suarez et al. 2005; Angulo-Valadez et al. 2007).

According to Tabouret et al. (2003), the presence of larvae in the frontal sinus and ethmoidal epithelium induces mainly a cellular immune response, with accumulation of leucocytes (B and T lymphocytes, macrophages) and granulocytes (eosinophils, mast cells). Specific IgG and IgA antibodies were found in mucus of infected lambs and this humoral response was mainly directed against salivary gland antigens and not to digestive tract targets (Tabouret et al. 2003b). The evaluation of the kinetics of the systemic IgG, IgM and IgA responses to *O. ovis* provides an important information about the chronobiology of the parasite, the period of foremost risk and the best time to administer an early treatment (Suarez et al. 2005).

2.4.5 Hypodermatinae

The Hypodermatinae includes nine genera (Zumpt 1965). Whereas the genus Hypoderma is well documented, only little information is available on the biology or lifecycle of the other genera. The Hypodermatinae described species are mainly restricted to the Holarctic region where they parasitize wild and domestic mammals and usually show strong host-specificity.

2.4.5.1 Genus *Hypoderma* (Latreille, 1818)

The genus *Hypoderma* includes six species which are commonly called heel flies, warble flies or cattle grubs. Each species is a host-specific parasite of a ruminant (e.g. *H. diana*-roe deer; *H. tarandi*-reindeer; *H. actaeon*-red deer, *H. bovis* and *H. lineatum*-cattle) and their distribution is restricted to the Holarctic region (Zumpt 1965). The disease induced by the development of the larval stages inside the host is called hypodermosis.

Cattle hypodermosis is the consequence of infestation by *H. bovis* (Linnaeus) or *H. lineatum* (de Villers) larvae. Both flies are responsible for major economic losses through affection of meat, milk and leather production (Scholl 1993) but they also affect the general health status of cattle. *H. bovis* and *H. lineatum* are widely distributed in the Northern hemisphere, i.e. at least 50 countries of North America, Europe, Africa and Asia (Scholl 1993). Cattle grubs are rare in the Southern hemisphere and generally introduced by imported cattle, but no indigenous populations are known (Zumpt 1965; Tarry 1986; Scholl 1993). Due to the economic impact of hypodermosis, many European countries organised control programs (COST 811) leading to a significantly decreased infestation level (e.g. in Switzerland (Charbon et al. 1995)) or even to eradication (e.g. in the U.K. (Tarry et al. 1992))(Boulard 2002).

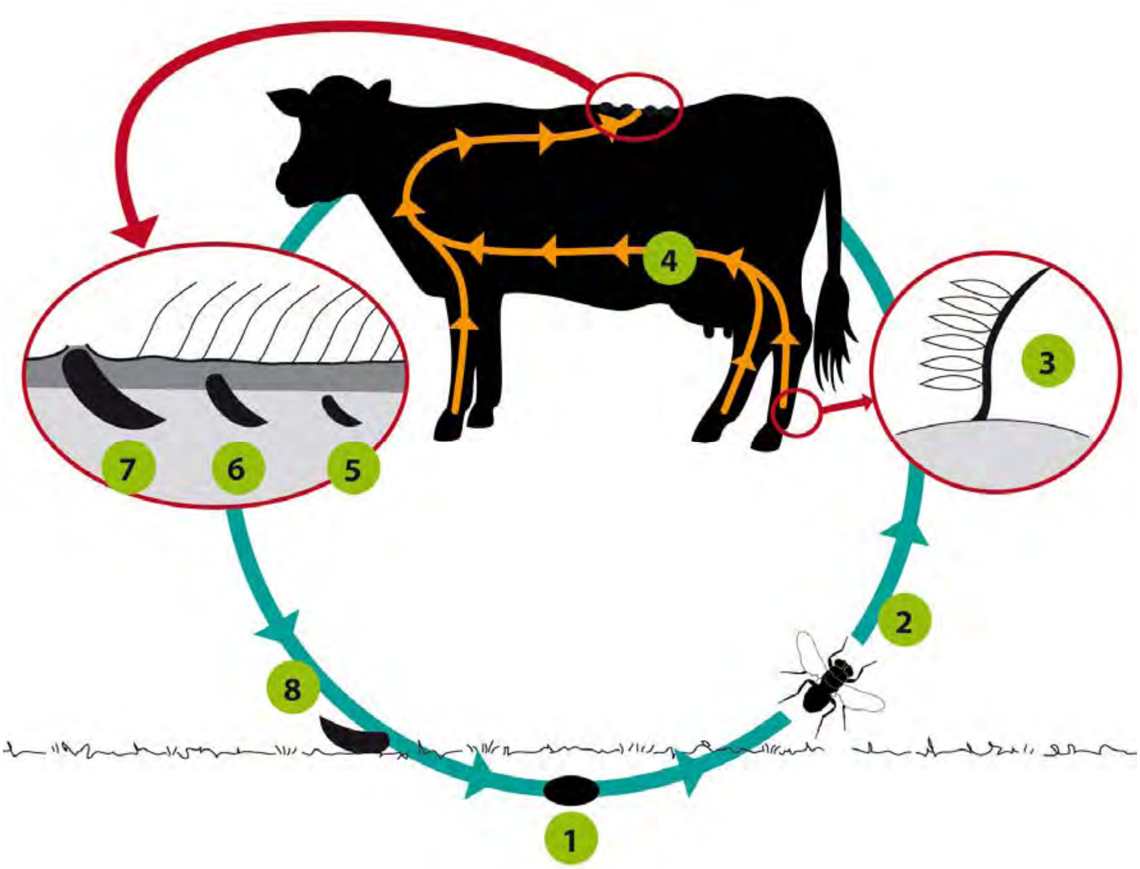


Fig.5. Life cycle of *Hypoderma* (1-8): after pupation (1) new adult emerge (2) and eggs are attached to host's hairs (3). The newly hatched larvae (L1) undergo a long migration within host body (4). The migration is achieved when larvae reach the middorsal region of their host. Larvae complete their development (L2-L3) in a subcutaneous swelling, or warble (5-7). Mature larvae leave the warble and pupate underground (8). ©S. Zanou, all rights reserved.

2.4.5.1.1 Life cycle of *Hypoderma* spp

Female *Hypoderma* deposit their eggs directly on the host (Fig.5 (2)) by using a specialized ovipositor. The oviposition behaviour differs between the two species (Scholl 1993; Medjitna 2003):

- *H. lineatum* attaches its eggs (connecting strings of 10-15 eggs) (Fig.5 (3)) to individual hairs of the host's pelage or skin surface, most often on the legs and lower body regions of the cattle.
- *H. bovis* deposits each egg on an individual hair shaft with a preference for the caudal areas of the animals (heel flies).

If *H. lineatum* generally oviposit on resting animals, *H. bovis* has a persistent activity which can lead to a dramatic escape response by cattle, called gadding (Hall and Wall 1995).

The larvae hatch after 3-7 days of incubation, migrate along the hair and penetrate the host skin. This first instars (L1) undergo a long migration of 8 to 10 months within the host tissue, in order to finally reach the subcutaneous tissue of cattle's dorsal area (Fig.5 (4)) (Scholl 1993). The routes of migration and sites of overwintering of the L1 depend of the species:

- *H. lineatum* larvae first migrate subcutaneously along the connective tissue, then between the facial planes of muscles of the thoracic cavity. After about 3-5 weeks they penetrate the submucosa of the oesophagus where they undergo for 5-6 months an overwintering period (Medjitna 2003; Colwell et al. 2006b).
- *H. bovis* larvae move to their overwintering site by migrating along the nerves to the spinal cord. They will remain 3-5 months in the epidural fat around the thoracic and lumbar vertebrae (Medjitna 2003; Colwell et al. 2006b).

During migration and the resting period, the L1 degrade host tissue and feed on it by ingestion and transcuticular absorption (Chamberlain et al. 1969). An important process is the reabsorption of the proteolytic enzymes, used to lyse the host's tissue, and their accumulation in larval midgut. A cellular "cork" blocks the excretion (Boulard et al. 1988).

The following spring, the L1 leave their resting sites and achieve their migration by moving to the middorsal region of their hosts where the first moult will occur. This moult induces structural modifications of the midgut which is now open at both extremities and releases the enzymatic mass accumulated during the L1 stage (Boulard et al. 1988). A subcutaneous swelling develops (warble) and the larva cuts a hole in the skin surface (Fig.5 (5-7)). The posterior spiracles are orientated at the opening, enabling the larvae to breathe while feeding on cellular debris and exudates inside the warble (Colwell et al. 2006b). A granuloma cyst begins to form around each larva, isolating them from host organism and the L2 moult into L3. These L3 dramatically increase in size, accumulating the energy resources to survive the non feeding free-living stages, and after a period of 30-60 days they leave the warble through the breathing hole (Fig.5 (8)). The mature L3 burrows actively into the grass or near the surface on loose soils and undergo pupal metamorphosis (Fig.5 (1)) (Scholl 1993). The pupation takes between 2-5 weeks depending on the climatic conditions (particularly temperature). After emergence, adult flies live 3-5 days (Boulard et al. 1988).

2.4.5.1.2 Pathogenesis

The frenzied attempt to escape the ovipositing adult female (gadding) is thought to be a potential cause of injury. Animals can hurt themselves by falling and/or running into fences. Spontaneous abortion and reduced milk production have also been associated with gadding behaviour but the losses are difficult to quantify (Colwell et al. 2006b).

The major symptoms associated with cattle hypodermosis are induced: by larval penetration and migration in host connective tissue; by anaphylactic reactions resulting from the production of larval antigenic substances; and by the immunosuppressive effects engendered by such substances (Medjitna 2003).

In the case of primary infestations, no significant lesions appear along the migratory pathway of first instar larvae. However, after repeated infestations, the penetration of the larvae through the skin or hair follicles can cause local inflammatory reaction and the migrating larvae L1 are surrounded by granuloma, which are mainly infiltrated by inflammatory cells. The connective tissue through which the larvae migrates is mostly degraded (Boulard 2006).

The overwintering of the L1 larvae at their respective sites (in the oesophagus submucosa for *H.* lineatum and in the epidural fat around the thoracic and lumbar vertebrae for *H. bovis*) can cause dysphagy with ptyalism or meteorism as well as temporary or persistent paresis and paraplegy (Boulard et al. 1988; Scholl 1993). Those clinical signs can appear consecutively to larval destruction after the application of delayed autumnal treatments. The release of massive larval antigens can induce allergic hypersensitivity reactions:

Hypersensitivity type I: mainly due to hypodermins A and B, inducing local oedema on specific locations on the host body in primo-infested animals or cardio-vascular collapses in sensitive animals

Hypersensitivity type III: due to the formation of immune complexes resulting in intense congestive syndromes with formation of oedema, haemorrhages and necrosis.

Hypodermins A and B induce an immuno-suppression condition in young animals during primo-infestations by *Hypoderma* larvae; therefore they might be exposed to several secondary infections (Araujo Jorge et al. 1994).

The most evident clinical manifestation of the *Hypoderma* infestation is the appearance of the warble on the host’s back (Fig.6). The second and third larval stages remain enclosed in this subcutaneous granuloma. When third instars complete their development, the warbles become very painful but there are no inflammatory reactions (Euzéby 1998).

Infestation of humans with *H. lineatum* and *H. bovis* larvae have been reported but they are not so common as described for *O. ovis*, probably because fewer people are closely associated to direct contacts with cattle than with sheep/goats and of the successful eradication programs of the past decades. Usually the development of the larvae is aborted but in some cases skin allergies, ophtalmomyiasis have been reported (Zumpt 1965; Boulard and Petithory 1977; Anderson 2006b).

Fig.6. Presence of subcutaneous warbles (arrows) in the dorsal, lumbar and lateral regions of a bovine. A mature third instar larva is emerging (encircled). (Charbon 1993)

2.4.5.2 Immunological aspects

Larval antigens

During the migration, i.e. the intra-host stage, the *Hypoderma* spp larvae interact with the host's tissue and thus with the host immune system. An involvement of enzymatic activities originating from the L1 midgut has been demonstrated. Three major larval enzymes, hypodermin A and B (31 KDa and 23 KDa, respectively characterized as serine proteases) and hypodermin C (24 KDa, characterized as collagenase) have been isolated (Boulard 1970; Lecroisey et al. 1979; Tong et al. 1981). These larval secretory enzymes are essential for the penetration and migration processes. The collagenolytic and fibrinolytic activity of hypodermin C (hyC) facilitate the progress of the L1 by digesting the conjunctive tissue and the inflammatory structures (Boulard 1975). The immunosuppressive effect of hypodermin A (hyA) and of hypodermin B (hyB) allows the L1 to evade the host immune system and consequently increases the larval survival (Boulard and Bencharif 1984; Boulard 1989; Chabaudie and Boulard 1992; Moire et al. 1994; Nicolas-Gaulard et al. 1995). The transitory alteration of the host immune response targets both non specific immune system such as the inflammatory processes (Nelson and Weintraub 1972; Boulard and Bencharif 1984), but also the specific immune system (Fisher et al. 1991; Chabaudie and Boulard 1992). For instance, it has been demonstrated that both hyA and hyB can inactivate complement components, in particular C3. The degradation of C3 may curtail the inflammatory response. Therefore the complete lack of an inflammatory response during a primo-infestation can be at least partially explained (Boulard 1989). HyA and hyB also affect the antigen-specific response (Fisher et al. 1991; Chabaudie and Boulard 1992; Moire et al. 1994; Nicolas-Gaulard et al. 1995).

In case of repeated exposure to cattle grubs, the development of resistance is observed. An intense inflammatory reaction inhibits or reduces the larval migration (Nelson and Weintraub 1972). Simultaneously, antibodies are produced against the larval secretions (Boulard 1975). As a consequence, protein extracts have been used in the search of effective vaccines for immunization of cattle (Magat and Boulard 1970; Baron and Weintraub 1986). The hyC is yet currently used in the development of antibody detection ELISA’s (Panadero et al. 1997), and the recombinant hyC with enzymatic activity (Casais et al. 1998) can be used for serodiagnosis of hypodermosis (Panadero et al. 2000; Panadero-Fontan et al. 2002). The hyC from *H. lineatum* has been described as antigenically similar to proteins secreted by other members of the subfamily, but no cross reactivity was found with other Oestridae such as *O. ovis* or *G. intestinalis* (Boulard et al. 1996).

Host-immune reaction

The immune response of naturally and experimentally infested cattle to *Hypoderma* larvae is well documented (Baron and Colwell 1991). The absence of inflammatory response during primo-infestation by first-stage larvae is a consequence of the secretion of larval enzymes, inhibiting host immunity. Cattle develop an acquired resistance after a successive exposure to *Hypoderma* larvae (Baron and Weintraub 1987a) and this factor can be essential in controlling grub populations. The decreasing rate of grubs is significant after the third consecutive infestation and sometimes total protection is reached in older animals (Gingrich 1982; Benakhla et al. 1999).

Circulating antibodies develop in response to L1 infestations and several studies measured the kinetic development of the humoral response in naïve, previously infested and vaccinated animals (Boulard 1975; Robertson 1980; Pruett and Barrett 1985). However no correlation was found between the development of antibodies and a resistance to grub infestation (Gingrich 1982). The high mortality in the early phase of infestation can be correlated with an increased antigen-specific responsiveness of animals previously exposed while this responsiveness occurs much later in naïve animals, i.e. when grubs reach the back (Baron and Weintraub 1987b). Additional evidence supports this hypothesis (Gingrich 1982) but still many aspects of the host immune and biological systems remain to be explored (Boulard 2006).

2.5 Gasterophilinae

The Gasterophilinae subfamily is divided in three genera; *Gasterophilus, Gyrostigma* and *Cobboldia*. The larvae of the different species are all obligatory parasites of the digestive tract of large herbivores.

2.5.1 Genus *Gasterophilus* (Leach, 1817)

Nine species of *Gasterophilus*, all causing gastrointestinal myiasis in equids are described:

Gasterophilus haemorrhoidalis	Linnaeus, 1758
Gasterophilus nasalis	Linnaeus, 1758
Gasterophilus intestinalis	De Geer, 1776
Gasterophilus pecorum	Fabricius, 1794
Gasterophilus inermis	Brauer, 1858
Gasterophilus lativentris	Brauer, 1858
Gasterophilus nigricornis	Loew, 1863
Gasterophilus ternicinctus	Gedoelst, 1912
Gasterophilus meridionalis	Pillers and Evans, 1926

2.5.2 Morphology

2.5.2.1 Imagines

A very detailed description has been made for most *Gasterophilus* imagines (Dinulescu 1932; Zumpt 1965). The main features to differentiate the *Gasterophilus* species are the morphology of the wings and the different coloration of thorax and abdomen.

The *Gasterophilus* are medium-sized (body length = 12-15mm), robust and extensively pilose flies. This pilosity associated to their coloration makes them resemble to honey bees. The colouring of *G. intestinalis* is highly variable as in other *Gasterophilus* species. A dark (Fig.7) and a pale form exist; the latter seems to occur mainly in warmer and drier parts of Africa. There is an alternation of black and yellow hairs on thorax and abdomen. Legs are also yellow-brown covered with appressed hairs. Wings have faint and ill-defined infuscations, forming a pattern of two dots at the apex and a vitta (broad longitudinal stripes) in the middle, which covers the whole width of the wing (Fig.7)(Wood 1987).

A particularity in female *Gasterophilus* is the fusion of the tergite and sternite 7 that forms a ring; these are separate in all the other oestrids. Dinulescu (1932) described the atrophied digestive system of these flies and noticed the absence of some diverticulum or organs such as the salivary glands, crop or gastric caecum. The proboscis, best developed in *Cobboldia* (Gasterophilinae) and in Cuterebrinae, are represented by nodules in *Gasterophilus* spp.

Fig.7. Adult fly of *G. intestinalis* from the collection of the Museum of Natural History of Neuchâtel. The wing shows the specific infuscations forming the pattern of the two dots (arrows) and the vitta which covers the whole width (star). ©L. Roelfstra.

2.5.2.2 Eggs

The characteristics of *Gasterophilus* eggs are presented according to Zumpt (1965).

The colour of the eggs depends on the species and can be either yellowish to brownish-black. All of them have a specific dorsal subterminal operculum and a ventral attachment organ intimately associated with the egg. The shape of the attachment organ and the position of the eggs vary among the species. During oviposition the organ is deformed for a better adhesion and an adhesive substance is added (Cogley and Anderson 1983). *G. pecorum* is an exception in the Gasterophilinae subfamily as females do not lay their eggs directly on their host but attach them to grass, leaves and stems of plants. Eggs hatch in response to a host stimulus or spontaneously.

Table 4. Main characteristics of *Gasterophilus* spp eggs and egg-hatching data. Details are unknown for *G. lativentris*, *meridionalis* and *ternicinctus*. From Zumpt (1965).

Gasterophilus species	Average size (mm)	Colour	Embryonic period (days)	Stimulation to hatch
G. haemorrhoidalis	1.5	brownish-black	2	Moisture from host
G. inermis	0.4	creamy-white	unknown	No external stimulation
G. intestinalis	1.25	yellowish	5	Application of moisture, self and mutual grooming and licking
G. nasalis	1.3	yellowish	5-10	No external stimulation
G. nigricornis	0.4	creamy-white	3-9	Unknown
G. pecorum	0.9	glossy black	5-8	Ingestion by host

2.5.2.3 First instar larvae (L1)

The first instars are small (1-2 mm in length), fusiform with a white coloration (Fig.8). They present prominent and robust mouth hooks (maxilla). Only a few species have been studied in detail.

Larvae are segmented and each segment is delineated by rows of spines, present on both ventral and dorsal surfaces (Dinulescu 1932). The number and density of spines decrease towards the terminal end, where they are generally lacking. L1 have small bilobed cephalic segments, followed by three thoracic and nine abdominal segments. The later has two prominent extensions that bear abdominal spiracles (Colwell and Scholl 1995).

All oestrids have cuticular sensilla distributed over the body. The number, structural diversity and type of sensilla vary among the subfamilies. The involvement of olfaction in migratory behaviour of *G. intestinalis* first instars has been examined. Cephalic olfactory sensilla appear to be present in larvae that must undergo a migration to find the appropriate site for its development inside the host. The presence of two types of trichoid sensilla, two types of coeloconic sensilla as well as a pit sensillum on thoracic and abdominal segments has been identified (Colwell and Scholl 1995).

Fig.8.Eclosion of *G. intestinalis* first instar larva. ©P. Brocard

The absence of olfactory sensilla from first instars Hypodermatinae or Oestrinae suggest that a reduction in sensory requirements of these larvae can be associated with their less demanding migration, but these aspects have not yet been well understood (Colwell 2006c).

2.5.2.4 Second instar larvae (L2)

The *Gasterophilus* second instars are cone-shaped with a slight increase in diameter, when approaching posterior segments (Leite and Scott 1999) (Fig.9). Body length varies between 11-16 mm. Spines are present in small numbers and are arranged in two or three rows except the last abdominal segment, which do not carry any. The morphology of the mandibles and maxillae, the posterior spiracles and the disposition of the spines are the major features used to identify the different *Gasterophilus* species (Zumpt 1965).

Fig.9. Different sizes of *G. intestinalis* second-instar larvae attached to a piece of a horse stomach. ©L. Roelfstra.

The different Oestrid subfamilies do not have the same sensory requirements. This difference mentioned for the first instars has also been demonstrated for the later larval stages. Some sensorial organs that seem to be essential for *Gasterophilus* species, which have to undergo a migration from mouth to stomach or to intestine, are limited or absent for other oestrid species (Colwell et al. 1998). Indeed, later stages of subcutaneous larvae which form cysts (e.g. Hypodermatinae or Cuterebrinae) do not need to further migrate or find nutrients, provided in quantities by the cyst. Consequently some sensorial organs can be considered as redundant and might be limited (Colwell 2006c).

Cephalic segment

The pseudocephalum bears an antenno-maxillary sensory complex formed by robust mouth hooks, or maxilla, flat mandibles and antennal lobes (Fig.10).

Variations have been noticed on the mouthhooks between *G. intestinalis* and *G. nasalis* (Cogley 1999). A study demonstrated that among six *Gasterophilus* species no substantial differences could be observed on the antennal lobes of third instar larvae (Colwell et al. 2007). According to these results it is possible to make the hypothesis that in second instar larvae there are also no major variations in the sensory structures present on the prominent antennal lobes. The sensory structures are; a large dome-shaped sensillum (or olfactory sensillum), a well defined cluster composing the complex sensillum (or gustatory sensillum) and at the periphery several accessory sensilla (basiconic or pit like sensilla) (Colwell et al. 2007) (Fig.11).

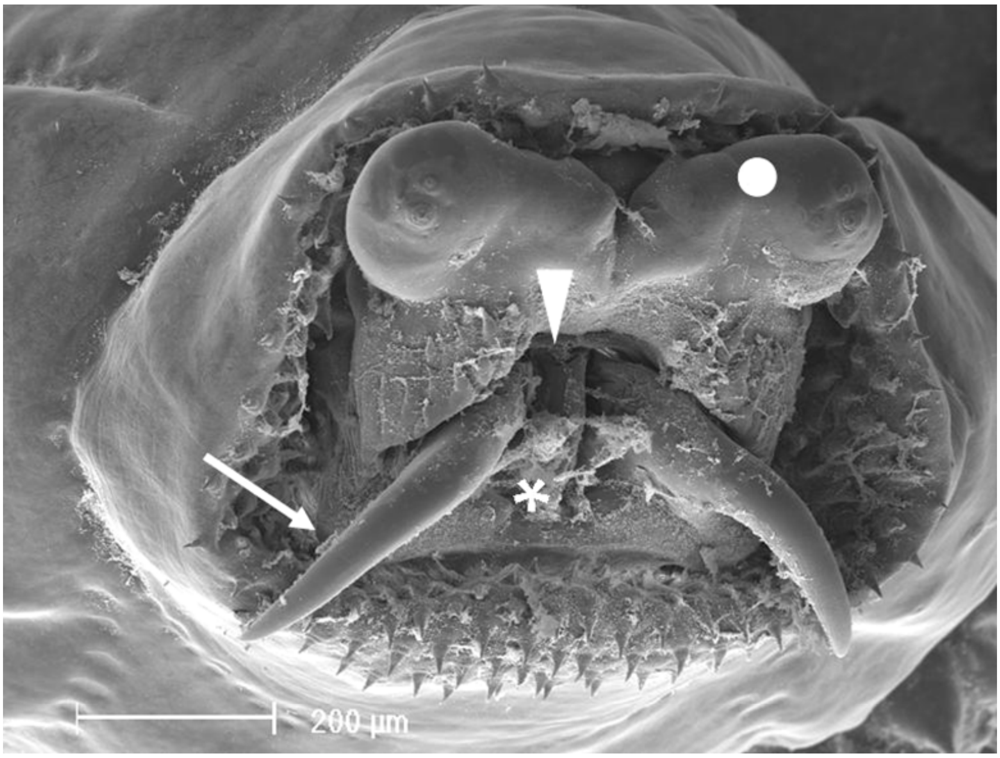


Fig.10. Scanning electron micrograph of the pseudocephalum of *G. intestinalis* second instar larva showing the maxillae (arrow), the mandibles (asterisk), the antennal lobes bearing a sensory complex (dot) and the oral opening (arrowhead). Scale bar = 200 µm. ©M. Vlimant.

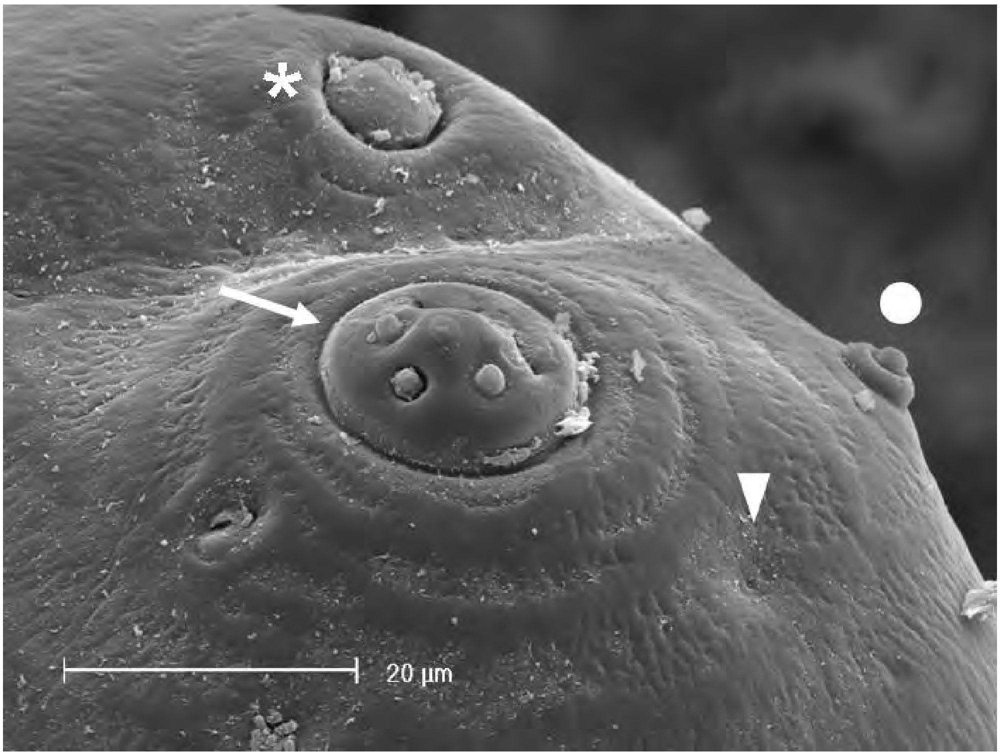


Fig.11. Scanning electron micrograph of the antenno-maxillary sensory complex on an antennal lobe of *G. intestinalis* second instar larva showing the large domed-shaped olfactory sensilla (asterisk), the gustatory sensory cluster (arrow), basiconic sensillum (dot) and pit like sensillum (arrowhead). Scale bar = 20 µm. ©M. Vlimant.

Thoracic and abdominal segments

The cephalic segment is followed by three thoracic and eight abdominal segments. They present cuticular depressions, are circled with spines (number of rows varies among the species) and surrounded by trichoid and campaniform sensilla (Leite and Scott 1999).
Gasterophilus larvae have to survive in gastro-intestinal tract of their host and therefore they require specific adaptation of the respiratory system; anterior thoracic and post-abdominal spiracles. Minute anterior spiracular openings are present on the first abdominal segment, but they do not exhibit the spiracular papillae as described in third instar larvae and are non functional (Principato and Tosti 1988). The terminal end of the eighth abdominal segment bears transverse cuticular folds that form a spiracular cavity, lateral tubercles and sensilla (Leite and Scott 1999) (Fig.12).

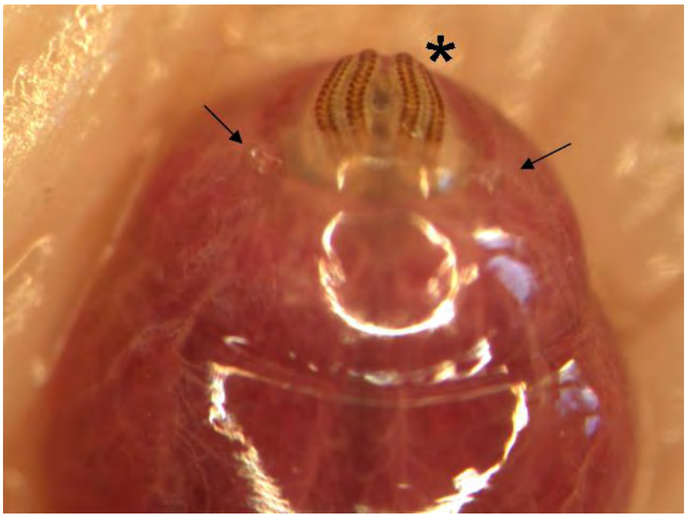


Fig.12. Light micrograph of *G. intestinalis* second instar larva showing the paired posterior spiracles (asterisk) and the lateral tubercles (arrows). ©L. Roelfstra.

A ventral and dorsal large cuticular lip opens and closes over the spiracles, isolating them from the outside. These spiracles are composed of two pairs of slit-like openings, slightly curved, which are surrounded by chitinous rima (Fig.13). The openings lead to a felt chamber containing a sponge-like mass of chitinous strands (Leite and Scott 1999).

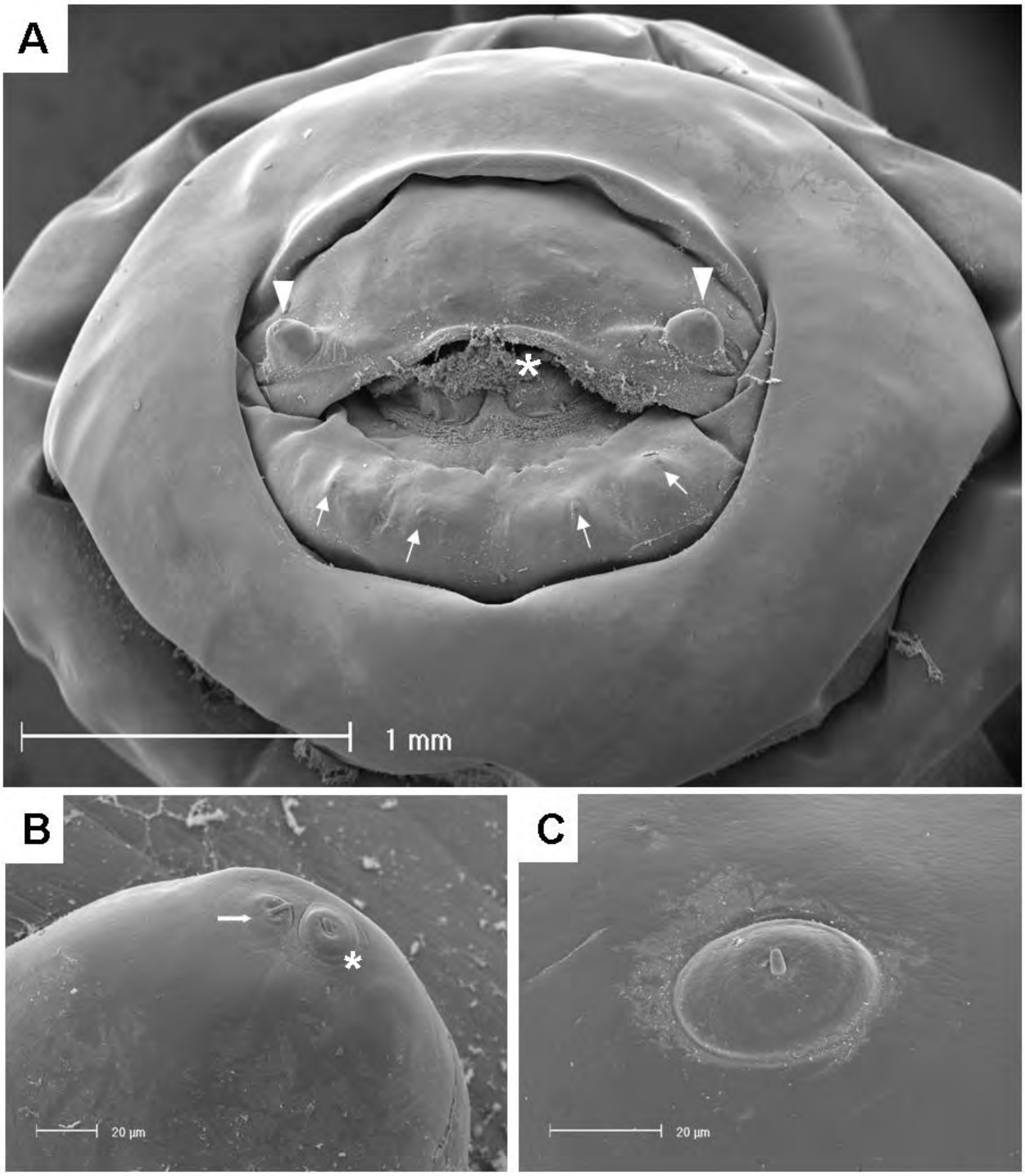


Fig.13. Scanning electron micrograph of the terminal abdominal segment (8th abdominal segment) of *G. intestinalis* second instar larva of showing (A) the lateral tubercles bearing sensilla, ventral to the spiracular plates (arrowheads), the spiracular cavity (asterisk) and the four trichoid sensilla, dorsal to the spiracular plates (arrows). Scale bar = 1 mm. (B) detail of a lateral tubercle showing a trichoid sensilla (arrow) and a basiconic sensilla (asterisk). Scale bar = 20 µm. (C) Detail of a trichoid sensilla. Scale bar = 20 µm. ©M. Vlimant.

2.5.2.5 Third instar larvae (L3)

Mature third instar larvae measure between 14-20 mm. The body shape is usually similar to second instar (Fig. 14) but in some species, it can be cylindrical with a very small cephalic segment. Third instar morphological features of different *Gasterophilus* species (*G. haemorrhoidalis, G. inermis, G. intestinalis, G. meridionalis, G. nasalis and G. pecorum*) have been well described by Colwell et al (2007). The morphology of cephalic and thoracic segments; the structure of the mandibles and maxillae; cuticular features (distribution and shape of the spines); the terminal abdominal segment; and the respiratory structures are characteristics that vary among the species with the exception of *G. haemorrhoidalis* and *G. intestinalis* that share all morphological characteristics (Colwell et al. 2007).

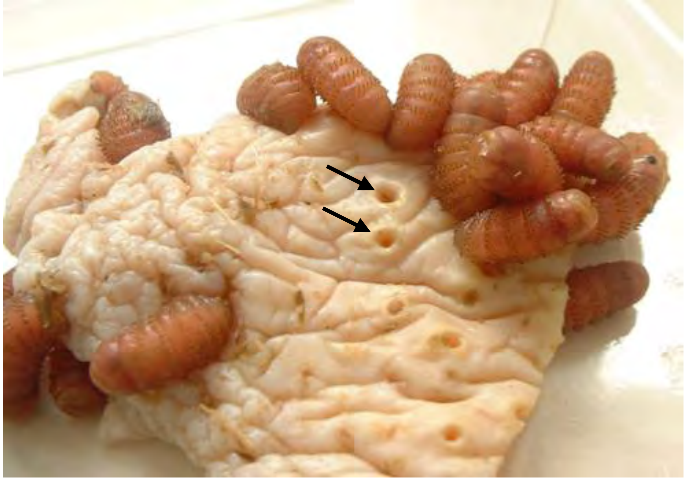


Fig.14. Picture of *G. intestinalis* third instar larvae attached to a piece of horse stomach. Arrows indicate damages left after removal of larvae. ©L. Roelfstra.

Cephalic segment

Two prominent antennal lobes are present on the cephalic segment (Fig.15), or pseudocephalum, of all species. They bear sensory structures composed of an olfactory sensillum, the gustatory sensilla and several accessory sensilla at the periphery. Similar structures have been observed for second instars (Fig.11).

Prominent maxillae are used for attachment to the mucosa of the different site inside the host and the flat mandibles are probably used to abrade host tissue for nutrition (Fig.15) (Colwell et al. 2007).

The maxillae and mandible exhibit variation among the six species, although *G. intestinalis* and *G. haemorrhoidalis* are very similar. Probably the robust maxillae are used for attachment and the flat mandibles are used to abrade host tissues to provide nutrients (Colwell et al. 2007).

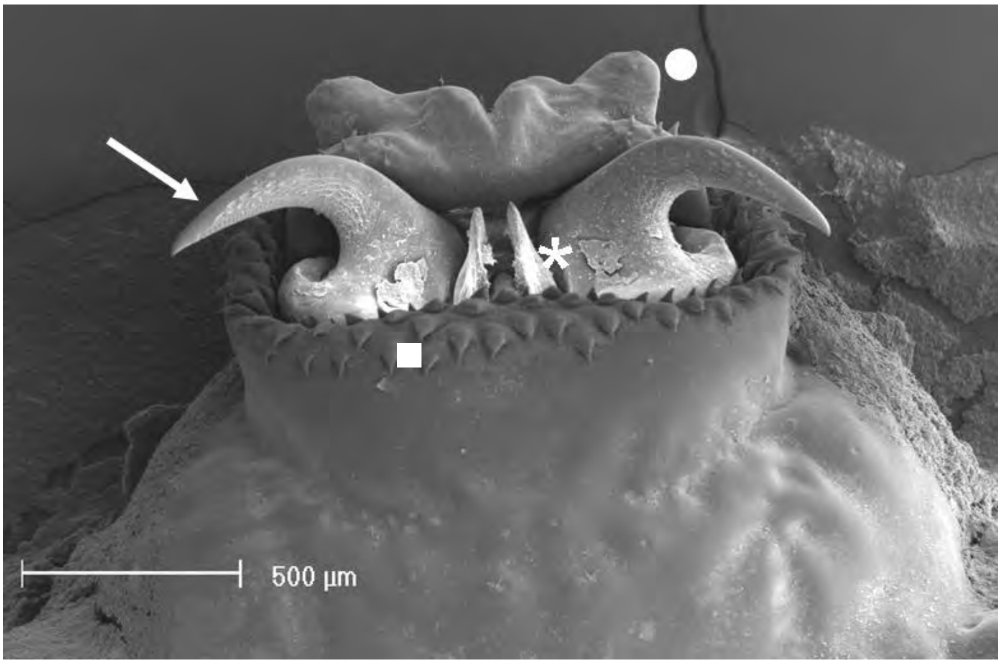


Fig.15. Scanning electron micrograph of the cephalic segment of *G. intestinalis* third instar larva showing the maxillae (arrow), the mandibles (asterisk), spines (square) and the antennal lobes (dot). Scale bar = 500 µm. ©M. Vlimant.

Rows of small simple spines surround the cephalic segment, around the mouth hooks and the antennal lobes.

Thoracic and abdominal segments

Spines on the thoracic and the abdominal segments are arranged in single or double rows and their number tend to decrease towards the terminal segments. The robust spines distributed on the thoracic and anterior abdominal segments help the larvae to maintain their position (Colwell et al. 2007). Trichoid, coeloconic sensilla and cuticular depressions are present around the larval body.

The terminal abdominal segment bears the spiracular plates (Fig.16A) and distinct cuticular sensilla are present on this segment:

- The compound sensilla are located laterally on the ventral lobes (Fig.16B) and are composed of two trichoid sensilla, each on a separate and supernumerary lobe (Fig.16D) and of one lateral pit sensillum.
- Four sensilla are located dorsal to the spiracular plates. Generally there are trichoid sensillae located on a small lobe (Fig.16C). The size of the lobe and the structure of the trichoid sensillum vary, but not consistently among the species (Colwell et al. 2007).

The size of the lobes and the structure of trichoid sensilla vary among the species (Colwell et al. 2007).

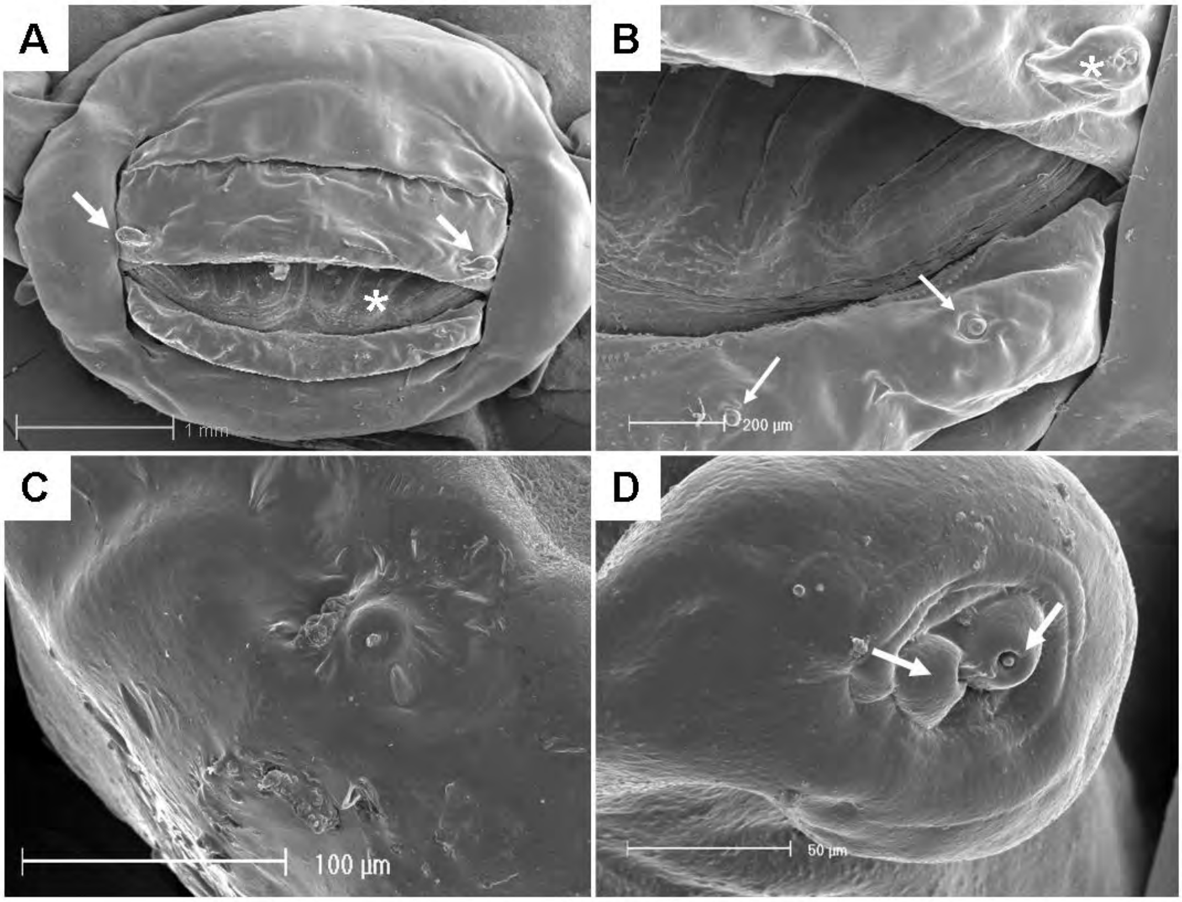


Fig.16. Scanning electron micrographs of the terminal abdominal segment of *G. intestinalis* third instar larva. (A) Terminal abdominal segment showing the two ventral lobes (arrows) and the spiracular plates (asterisk). Scale bar = 1 mm. (B) Ventral lobe bearing the compound sensilla (asterisk) and two individual sensilla located dorsally to the spiracular plate (arrows). Scale bar = 200 µm. (C) Dorsal sensilla on a cuticular lobe showing trichoid sensillum. Scale bar = 100 µm. (D) Detail of the compound sensilla showing two secondary lobes (arrows) bearing two trichoid sensilla. Scale bar = 50 µm. ©M. Vlimant.

Respiratory system

The larvae have to survive in difficult environmental conditions such as the high internal concentrations of carbon dioxide, the gastric juices and the food swallowed that tend to overwhelm them. To prevent the entrance of extraneous material and to capture and store the maximum quantity of oxygen, the respiratory system has been adapted (Principato and Tosti 1988) and two major structures are present:

1. Anterior spiracles (prothoracic spiracles): the two thoracic spiracles consist of a chitinous tube or felt-chamber (Keilin 1944) which at one end extends into the larvae and at the other end opens towards the outside. Several features of these structures vary among the different species which have been studied. These spiracles remain non functional during larval stages meaning that larvae are metapneustic. The anterior spiracles extrude at the end of third instar, and become functional when larvae pupate.

2. Posterior spiracles (post-abdominal spiracles): they are located at the posterior end of the larval body. The terminal abdominal segment bears the spiracular plates within the respiratory chamber. A preliminary protection is exerted by the mobile cuticle covering and uncovering the spiracles (Fig.17).

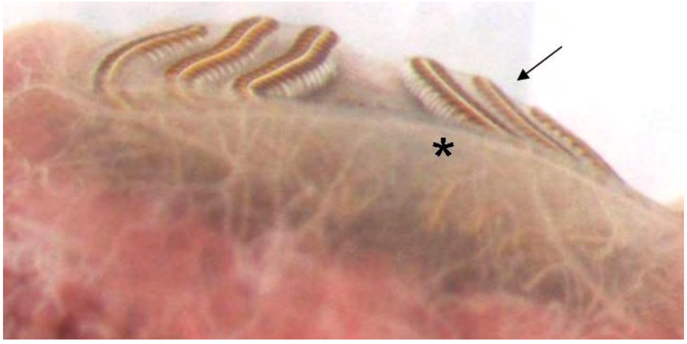


Fig.17. Light micrographs of the terminal abdominal segment of *G. intestinalis* third instar larva showing the spiracular plates composed of three slit-like openings (arrow) and the mobile cuticle (asterisk). ©L. Roelfstra.

The two spiracular plates are joined together; both are composed of three slit-like narrow openings (or peritremes after Zumpt, 1965) (Fig.17) surrounded by a cuticular rim (serrated side) and internally supported by a number of Y-shaped stigmatic sclerites (Fig.18). The serrated sides of the openings function as a preliminary protection when they close by forming a hermetic seal and thus preventing communication with the outside and isolating the whole respiratory system. The trabecular reticulum inside the spiracles can work as an additional filter by preventing material from entering the tracheal system (Principato and Tosti 1988).

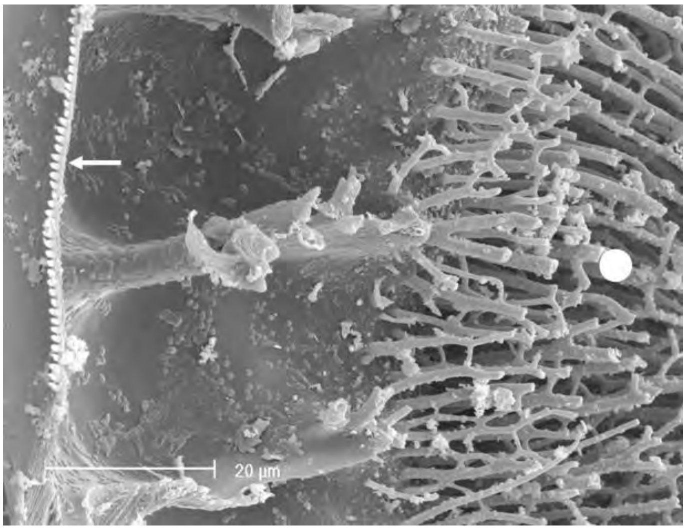


Fig.18. Scanning electron micrograph of the interior of the post-abdominal spiracle of *G. intestinalis* third instar larva showing the trabecular tissue (dot), a serrated side of an opening (arrow). Scale bar = 20 µm. ©M. Vlimant.

The oxygen available for the larvae has been swallowed by the host animal while feeding. The oxygen molecules are absorbed through the post-abdominal spiracles. The distal end of the spiracular chamber receive five pairs of tracheae of unequal diameter and length (Fig.19A, B). The pair of smaller diameter represents the main tracheal trunk which communicates anteriorly with the prothoracic spiracles and supplies the tracheae to all organs of the larvae. The remaining four pairs of tracheae are exceptionally wide at their base but rapidly decrease and become conical. All along their surface they give off numerous branches of smaller tracheae which ramify and finally break up into numerous tracheoles filling the large tracheal cells (Keilin 1944). These large tracheal cells almost completely fill the posterior third of the larvae’s body (Figs 20, 21).

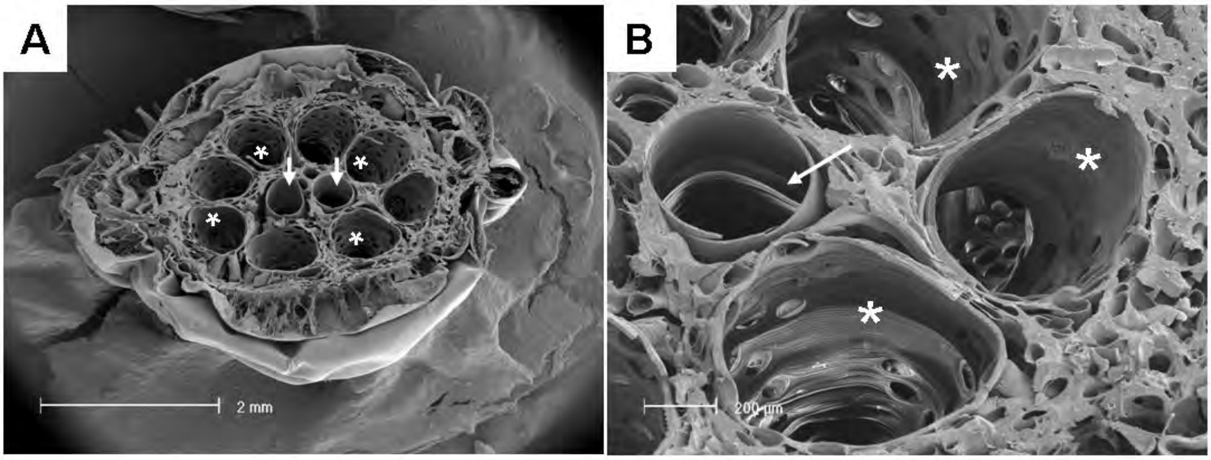


Fig.19. Scanning electron micrographs of the tracheal system of *G. intestinalis* third instar larva (A). Transverse section of the posterior end of the larvae showing the four pairs of tracheae of unequal diameter and length (asterisks); and the main tracheal trunk composed of two smaller tracheae (arrows). Scale bar = 2 mm. (B) Detail inside the large tracheae (asterisks) showing the ramification with the smaller tracheae; main tracheal trunk (arrow). Scale bar = 200 µm. ©M. Vlimant.

The gradual colour change from the white first instar larvae to the red coloration observed in second and third instars is attributed to the physiological development of the tracheal system and represents an increase in hemoglobin content (Cogley et al. 1982). It was observed that early first instars are devoid of hemoglobin and that the red coloration of second instar larvae is a consequence of the presence of their own hemoglobin throughout the tissue (Keilin and Wang 1946). In early instars the larvae contain hemoglobin dissolved throughout the fatbody, but in third instar larvae it becomes concentrated in the tracheal cells (Chapmann 1982). The molecule of hemoglobin has been purified and characterized and the high oxygen affinity was demonstrated (Dewilde et al. 1998).

The morphological changes in the respiratory structures associated with the tracheal organ formation occurring during the first moult prepare the larvae for its subsequent growth in the low tension environment of the stomach (Cogley et al. 1982).

The tracheal cells and the complex respiratory system with its protective systems reflect a perfect adaptation of the larvae to their parasitic life (Principato and Tosti 1988).

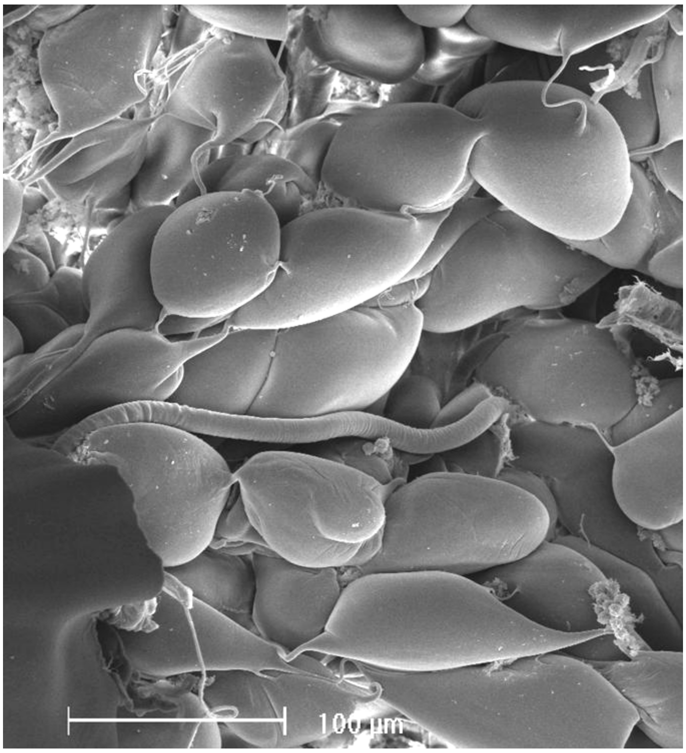


Fig.20. Scanning electron micrographs of the tracheal cells of *G. intestinalis* third instar larva. Scale bar = 100µm. ©M. Vlimant.

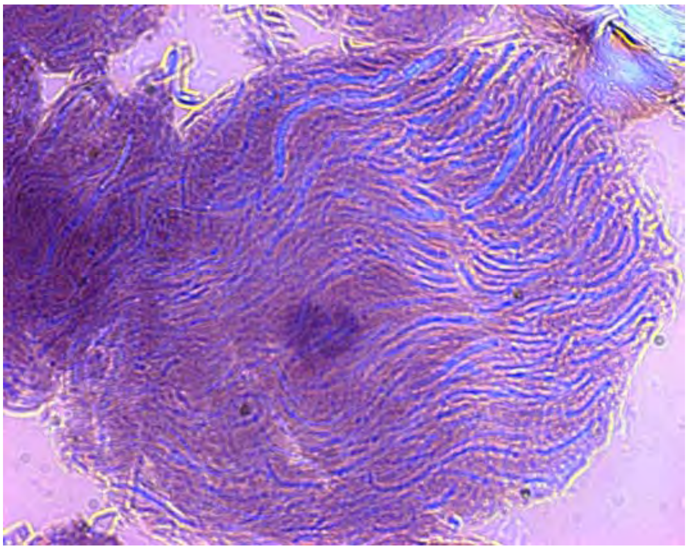


Fig.21. Detail of a tracheal cell. Light microscopy, x 400, hematoxylin and eosin staining. Centre for Integrative Genomics, Lausanne. ©L. Roelfstra.

Internal structure

The study of internal organs has been limited in Oestrids to several species of high veterinary importance (*H. bovis, H. lineatum, O. ovis,* and *D. hominis*). Therefore it is extremely difficult to make generalities and comparisons between the different subfamilies as well as between species. However, organs like the salivary glands or the midgut have been of major concern in the mentioned species to study the parasite-host immune interaction, and to determine the location of specific antigens that could induce host immune responses. Little is known for the different *Gasterophilus* species. Fig.22 shows some of the internal organs of the anterior section of a third instar *G. intestinalis*.

A detailed description of the digestive system and salivary glands of *G. intestinalis* is presented in chapter 4.2.1

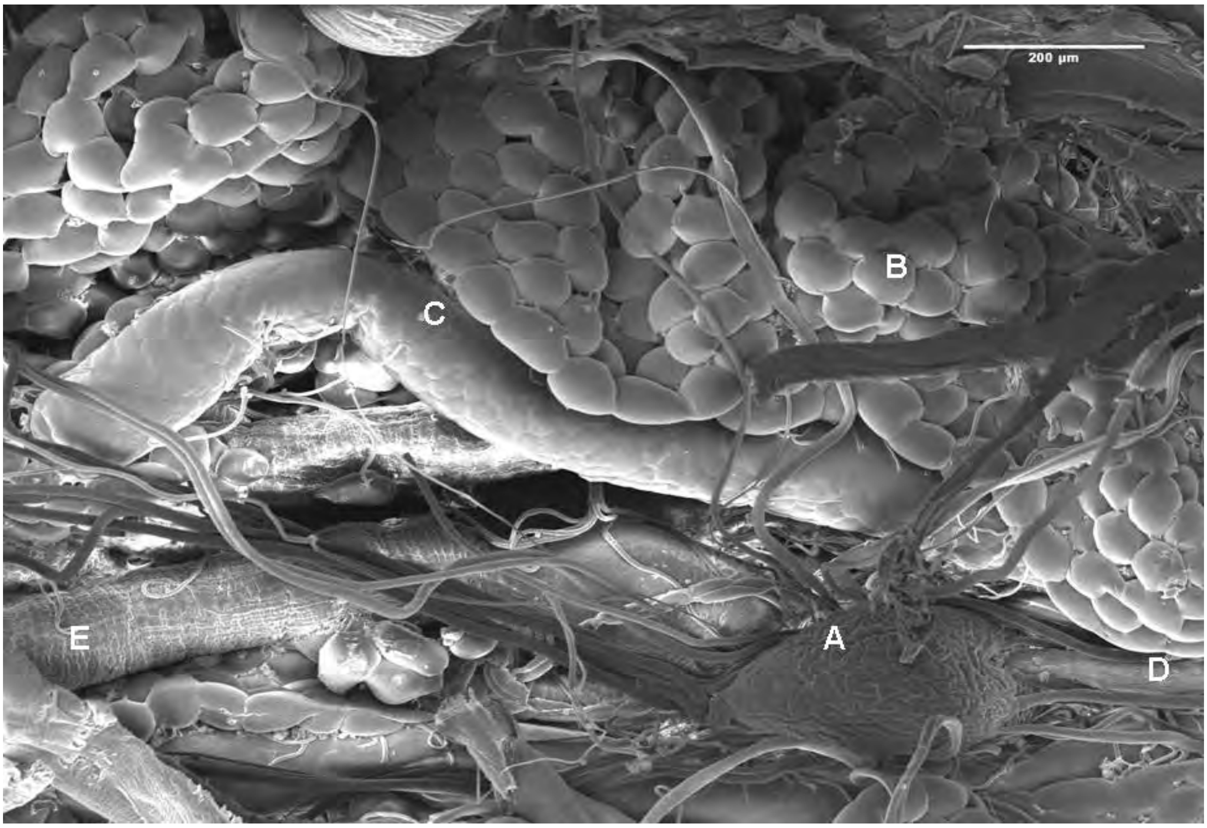


Fig.22. Scanning electron micrographs of dissected *G. intestinalis* third instar larva showing the internal organs. (A) Cephalic ganglia; (B) Fat body; (C) Salivary gland; (D) Oesophagus; (E) Midgut. Scale bar = 200µm. ©M. Vlimant.

2.5.3 Life cycle of *Gasterophilus* spp

2.5.3.1 Endogenous life cycle of *G. intestinalis*

One year is necessary for completing the life cycle of *G. intestinalis*, producing thus one new generation per year. The different stages of the life cycle are highly dependent on external environmental conditions. In Switzerland, eggs are found on horses between July and November with a maximum in September and October. Accordingly, L2 can be found between September and December with a maximum in October and November. L3 begin to be present onwards October/November and until next spring. Generally no larvae are found in horse stomachs between July and August (Brocard 1991).

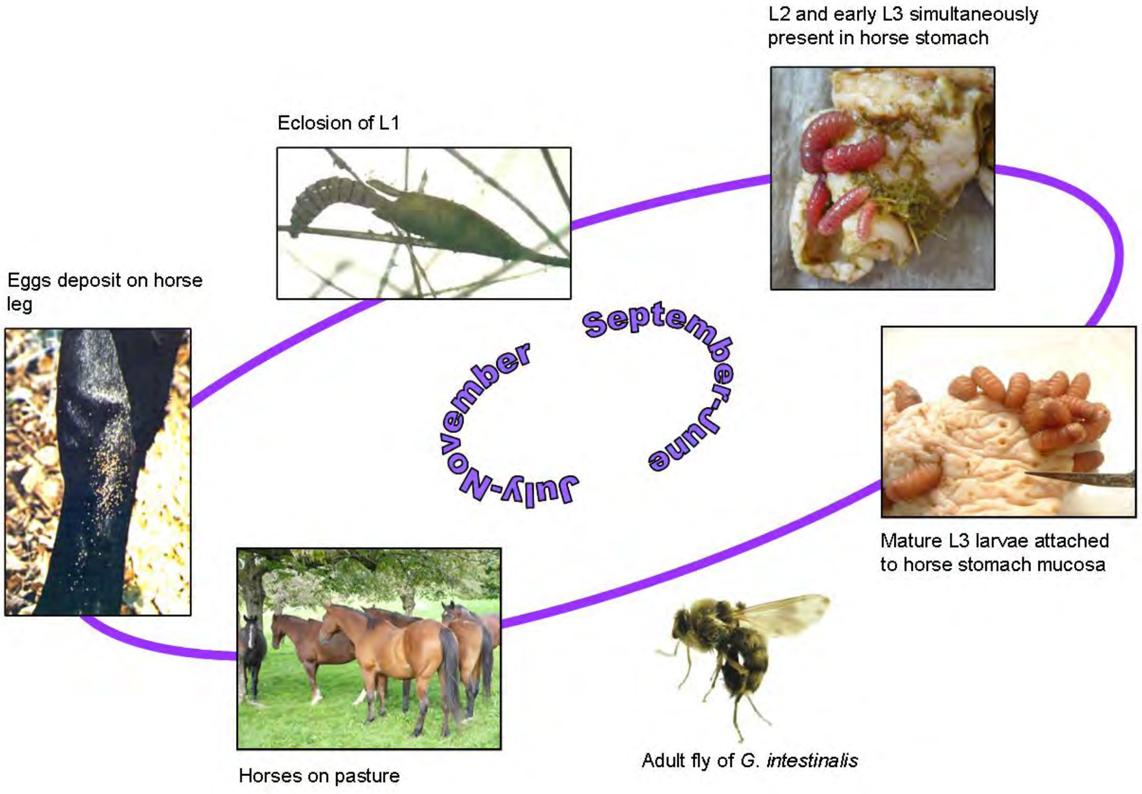


Fig.23. Life cycle of *G. intestinalis*

The life cycle of *G. intestinalis* (Fig.23) mainly refers to Cogley (1989). Females lay their eggs on specific sites on their host, mostly on forelegs. Eggs are ingested by grooming and licking behaviour, and hatch by the host's stimulation (moisture). L1 in the oral cavity rapidly enter the tongue epithelium in areas of natural breaks and depressions. Larvae form epithelial burrows by excavating through the *stratum germinativum* and thereby periodically produce air holes (openings to the surface of the tongue). During migration through the tongue, the larvae grow and move to the upper molars. The larvae will form pockets at the junction of the interdental and lingual gingiva, and it is exclusively in this space that the L1 will moult into L2. The cephalic portion is embedded within the gingiva and the posterior portion, bearing the stigmata is exposed to the exterior. The larvae grow continuously and when the interdental space becomes confined, they leave the area and move to the root of the tongue (*isthmus faucium*) where they attach themselves. Only the cephalic segment end is embedded in tissue, the rest of the body remains exposed. The time during which *G. intestinalis* remains in its host oral cavity probably varies between 21 to 28 days.

The migration of the L2 from the root of the tongue to the stomach has not been studied and information about this part of lifecycle is lacking.

In the stomach, L2 moult into L3 which remain attached to the wall of the stomach, grouped around the interface of the glandular and non glandular epithelia (near the margo plicatus) for 8-10 months. Mature L3 are expelled via faeces and these larvae burrow into the soil where pupation will take place. Adults emerge from the puparium after 22-28 days. According to external environmental conditions (T°, humidity), the duration of pupation can vary (Dinulescu 1932). Mating takes place a short time after emergence; males have even been observed to attempt to copulate with not fully emerged female (Cogley and Cogley 2000).

Life history features of the other *Gasterophilus* species (Zumpt 1965; Colwell et al. 2006b)

G. nasalis: eggs are laid on the hair under the chin. They hatch spontaneously and migrate superficially to the interdental spaces where they moult into L2. These L2 migrate to the duodenum and moult into L3 which will remain attached to the duodenal wall (near the pylorus) for several months (Drudge et al. 1975).

G. haemorrhoidalis: eggs are laid on single hairs of the lips and host stimulation makes them hatch. They penetrate the skin at the hatching site and migrate subcutaneously in the host mouth, away from cheeks and tongue. L2 are found in the stomach and the duodenum where they moult into L3. Before reaching maturity, L3 migrate to the rectum and reattach near the rectum.

G. inermis: eggs are laid on hairs on cheeks where they hatch spontaneously and penetrate the skin. The L1 migrate subcutaneously towards the corner of the mouth before continuing their migration in the inner parts of the cheeks. A first moult occurs between the interdental spaces. The L2 and L3 are found in the rectum.

G. nigricornis: eggs are laid on the hairs of cheek or neck, where they hatch spontaneously and penetrate into the host at the hatching site. L1 of *G. nigricornis* have the same migratory route as *G. inermis* L1. Moulting to the L2 occurs in the mouth and thereafter they migrate to the duodenum wall where they develop into L3. L3 are attached more superficially than the L2 which develop in deep duodenal swellings.

G. pecorum: eggs are laid on grass and vegetation; they hatch when ingested by the host and then penetrate into the mucosa of the lips, cheeks, tongue, gums and hard palate, from where they burrow towards the root of the tongue and the soft palate. The larvae remain in the oral cavity for up to 10 months, thereby moulting into L3. Generally, a large accumulation of larvae is found on the soft palate and at the root of the tongue. L3 usually move to the stomach to reach maturity. In case L1 are swallowed, the may complete development within the walls of the pharynx, oesophagus or stomach.

No data are available about the life cycle of *G. lativentris,* whereas the only data available in *G. meridionalis* and *G. ternicinctus* is the fixation of the L3 to the host stomach mucosa.

2.5.3.2 Exogenous life cycle of *Gasterophilus* spp

Adults appear when climatic conditions become appropriate generally during summertime. However, published observations of adult Gasterophilinae flies are rare and few report on the behaviour of *Gasterophilus* spp in their natural environment. As explained by Cogley and Cogley (2000), the period of emergence occurs only a few months each year and it seems that the population tends to concentrate temporally and spatially to enhance the chance for both sexes to meet. This lack of investigations is understandable when the hosts involved are rhinoceroses or elephants but the gentle nature of horses should allow a safe approach. The parahost behaviour study of adult *G. intestinalis* made by Cope and Catts (1991) and the 10 year field observations of *G. intestinalis* made by Cogley and Cogley (2000) provide interesting information for this species.

Male behaviour (Cope and Catts 1991)

The urgency in mating and oviposition seems to be dictated by the short life span. An in-flight search and intraspecific aggressive behaviour is characteristic of male parahost flight. This behaviour seems to be density dependent. When density is low (1-2 flies/20 horses), they fly rapidly from horse to horse until meeting a female. In case of higher density (1 fly/5 horses), males remain a bit longer near each horse and closer to the ground. The proximity of two or more males near a horse results in an aggressive interaction (pursuit, audible in-flight contact, grappling and falling to the ground). Intraspecific aggression has never been observed between females.

Mating

Mating takes place rapidly after emergence without directed activity of females. The identification of a specific mating location is not totally elucidated but it seems that hilltop aggregation (Catts 1979) as well as the designation of horses as a congregation site for mating (Cope and Catts 1991) are unlikely. Cogley and Cogley (2000) proposed that faecal piles of the host were the most likely mating sites.

Host finding

After mating a period is necessary for female to become interested in finding hosts, estimated to be less than 20 hrs. Several field observations suggest that both olfaction (Mock 1973) and visual cues are used by the female fly to orientate itself to a horse. The attraction to high-contrast silhouettes is a behaviour already reported for other Diptera and can explain the disinterest of *G. intestinalis* in horses in dark areas or in stables (Cogley and Cogley 2000). The production of a finer silhouette of darker horses compared to lighter ones would explain the preference of the flies for the former as observed by Brocard and Pfister (1991).

Oviposition

Female *G. intestinalis* are active during the day but midmorning and late afternoon appear to be the favourable periods for ovipositing. They demonstrate a huge tenacity as they can lay their eggs on walking, trotting or galloping horses by pursuing them. Horses can be irritated by intense fly activity and try to escape but flies resume oviposition immediately when horses stop. Each *Gasterophilus* female has the potential to produce a certain amount of eggs which are deposited at specific locations according to the species (data are summarized in Table 5). Female *G. intestinalis* can deposit their total load of eggs (400-1000) within one hour and they demonstrate a spasmodic behaviour moving from one horse to another probably to ensure the survival of offspring if one host isn't suitable enough (Cogley and Cogley 2000). The production of large amount of eggs as it is the case for *G. pecorum* and *G. intestinalis* might be an adaptive behaviour. Indeed, *G. pecorum* is the only species that deposits its eggs away from the host, i.e. on vegetal substrate, and even if the larvae develop in 5-8 days they can remain dormant within the egg for several months until ingested by the host (Colwell 2006a). *G. intestinalis* is the only species which do not deposit its eggs close to the host's mouth and therefore producing a larger number of eggs might increase the chance of the larvae to be ingested (Colwell 2006a).

Table 5. *Gasterophilus* species and oviposition data. Details are unknown for *G. lativentris, G. meridionalis* and *G. ternicinctus* (From Zumpt, 1965).

Gasterophilus species	Oviposition site	Number of eggs
G. haemorrhoidalis	Lips, mainly the upper lips	50-200
G. inermis	Cheeks	320-360
G. intestinalis	Mainly lower forelegs, also back and flanks	400-1000
G. nasalis	Under the chin in the groove between the halves of the lawer jaws	300-500
G. nigricornis	On cheeks, rarely on nasal region	330-350
G. pecorum	Leaves and stems of plants, mainly grasses	1300-1500

2.5.4 The hosts

The different species of this genus show strong host specificity for equids (Table 6).

Cases of accidental myiasis by *Gasterophilus* larvae have been reported in man, causing cutaneous (creeping myiasis), oral infections or ophtalmomyiasis (Zumpt 1965). Very seldom *Gasterophilus* larvae have been found in digestive system of scavengers, e.g. vultures (Cooper and Housten 1972) or in carnivores, e.g. lions (Battisti et al. 1997), probably as a consequence of the ingestion of previously infested animals. Generally, the larvae die as the L1 and cannot complete their development.

Table 6. *Gasterophilus* spp and their hosts. Data are unknown for *G. lativentris*.

Gasterophilus species	Host
G. haemorrhoidalis	Horse, donkey, *Equus burchellii*, *Equus zebra*
G. inermis	Horse, *Equus burchellii*
G. intestinalis	Horse, donkey
G. nasalis	Horse, donkey, *Equus burchellii*
G. nigricornis	Horse, donkey
G. pecorum	Horse, donkey, *Equus burchellii*
G. ternicinctus	*Equus burchellii*
G. meridionalis	*Equus burchellii*

2.5.5 Digestive tract of horses

Horses are nonruminant herbivores; they do not have a multi-compartmented stomach as bovines. The swallowed food passes from mouth to stomach via the oesophagus, a simple muscular tube between the pharynx and the stomach. Two sphincters are found at either end of the oesophagus and under resting conditions the oesophagus is collapsed and both sphincters are closed.

The horse stomach (Fig.24) is typically J-shaped and can anatomically be divided into four regions; the area surrounding the oesophageal sphincter is the cardia; the fundus represents the rounded area at the top; the antrum is the funnel-like area at the bottom of the stomach; whereas the corpus is the area found between the fundus and the antrum, also called the body of the stomach. The duodenum is separated from the stomach by the pyloric sphincter. The volume of the stomach is small compared to the horse size and has a capacity of 8-15 litres. The stomach secretes gastric acid and plays an important role in the enzymatic digestion.

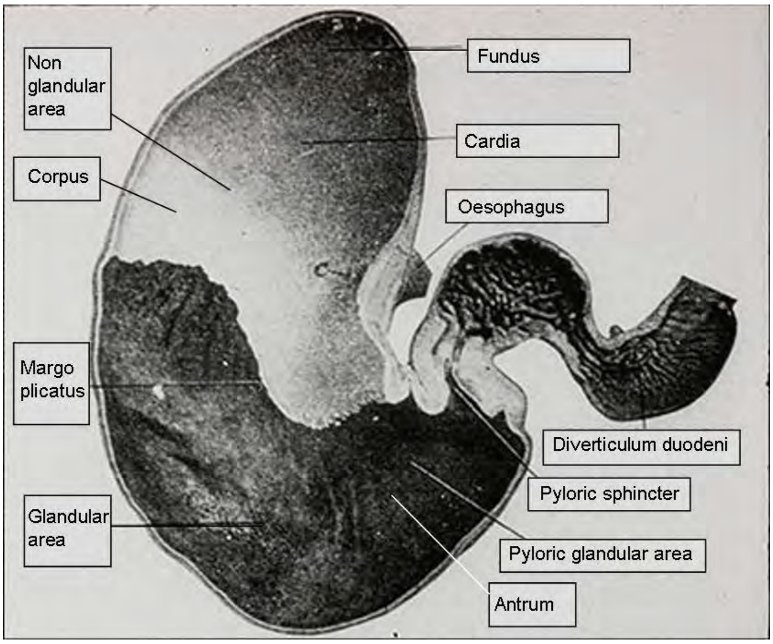


Fig.24. Section of a horse stomach showing the different regions: cardia, fundus, antrum and corpus. Glandular and non glandular parts are clearly delimited by the margo plicatus. (Adapted from Sisson, The Anatomy of Domestic Animals, 1975, Ed. R. Getty)

The muscular walls of the stomach can dilate in order to accommodate to large amounts of food. The peristaltic movements mix the digestive juices and food to form a semi-fluid substance called chyme; which reaches the small intestine via the pyloric sphincter.

The small intestine (Fig.25) measures about 21 meters and is divided into three segments: Duodenum, Jejunum and Ileum. It is the major digestive organ for the digestion and absorption of nutrients. Pancreatic enzymes contribute to digest food and bile constantly flows into the small intestine from the liver as horses do not have a gall bladder.

The Ileum is linked to the caecum by the ileo-caecal junction who represents a sort of limit between the enzymatic and microbial digestion. The caecum is a blind sack of approximately 1.20 meters and both entrance and exit are situated at the top of the organ. It can be compared to a microbial inoculation vat where the food from the small intestine is decomposed by bacteria, brewed and mixed before being sent, by muscular contractions into the large intestine.

The colon measures about 3.5 meters and is segmented into a right ventral, a left ventral and a dorsal colon. It represents the most voluminous part of the digestive tract of the horses. The microbial digestion continues in the colon and most of the nutrients produced through microbial digestion and water are absorbed at this level. The faecal balls are produced and are expelled via the rectum.

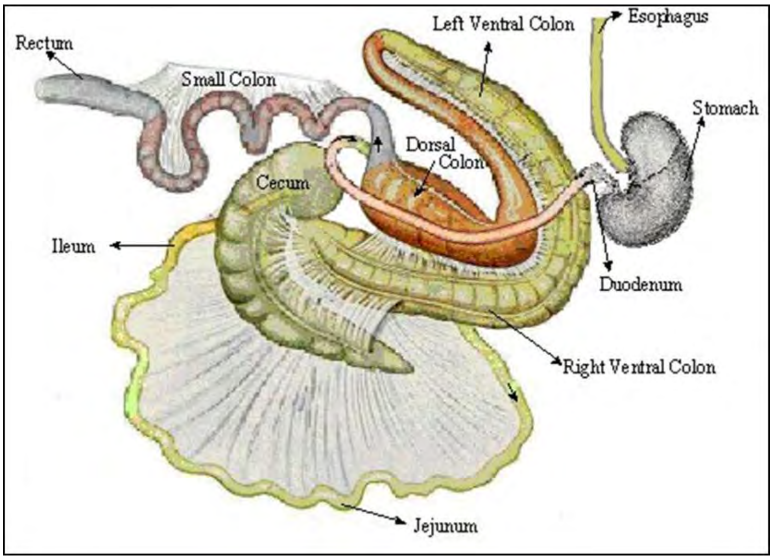


Fig.25. Digestive tract of horses (Adapted from the Atlas of Topographical Anatomy of the Domestic Animals, P. Popesko, 1985, Ed. W.B. Saunders)

2.5.6 Distribution

The *Gasterophilu*s flies have their origin in the Palaearctic and Afrotropical regions and they were probably introduced to other regions in association with their hosts (Zumpt 1965).

Nowadays, representatives of the genus are distributed worldwide. While *G. intestinalis* and *G. nasalis* are distributed worldwide and are often the only species reported in many parts of the New World; e.g. in the USA (Drudge et al. 1975; Tolliver et al. 1987; Lyons et al. 2006); or in New Zealand (Kettle 1974), the remaining species are only reported in very limited areas of Europe, Eastern Countries and Africa (Zumpt 1965).

Studies focused on the species present in donkeys and both *G. intestinalis* and *G. nasalis* were the only species detected in Morocco (Pandey et al. 1992; Pandey and Cabaret 1993); in Egypt (Hilali et al. 1987) and in Jordan (Mukbel et al. 2001).

G. nasalis is the most common species in the Neotropical region, and it is the only species reported in Brazil (Sequeira et al. 2001). Although *G. intestinalis* was previously introduced; it could not be established and was no more reported (Guimarães and Papavero 1999).

In many areas of Europe, the examination of horse stomachs after necropsy allows to clarify the distribution of the different *Gasterophilus* species. The studies made in Northern England and Wales (Edwards 1982) and in Southwest England (Lyon et al. 2000), demonstrated that, even if *G. nasalis* has been occasionally detected, *G. intestinalis* is the only reported species in infested animals. In northern France, three different *Gasterophilus* species have been detected; *G. intestinalis*, *G. nasalis* and *G. haemorrhoidalis* (Bernard et al. 1994). A very specific situation has been observed in central and southern Italy where five different *Gasterophilus* species; *G. intestinalis, G. nasalis, G. haemorrhoidalis, G. inermis* and *G. pecorum* have been reported (Principato 1989; Otranto et al. 2005). Except these examples, the only species recorded in Western, Central and Northern Europe is *G. intestinalis*; i.e. Germany (Cirak et al. 1996), The Netherlands (Borgsteede and van Beek 1998), Sweden (Hoglund et al. 1997), Poland (Gawor 1995) and Switzerland (Brocard and Pfister 1991).

2.5.7 *Gasterophilus* in Switzerland

Brocard (1991) made a study in several areas in Switzerland to analyse the distribution and prevalence of *Gasterophilus* spp. Only *G. intestinalis* has been detected with a prevalence of 64.6%. The western part of Switzerland seems to be considerably more contaminated compared to the other areas of the country.

L2 are found in horse stomachs between the end of September and the end of November. At the same period and until to following summer, L3 could be detected (personal observations). Several authors observed the same pattern in different areas (Drudge et al. 1975; Principato 1989; Brocard and Pfister 1991).

2.5.8 Pathogenesis

Different lesions associated to the migration and maturation stages of the three larval instars of *Gasterophilus* spp have been well described for *G. intestinalis* and *G. nasalis*. Principato

(1987) made a classification of the major macroscopic lesions of the whole digestive tract (oral, gastric and intestinal) produced by the larvae of the five *Gasterophilus* spp occurring in Southern Europe: *G. intestinalis, G. nasalis, G. pecorum, G. inermis* and *G. haemorrhoidalis*. The pathogenic potential of the larvae varies with the species and depends principally on the route of migration and the localization of the larvae, their morphological characteristics, the sizes of the mouthhooks and the reaction of the gastroenteric mucosa of the host (Principato 1988).

Description of some major lesions

1. Oral cavity lesions (Principato 1988)

   The L1 migration through the tongue epithelium can cause petechial lesions. The superficial burrows look like haemorrhagic impressions.

   Large holes, containing 15-20 larvae, can be found between the upper molars. Gum interdental lesions are represented by large holes found between upper molar teeth. An average of 15-20 larvae per lesion was observed. After emergence of the larvae, internal infection of the upper arcade can occur.

   Glossitis and peridontitis are the major pathologies associated to the two first larval stages of *G. intestinalis*. The subcutaneous migration of G. inermis can induce a condition known as “summer dermatitis of the cheek of horses” (Zumpt 1965).

   Early instars (L1) are generally responsible for masticatory and/or swallowing problems. Problem to accept the bit has been reported (Griss and Simhofer 2006).

2. Oesophageal lesions (Griss and Simhofer 2006)
   Oesophageal obstruction has been diagnosed as a consequence of L2 migration.

3. Gastric and intestinal lesions (Principato 1988)

   G. intestinalis L3 can cause crater-like gastric lesions (Fig.26) and in case of strong infestation levels the gastric mucosa can thicken drastically. The detachment of portions of mucosa provoking sometimes haemorrhages has been observed as well as unusual gastric mucosal reactions inducing atypical pit-like gastric lesions.

Most of the intestinal lesions are associated to *G. nasalis*, *G. haemorrhoidalis* (duodenal) and *G. haemorrhoidalis*, *G. inermis* (rectal) L2 and L3. However, erratic second or third instar *G. intestinalis* can cause small lesions along the whole intestine.

Fig.26. Crater-like lesions (circles) produced by *G. intestinalis* third instar larvae (arrow) in the cardiac region of the stomach of a horse. ©L. Roelfstra.

Gasterophilus spp are implicated in digestive disturbances, including colic, pyloric obstruction and ulcers. Therefore, the general health of infested animals can be affected. Occasionally peritonitis, gastric rupture and perforating ulcers have been observed in some cases (Dart et al. 1987; Cogley and Cogley 1999; Lapointe et al. 2003). However, the degree of ulceration varies with the species and generally, the proliferation of fibrotic tissue below the ulcer prevents larval penetration into the stomach and duodenal wall, as observed in *G. intestinalis* and *G. nasalis*. The tissue proliferation that produces a thicker wall, considered as beneficial for both host and parasite by eliminating the risk of peritonitis, can be seen as a regulatory mechanism in regard to *Gasterophilus* infections (Cogley and Cogley 1999).

2.5.9 Host-parasite interaction

Only little information is available on the *Gasterophilus*-host interaction. The host immune response to the migration and maturation stages of the larvae of the different *Gasterophilus* species is poorly documented although the pathologies associated to these larvae are well characterized. The immunogenic properties of the three larval stages and the characterization of major immunogens have not been reported. However, early studies demonstrated the presence of several digestive enzymes in *G. intestinalis* larvae and tried to clarify the way of nutrition of this myiasis.

2.5.9.1 Larval enzymes

At the beginning of the past century many studies concentrated on the nutrition and digestion capabilities of insects. During that period, Roy (1937) focused on the endoparasitic forms of *G. intestinalis* and demonstrated the presence of an amylase, a proteinase and a lipase in the larvae. Tatchell (1958) made a more detailed study of the digestive enzymes present in this species and found new digestive enzymes in the midgut (maltase, invertase, polypeptidase and di-peptidase); in the salivary glands (amylase, maltase, invertase) and in the haemolymph (lipase and amylase). During his study, Tatchell (1958) also checked for the presence of freshly ingested blood in the larvae and concluded that the L2 and early L3 sometimes had fresh blood in their midgut. But the tissue exudates seem to be the main food of the larvae, thus *G. intestinalis* larvae could only be considered as occasional blood feeders. An interesting finding was the identification of an anticoagulin in the secretion of the salivary glands, confirming the observations made by Dinulescu (1932). Generally it is in the saliva of many blood-sucking insects that anticoagulin can be detected (e.g. *Glossina*, *Rhodnius, Triatoma)* (Wigglesworth 1972). Similar anti-coagulation properties were also noticed with crude midgut preparations although *Gasterophilus* larvae seldom seem to ingest blood. Even if these latter aspects could provide useful information, specifically regarding a potential influence on the host immune response, no further studies have been undertaken.

The presence or absence of microorganisms in the *Gasterophilus* larvae midgut has been an intriguing question. If it seems clear that the larvae do not feed on the stomach content of their host, and consequently do not ingest the extremely rich bacterial flora present in equids stomach, how to explain the marked bactericidal activity found by Vogelsang et al (1955) in the *G. intestinalis* midgut. Recently, microorganisms have been recognized as widespread in insects, and the development of molecular techniques for the study of uncultured bacteria allowed the extensive study of this association between insects and symbiotic bacteria (Gil et al. 2004). The association of *Drosophila* species with endosymbionts is well documented (Ebbert et al. 2003; Harcombe and Hoffmann 2004; Mateos et al. 2006; Montenegro et al. 2006), but the presence of specific symbiotic bacteria has also been described in other insects;

e.g. Diptera, *Tephritinae* (Mazzon et al. 2008); Orthoptera, *Grillinae* (Ulrich et al. 1981). The identification of bacteria in *Gasterophilus* spp and the analysis of their nature and function are of main interest, with regard to their influence on the parasite biology.

2.5.9.2 Monitoring by serodiagnosis

The detection of myiasis-causing larvae inside their host, using serological tests, has been possible for several species: e.g. *Hypoderma* in cattle (Boulard 1975), *Oestrus ovis* or *Lucilia cuprina* in sheep (O'Donnell et al. 1980; Bautista-Garfias et al. 1988).

In previous years, only little work was conducted to improve the immunological diagnosis of gasterophilosis. Viviers et al (1973) were among the first to experimentally study the immune reaction between horse sera and third instar larval extracts of *G. intestinalis* and *G. nasalis*. A variation of serum antibody levels was detected using indirect hemagglutination. Antibodies were present between third and twelfth week post-infestation in case of *G. intestinalis* and between seventh and twelfth week using *G. nasalis* extracts. The complement fixation and the double diffusion techniques did not give satisfactory results (Viviers et al. 1974). However, these results have never been further confirmed.

Immunological techniques, including counterimmunoelectrophoresis, indirect hemagglutination and intradermal injection, using antigen made from larvae of six *Gasterophilus* species, were tested by Ribbeck et al (1984). However, even if the three techniques produced positive results and the intradermal injection induced an immediate reaction, the correlation between the degree of infestation with *Gasterophilus* larvae and the intensity of the immune reaction could not be explained (Ribbeck et al. 1984).

A comparison of five tests for the serological diagnosis of *Gasterophilus* spp in horses and donkeys was made by Escartin-Pena and Bautista-Garfias (1993). Double immunodiffusion (DD), counterimmunoelectrophoresis (CIE), indirect hemagglutination (IH), thin layer immunoassay (TIA) and diffusion-in-gel ELISA (DIG-ELISA) were tested to detect circulating antibodies in horse and donkey sera against crude somatic antigens prepared from third instar larvae of *G. intestinalis* and *G. nasalis*. The results indicate the possibility to detect, with a varying sensitivity between the tests, circulating antibodies to *Gasterophilus* spp in naturally infested horses and donkeys (Escartin-Pena and Bautista-Garfias 1993). The TIA and DIG-ELISA, gave to most promising results with regard to sensitivity, however false positives were detected.

All the mentioned techniques were only tested by using crude larval extracts of third instar larvae of *Gasterophilus* spp. However, no study focused on the first and second instar larvae. Therefore it seems necessary to identify larval stage-specific antigens in order to get precise information about the specificity of the host immune response. Indeed, an important feature in *G. intestinalis* is the probable use of enzymes in tongue burrowing. The secretion of enzymes to penetrate the host tissue has already been demonstrated in several other myiasis-producing flies (Nelson and Weintraub 1972; Aurstad and Skeie 1973). Although enzymes have not yet been associated to first instar larvae of *Gasterophilus* spp, the presence of precise impressions in the burrow immediately surrounding the cephalic portion of larvae should be associated to an enzymatic activity and not to an intensive dug and tore behaviour (Cogley et al. 1982). Similar hypotheses can be made for the second instar larvae that have to migrate from the basement of the host tongue to stomach (Brocard and Pfister 1991).

Additionally, the evaluation of the potential cross-reactivities with antibodies to other parasites present in equids, or even the reactivity of residual antibodies from previous *Gasterophilus* infestations are also of primary concern with regard to the reliability of these methods. Further investigations are thus needed to understand both specific and nonspecific immune responses of hosts to infestations by myiasis-causing larvae and to facilitate the preparation of a reliable serodiagnosis tool.

2.5.10 Genus Gyrostigma (Brauer, 1884)

This genus contains three species (Table 7) whose larvae parasitize the stomach of Asiatic and African rhinoceroses (Zumpt 1965).

Table 7. Summary of *Gyrostigma* species, their hosts and distribution

Species	Hosts	Distribution
G. rhinocerontis (or pavesii)	Black and white rhinoceros	Sub-Saharan Africa
G. conjugens	Black rhinoceros	Sub-Saharan Africa
G. sumatrensis	Asiatic two horned rhinoceros	Sumatra

The Asiatic species, *G. sumatrensis* is only known from the third larval stage recovered from a captive rhinoceros in Germany but the adult flies of both African species have been

described by Zumpt (1962). One of them, *G. conjungens* seem to be exceptionally rare. Most available information comes from *G. rhinocerontis,* the largest fly species known in Africa.
The world rhinoceros population drastically declined during last century (poaching, destruction of their natural habitats) and probably would have been close to extinction without protection campaigns. Although the conservation efforts seem successful, most rhinoceros species are still endangered. The fluctuation of this population obviously affected the *Gyrostigma* species entirely dependent on their host survival (Barraclough 2006).

The complete lifecycle of *Gyrostigma* has not been described. Female deposit their eggs on hosts skin, mainly on the head, neck and shoulders, but what happens until the second and third instar larvae are attached to the hosts' stomach remains unknown. The mature third instars are expulsed with the faeces and pupation occurs in the soil. Adults are huge flies (*G. rhinocerontis* = 24-35 mm) with astonishing red, yellow, orange coloration (Zumpt 1965).

According to the scant documentation it is difficult to describe any disease associated to the presence of the larvae. As no major damages have been observed in the host stomach, the larval impact is considered as benign (Zumpt 1965).

2.5.11 Genus Cobboldia (Brauer, 1887)

Three species are described in this genus (Table 8) and their larvae are parasites in the stomach of Indian or African elephants.
As for *Gyrostigma*, very little information is available about the biology, lifecycle and prevalence of the different *Cobboldia* species.

Table 8. Summary of *Cobboldia* species, their hosts and distribution

Species	Hosts	Distribution
C. elephantis	Indian elephants	India
C. loxodontis	African elephants	Sub-Saharan Africa
C. roveri	African elephants	Central Africa

Imagines of each species have been described as having bright orange colours on their heads and the coloration of the thorax and abdomen varies following the species (Zumpt 1965);

uniform metallic dark blue with deep black legs for *C. loxodontis*; bright metallic green or bluish-violet depending on light and metallic dark blue legs with yellow knees for *C. roveri*; and a shiny black thorax, abdomen and legs for *C. elephantis*.

Females glue their eggs to the base of tusks and the three larval stages are found simultaneously in the host stomach. The larvae are not, unlike *Gasterophilus* or *Gyrostigma*, attached to the stomach wall but they move freely between the wall and the partly digested food. Mature third instar crawl to elephant mouth, exit the host and pupation takes place in the soil. Adults emerge after 2-3 weeks. There seems to be no seasonal restrictions as the different larval stages can be found together and adults can be observed any time (Zumpt 1965).

No pathologies associated with larvae have yet been reported.

2.6 Treatments

The macrocyclic lactones are the most widely used antiparasitics for the treatment of oestrid flies and an efficacy of nearly 100% against the larvae of different species, when administered at recommended doses, has been recorded, e.g. Cuterebrinae (Scholl 2006), Oestrinae (Dorchies et al. 2001; Dorchies et al. 2006; Colwell et al. 2006b).

Currently, the avermectins are used because of their spectrum and excellent efficacy. However, these products are strictly forbidden to treat dairy cattle as they persist for a long period in the milk and tissues. Therefore, the use of micro-doses of ivermectin was recommended (Drummond 1984). More recently eprinomectin, corresponding to the last macrocyclic generation, has been recommended for the treatment of hypodermosis. Indeed this substance is highly efficacious and no residues are accumulated in the milk (Holste et al. 1998; Medjitna et al. 2001).

Ivermectin derived from avermectins (products of the fermentation of *Streptomyces avermitlis*) and moxidectin belonging to the milbemycin group (combination of fermentation of *Streptomyces cyaneogriseus* and chemical synthesis), are the two only macrocyclic lactones available for use in horses. If both product seem to be highly efficient (Lyons et al. 1992; Costa et al. 1998; Scholl et al. 1998; Reinemeyer et al. 2000) some results could not clearly demonstrate the effect of moxidectin against *G. intestinalis* (Xiao et al. 1994; Eysker et al. 1997). Therefore moxidectin might appear less potent than ivermectin against *Gasterophilus* spp.

3 Material & Methods

3.1 Authorization

All procedures were approved by the local veterinary authorities.

3.2 *Gasterophilus intestinalis* collection

The eggs and larvae of *G. intestinalis* were collected in the Swiss Jura (Fig.27). This small region (838 km^2) presents one of the highest densities of equids per km^2 in Switzerland (12.4 equids/km^2), with about 4580 horses distributed in 500 farms. Statistics from OFS, Reflets de l’agriculture. (www.bfs.admin.ch).

The high level of endemicity of *G. intestinalis* in the Jura has been previously described (Brocard and Pfister 1991).

Fig.27. Map of Switzerland. The area of the Swiss Jura is encircled. ©2009 Google Map – Cartographic data © Tele Atlas.

3.2.1 Egg collection

Eggs of *G. intestinalis* were collected from end July till end October on horses living in nine different pastures (Fig.30), all located in the Cantons of Neuchâtel and Jura. Legs of horses and ponies were shaved with a razor blade and hairs with glued eggs were collected in envelopes. Eggs have been conserved 2-3 days at 4°C before being processed.

3.2.2 Larvae collection

G. intestinalis second and third instar larvae were collected in three slaughterhouses located in Courrendlin, Les Bois and Les Breuleux (Fig.30).

3.2.2.1 First instar larvae

First instar larvae (L1) were extracted manually from freshly collected eggs (Fig.30). Eggs were deposited in a Petri dish and the operculum opens after stimulation with a scalpel. L1 larvae were extracted, washed in sterile PBS and stored at -20°C.

3.2.2.2 Second instar larvae

Second instar larvae (L2) were recovered from freshly slaughtered horse stomachs between the end of September and the end of December. The L2 were found moving freely on the surface of the horse stomach mucosa (Fig.28). The collected larvae were immediately washed in sterile PBS and stored at -20°C.

3.2.2.3 Third instar larvae

Third instar larvae (L3) were recovered from the stomach of freshly slaughtered horses, between the end of September and late May. Larvae were recovered from the stomach mucosa to which they were strongly attached. Between September and December, it was not unusual to find simultaneously L2 and L3 (Fig.29). Larvae were immediately washed several times in sterile PBS and stored at -20°C.

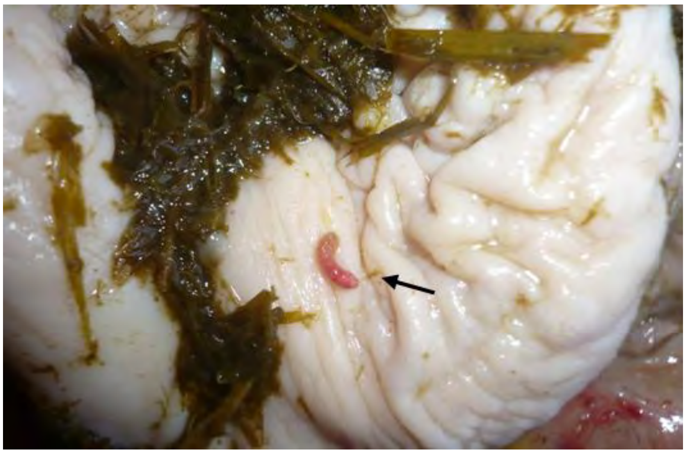


Fig.28. *G. intestinalis* second instar larva (arrow) on the stomach mucosa of a freshly slaughtered horse. ©L. Roelfstra.

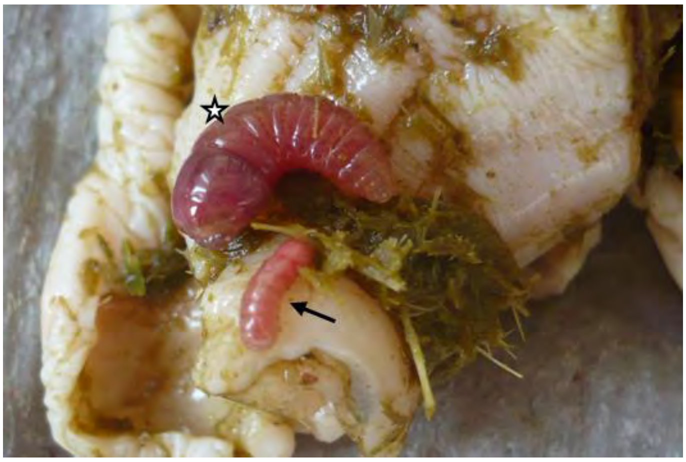


Fig.29. A second instar larva (arrow) and an early third instar larva (star) simultaneously present on a piece of horse stomach freshly slaughtered. ©L. Roelfstra.

3.2.2.4 L2 and L3 collection for microscopy

In case larvae were destined for microscopic analysis, the specimens were not recovered from freshly slaughtered horse stomach, but the entire piece of the stomach bearing the larvae was collected and transported to the laboratory. The piece of stomach was conserved in a wet chamber in an incubator at 37°C. Larvae have to be processed within a few hours for microscopy.

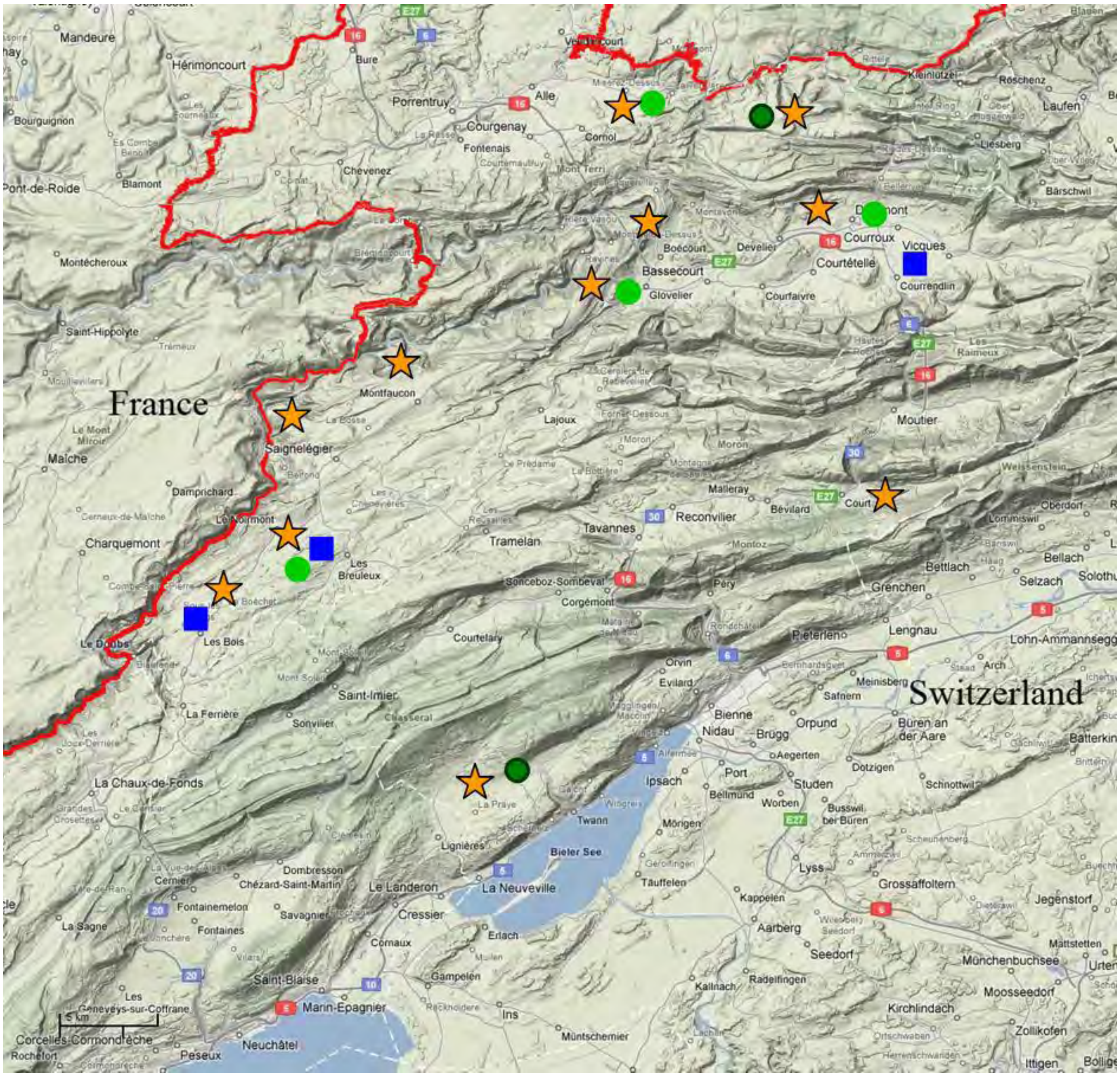


Fig.30. Detailed map of the districts of Neuchâtel and Jura. Site of *G. intestinalis* egg collection (orange stars), location of the slaughterhouses (blue squares) and location of the farms selected for horse blood sampling (green dots). Both light and dark green dots indicate the farms that were selected for the first blood sampling campaign. The dark green dots indicate the location of the two farms selected for the second blood campaign. ©2009 Google Map – Cartographic data © Tele Atlas.

3.3 Horses

3.3.1 Switzerland

Blood samples were collected from horses originating from six different farms located in the Jura Mountains (Fig.30). Two blood sampling campaigns were realized as follows:

- Between November 2003 and May 2004, blood samples were taken monthly from 36 horses originating from the six farms (Fig. 30: light and dark green dots).
- Between July 2005 and August 2006, blood samples were taken monthly from 15 horses originating from two farms (Fig. 30: dark green dots).

The blood samples were centrifuged at 3500 *x g* for 15 minutes at room temperature and the sera were stored at -20°C

3.3.2 France

Blood sample were collected from horses originating from the region of Nantes (Fig.31) by Prof. D. Tainturier, Ecole Nationale Vétérinaire de Nantes. This region is known to be endemic to *Gasterophilus* spp (personal communication).

Fig.31. Map of France. The area of Nantes is encircled. ©2009 Google Map – Cartographic data © Tele Atlas.

3.3.2.1 Blood samples

Blood samples were taken from nine mares and from their respective foals at birth. The foal blood samples were collected before the newborns could uptake the first colostrum.
The umbilical blood was collected from seven mares at the same time.

The blood samples were centrifuged at 3500 *x g* for 15 minutes at room temperature and the sera were stored at -20°C.

3.3.2.2 Collection of colostrum samples

Samples of colostrum were collected from nine mares originating from the region of Nantes (Fig.31) by Prof. D. Tainturier, Ecole Nationale Vétérinaire de Nantes, after foals' birth.

Colostrum was centrifuged at 3500 *x g* for 20 minutes at room temperature. The aqueous phase was collected. This operation was repeated 2 times when the colostrum was sticky. The colostrum serum samples were stored at -20°C.

3.3.3 Greenland

The livestock population of Greenland is very small and an estimation was made in 1998 (Report 1998). The most abundant livestock species are sheep with approximately 22000 individuals. Sheep farming is concentrated in the southwest area of Greenland (Fig.32). Other farm animals are only little represented with approx. 200 poultry and 200 horses. No pigs or goats have been reported.
Semi-domesticated and wild-life species are more important with approx. 2500 Reindeer, approx. 20000 Wild reindeer and approx. 14000 Musk-ox.

Fig.32. Map of South Greenland. ©2009 Google Map – Cartographic data © Tele Atlas.

The blood samples collected by Dr Marner Nolsøe (state veterinarian) came from four horses living in Qaqortoq and Qanisartut (Fig.33). These animals were born and lived exclusively in Greenland.

Fig.33. Area of horse blood collection in Qaqortoq and Qanisartut is encircled. ©2009 Google Map – Cartographic data © Tele Atlas.

Qaqortoq is a small village (Fig.34) where only two horses were reported. Qanisartut is a sheep farmer place and 10 horses live in that village. The horses are used during autumn to gather the sheep from the mountain. During winter time, the horses are stabled and fed mainly hay and silage. They live in close contact with sheep.

There has not been any survey on horse parasites in Greenland, therefore the status is unknown. Studies made on sheep have shown that they are infected with many nematodes (Rose 1990).The ectoparasites diagnosed in sheep were: sheep ked, *Melophagus ovinus*; lice, *Damalinia ovis*, *Linognathus ovillus, Linognathus pedalis* and the sheep scab mite *Psoroptes communis ovis*. Mosquitoes and flies are present but no studies focused on these insects (Dr Marner Nolsøe, personal communication).

Fig.34. Picture of Qaqortoq. Photo: Creative Commons / Jens Buurgaard Nielsen.

3.4 Preparation of *G. intestinalis* crude larval extracts

3.4.1 Preparation of crude larval extracts for ELISA-tests, Western blots and Electrophoresis

Preparation of crude larval extracts adapted from Medjitna (2003).

Larvae were weighted before preparation of the solutions.

Frozen larvae are crushed into powder with a pestle and mortar, under sterile conditions. N_2 is regularly added to prevent powder to become liquid and help the crushing. Extraction buffer is added, approximately 1ml/g of larvae (carbonate buffer (0.1M, pH 9.6) + 1mM phenylmethanesulfonyl fluoride (PMSF, stock 20mM) (Fluka) + 5mM ethylenediaminetetraacetic acid (EDTA, stock 200mM) (Fluka)). Additionnally 10% of Protease Inhibitor Cocktail (Sigma-Aldrich) is added to this solution.

The solution is homogenized overnight at 4°C, under agitation, and aliquots are centrifuged for 20 minutes at 20000 *x g*. The supernatant is collected and the final protein concentration is determined by spectrometry (Bradford method, BioRad). Samples are frozen and stored at -20°C.

3.4.2 Lyophilization

This technique was used to preserve the larval samples during their transport to the Academy of Science of the Czech Republic, in Ceske Budejovice, and to the Institute of Physiology, in Munich, where the chromatography and the proteomic analyses were respectively performed. Usually 24 hours were necessary to lyophilize 10 ml of larval sample. Frozen samples were sublimed directly from the solid phase to gas.

3.4.3 Organ extraction

Different organs were extracted from second and third instar larvae (Fig.35). Larvae were kept attached to host stomach and incubated (37°C) in a wet chamber until dissection. Organs were removed (under binocular), cleaned in sterile PBS (pH 7.4) and stored in small sterile falcon tubes (1 ml).

The extraction of the different organs being very difficult needs to be done carefully. It might be difficult to remove all the fatbody or tracheal cells, highly represented in third instar larvae.

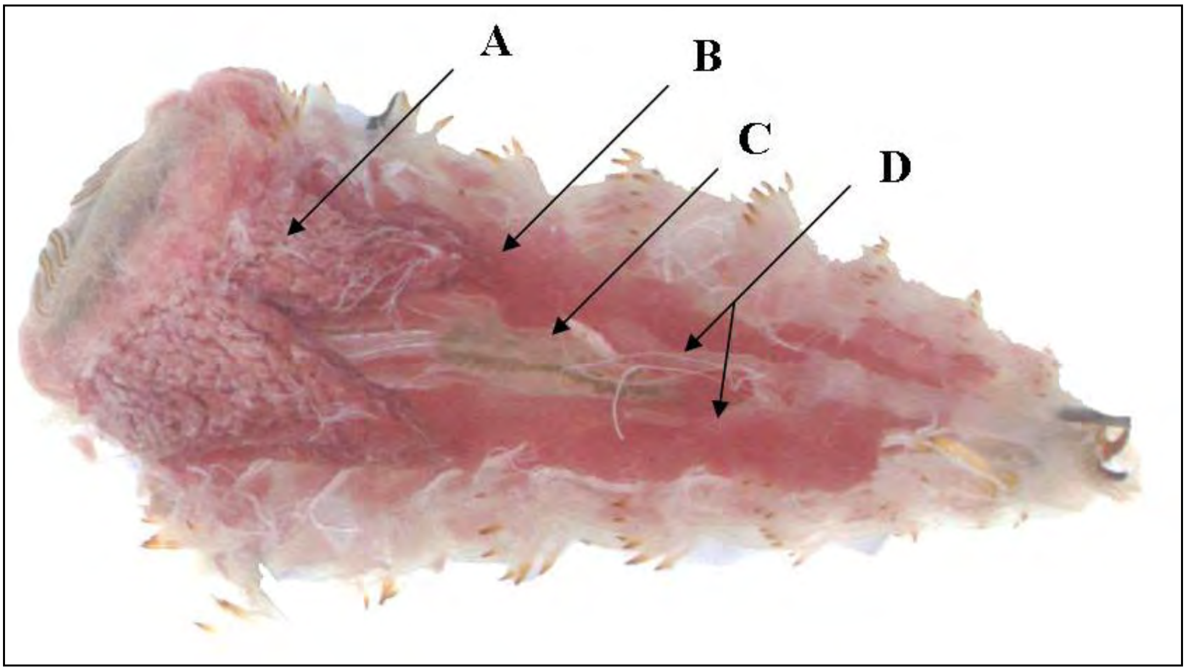


Fig.35. Dissected *G. intestinalis* third instar larva, showing tracheal cells (A), fatbody (B), midgut (C) and salivary glands (D). ©M. Vlimant.

The protocol used to prepare the different larval extracts is similar to the protocol used to prepare the crude larval extracts. Each extract is prepared separately and material has to be cleaned and sterilized between consecutive uses.

The final protein concentration is determined by spectrometry (Bradford method, BioRad).

3.5 Immunisation of mice

Balb/C mice are immunised with crude larval extracts of the three larval stages of *G. intestinalis* (L1, L2, L3) and with a purified recombinant mutant *Gasterophilus* hemoglobin (Hb).

Three different trials were made to generate anti-*Gasterophilus* antibodies in Balb/C mice (Table 9).

Table 9. Summary of the anti-*Gasterophilus* antibody production

Dates (4 immunisations)	Balb/C Mice	Antigen
December 2005 - February 2006	2 2 2 2	L1 L2 L3 Negative control
April - June 2006	8 2	L3 Negative control
January - March 2007	4 2	Hb Negative control

3.5.1 Preparation of crude larval extracts for immunisation

Frozen larvae are crushed into powder (with N_2), under sterile conditions. Powder is weighted and homogenised in PBS (pH 7.4) before centrifugation (20 minutes at 20000 *x g*). The supernatant is collected and final protein concentration is determined by spectrometry (Bradford method, BioRad).

Aliquots of antigen are prepared at a concentration of [200µg/200µl]. An equal volume of Incomplete Freund’s adjuvant (Sigma-Aldrich) was added to each sample before immunisation (200 µl). The samples need to be carefully emulsified with a syringe and each sample was used to immunise two mice.

A dose of 100µg protein/mouse, in a volume of 200 µl, is injected by intramuscular route every 2 weeks, according to the scheme described in Fig.36.

A total of four immunisations were necessary to have optimum anti-*Gasterophilus* antibodies produced by Balb/C mice.

3.5.2 Preparation of the samples with mutant *Gasterophilus* hemoglobin

1 ml of purified recombinant mutant *Gasterophilus* Hb [30µg/µl] was gratefully received from Professor Luc Moens (Department of Biomedical Sciences, University of Antwerp, Belgium).

In this molecule the Cysteine residue is mutated to a Serine.

As for the crude larval extracts, aliquots containing [200µg/200µl] of recombinant Hb in PBS (pH 7.4) are prepared and stored at -20°C.

3.5.3 Anesthetization of mice

Before each manipulation mice must be anaesthetised

Anaesthesia: 0.04 ml of (0.5 ml Ketamine (Ketalar, Parke-Davis) + 0.8 ml Xylazinum (Xylasol, Graeub AG)) was injected intramuscularly into the hind leg.

The average duration of the anaesthesia is approximately 30 minutes.

3.5.4 Scheme of immunisation and blood sampling

Immediately prior to immunisation, samples are emulsified in 200 µl of Incomplete Freund's adjuvant to obtain a final volume of 400 µl which will be used to immunise two mice.
Control mice are inoculated with a combination of PBS (pH 7.4) and Incomplete Freund's adjuvant in equal volumes, according to the same immunisation scheme (Fig.36).

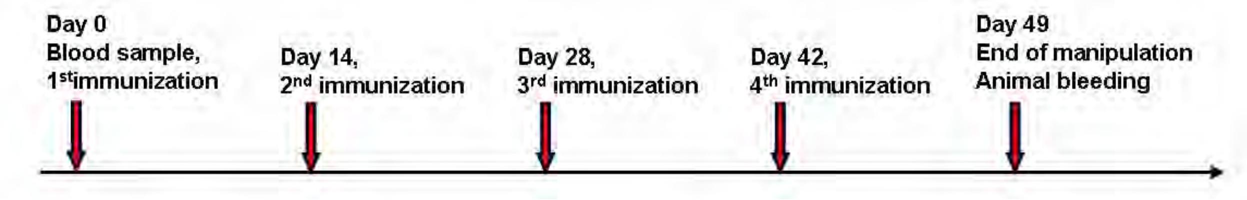


Fig.36. Scheme of immunisation and blood sampling

3.5.5 Collection of blood samples

Blood is collected from the orbital sinus. The retro-orbital blood collection can only be performed by qualified personnel.
On day 0, 100 µl of blood is collected and animals are bled 7-8 weeks after the first immunisation, usually at day 49.
Blood samples are centrifuged at 3500 *x g* for 15 minutes, and sera stored at -20°C.

3.6 Chromatography

Fast performance liquid chromatography (FPLC) (Superose 6 and MonoQ columns) was performed to separate soluble fractions of L3 crude extracts.

3.6.1 Preparation of crude larval extracts for chromatography

Three third-instar larvae were homogenized in a homogenization buffer (0.05 M Tris-HCl, pH 8, containing 10% of Protease Inhibitor Cocktail (Sigma-Aldrich)).

After 15 minutes at 3500 *x g*, the supernatant was collected and the pellet was resuspended in the homogenization buffer containing detergent (1% triton X100, 0.1% SDS, 0.5% deoxycholic acid) to solubilise the membrane proteins and centrifuged 15 minutes at 3500 *x g*

The supernatant was filtered (Ø 45 µm) to remove the lipidic substance as much as possible and centrifuged again for 15 minutes at 13000 *x g*.

The final protein content was determined by spectrometry (Bradford method, Bio-Rad).

3.6.2 Superose 6 column

The FPLC analysis was performed at room temperature, using FPLC System (Pharmacia). The larval protein separation occurs on a Superose 6 HR10/30 column (GE Healthcare). Superose 6 column is ideal to fraction proteins between 10 to 500 KDa. The column was equilibrated with a carbonate buffer (0.1 M Na-carbonate, pH 9.6) and 500 µl of larval solution was injected. Standard flow was 0.5ml/min.

3.6.3 MonoQ column

A MonoQ column (GE Healthcare) has been used to separate more specific larval fraction. The column was equilibrated with: solution A: 25 mM Tris-HCl, pH 7.5, 1 M NaCl and solution B: 1 M NaCl.

3.7 One-dimensional polyacrylamide gel electrophoresis

The crude larval extracts from whole larvae (L1, L2 or L3), and the extracts of different larval organs (midgut, salivary glands, fatbody, tracheal cells, and hemolymph) have been analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (MiniGel: Bio-Rad).

The samples were denatured under reducing conditions, by boiling them for 5 minutes in sample buffer (0.5 M Tris-HCl, pH 6.8, 10 % glycerol, 1 % SDS, 5 % 2-β-mercaptoethanol, 0.01 % bromophenol blue), before SDS-PAGE fractionation.

Depending on the extract and the experiment, different amounts of proteins were loaded per well (always between 5-15 µg/well).

A broad range protein marker was always loaded (5 µl/well) in each gel (Broad range molecular marker, Bio-Rad).

Table 10. Polyacrylamide gels preparation (2 x 1.5 mm gels)

Substance	4.5 % stacking gel	12 % running gel
30 % acrylamide (Chemie Brunschwig)	1.2 ml	9.6 ml
ddH_2O	4.8 ml	8.4 ml
stacking gel buffer (0.5 M Tris-HCl, pH 6.8)	2 ml	-
running gel buffer (1.5 M Tris-HCl, pH 8.8)	-	6 ml
10 % SDS	100 µl	200 µl
10 % Ammoniumpersulfate (APS)	100 µl	100 µl
TEMED	10 µl	10 µl

The gels were run at a constant voltage of 14V/cm until the bromophenol blue entered the running gel after which the voltage was increased to 24V/cm, until the loading buffer reached the end of the gel.

After electrophoresis, gels were stained (Coomassie blue or silver staining) or transferred onto nitrocellulose membrane for immunoblotting (Western-blot).

3.8 Gel staining

3.8.1 Coomassie blue

Adapted from Simpson (2003)

Solutions

Coomassie blue solution:	1 g Coomassie CBR-250, 500 ml MeOH, 100 ml AcOH in 400 ml ddH_2O
Destain solution:	120 ml MeOH, 70 ml AcOH in 810 ml ddH_2O

After completion of electrophoresis

Place the gels in the staining solution and agitate for 5-20 minutes at room temperature

Discard used coomassie solution

Add destain solution and agitate until a suitable background is achieved

Storage

Gels can be stored in a solution containing 5 % AcOH (=5 ml AcOH + 95 ml ddH_2O)

3.8.2 Silver staining

Adapted from Blum et al. (1987)

Solutions

Solution A:	50 % MeOH, 10% AcOH in ddH_2O to 100 ml.
Solution B:	5 % MeOH in ddH_2O to 100 ml.
Solution C:	0.02 % $Na_2S_2O_3$ in ddH_2O to 1 litre.
Solution D:	dissolve 200 mg $AgNO_3$ in 100 ml ddH_2O.
Solution E:	Mix 3 g Na_2CO_3 + 50µl HCOH (37%) + 2 ml of solution C in 100 ml ddH_2O.
Solution F:	Dissolve 1.4 g Na_2-EDTA in 100 ml ddH_2O.

After completion of electrophoresis

Fix	Incubate gel 30 minutes in solution A
Wash	Wash 15 minutes in solution B

Rinse	Rinse 3 x 5 minutes in ddH_2O
Pretreat	Incubate 2 minutes in solution C
Rinse	Rinse 3 x 30 seconds in ddH_2O
Impregnate	Incubate 25 minutes in solution D.
Rinse	Rinse 3 x 1 minute in ddH_2O
Develop	Incubate 5-10 minutes in solution E.

When bands or spots appear, stop incubation as coloration continues for a few seconds after removing from solution E

Stop	Incubate 10 minutes in solution F
Wash	Rinse in ddH_2O

Storage

Gels may be stored one week at 4°C in 50% MeOH.

Gels may be prepared for drying as follow:

Wash

Wash

Dry for 1 hour at 80°C between sheets of porous plastic

3.9 Western-blot

Electrophoretically fractioned proteins are transferred onto nitrocellulose membrane (GE Healthcare), using the semi-dry mini blot transfer cell-system (Bio-Rad): 1 hour, 120mA/gel, in a transfer buffer (25mM Tris-HCl, 192mM glycin, 20% MeOH, 0.1% SDS).

After blotting, membranes are stained (reversible) with Ponceau S red solution (0.5 g Ponceau S (Sigma-Aldrich) + 1 ml AcOH into 100 ml ddH_2O). Membranes are rinsed with ddH_2O and incubated overnight in blocking buffer (TBS, pH 7.4, 0.1 % tween-20.5 % milk powder).

After washing the membranes 2 x 10 minutes in washing buffer (TBS, pH 7.4, 0.1 % Tween-20), they are incubated 1 hour in antibody solution diluted in blocking buffer (horse or mice sera diluted 1/100).

The membranes are washed 2 x 10 minutes and 3 x 5 minutes in the washing buffer and are incubated 1 hour with the secondary antibodies (Table 3) diluted in blocking buffer.

Table 11. Secondary antibodies used for Western-blot.

Antibody	Source	Manufacturer	Dilution
Anti-horse	Rabbit IgG (whole molecule), horseradish peroxidase labelled	Sigma-Aldrich	1:1000
Anti-mouse	Goat IgG (H+L), horseradish peroxidase labelled	Nordic Immunology	1:2000

The membranes are washed 2 x 10 minutes and 3 x 5 minutes in washing buffer.
Detection of bound antibodies is carried out by chemiluminescent detection using an ECL detection kit (Amersham). The membranes were incubated for 1 minute in a freshly prepared mixture from reagent 1 and 2 of the ECL detection reagents, followed by exposure to Hyperfilm ECL, according to company's instructions.

The chromogenic reaction with 4-chloro-1-naphtol, or with 3.3-diaminobenzidine (DAB) to visualize the immunocomplexes did not give satisfactory signal in experiments using *G. intestinalis* larval extracts.

3.10 Enzyme-linked immunosorbent assay (ELISA)

The presence of antibodies in horse and mice sera against *G. intestinalis* crude larval extracts (L1, L2 and L3) and larval fractions (L3 salivary glands, midgut, fatbody, tracheal cells, hemolymph, and hemoglobin) were analyzed using an indirect-enzyme-linked immunoassay test.

3.10.1 Experimental procedure

The antigen, the primary and the secondary antibodies varied according to the experiment. However the same procedure, adapted from Boulard (1985), has been applied for each ELISA.

Larval extracts are diluted in carbonate buffer (pH 9.6) at 2.5, 5, 10 µg mL^{-1} and distributed in 96 well plates (Nunc) in volumes of 100 µl. The plates are incubated at 37°C for one hour and then kept at 4°C overnight.

The plates are washed 4 x with PBST (0.01M phosphate, 0.15 M sodium chloride, pH 7.4, 0.1 % Tween-20) and blotted dry. Then 200 µl of blocking solution is added (carbonate buffer, pH 9.6, 0.5 % gelatine) and the plates are incubated at 37°C for half an hour.

The blocking solution is removed and 100 µl sera (horse, mice) diluted 1/100 (eventually 1/200) with PBST-gelatine (0.01 M phosphate, 0.15 M sodium chloride, pH 7.4, 0.1 % Tween-20, 0.5 % gelatine) is added in the wells. The wells at the edge of the plate: e.g. A1 – A12 or H1 – H12 (Fig.37) do not contain the diluted sera, but only PBST-gelatine and serve as control. Plates are incubated 1 hour at 37°C and washed 4 x with PBST.

The secondary antibody is added: 100 µl per well of horseradish peroxidase conjugate rabbit anti-horse IgG (whole molecule) (Sigma-Aldrich) diluted 1:10000 or horseradish peroxidase conjugate goat anti-mouse IgG (H+L) (Nordic Immunology) diluted 1:20000 in PBST-gelatine, for 1 hour at 37°C. Plates are incubated 1 hour at 37°C and washed 4 x with PBST.

Plates are washed 4 x with PBST and 2 times with PBS before addition and incubation at 37°C for 1 hour of 100 µl per well of the ABTS Peroxidase Substrate System (KPL). Colour reaction is stopped after 1 hour by adding 100 µl of sodium dodecyl sulfate 1% (SDS).

The optical density (OD) of every reaction is recorded at 405nm using ELISA reader (Synergy HT, Biotech).

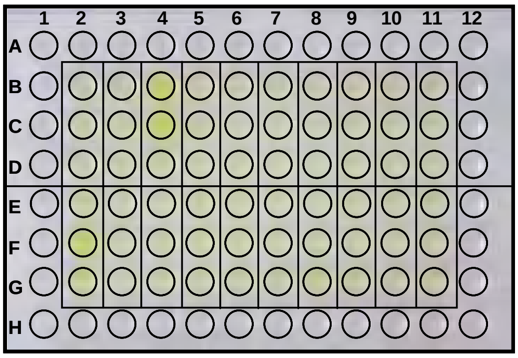


Fig.37. ELISA plate

3.10.2 Comments:

Many trials were necessary to establish an appropriate protocol to demonstrate an immune reaction between horses and *G. intestinalis* larvae, using ELISA tests, and the following points are important:

1. Microtiter plates from different companies showed high variation in adsorption values when tests were realised under similar conditions.
2. Besides the quality of the plates, the blocking solution plays an important role and it was noticed that the bovine serum albumin (BSA) usually used to block the wells did not give satisfactory results, therefore only PBS-tween-gelatine was used as blocking solution.

4 Results

4.1 Publications

The results of the analysis of the crude larval extracts of *Gasterophilus intestinalis* second and third instar larvae, the study of the horse immune reaction and the microscopic analysis of *G. intestinalis* salivary glands and midgut are presented as two publications and submitted to peer-reviewed journals.

4.1.1 Publication 1

Protein expression profile of *Gasterophilus intestinalis* larvae causing gastric myiasis and characterization of horse immune reaction

Liselore Roelfstra*[1], Cornelia A Deeg[3], Stefanie M Hauck[2,3], Christina Buse[3],
Mathieu Membrez[4], Bruno Betschart[1] and Kurt Pfister[4,1]

Parasites and Vectors

Submitted: October 27th 2008
Accepted for publication: January 8th 2009

[1] Institute of Biology, University of Neuchâtel, Switzerland
[2] Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Neuherberg, Germany
[3] Institute of Animal Physiology, Ludwig Maximillians University, Munich, Germany
[4] Institute of Tropical Medicine and Parasitology, Ludwig Maximillians University, Munich
* Corresponding author

PDF File available from:
http://www.parasitesandvectors.com/content/2/1/6

Protein expression profile of *Gasterophilus intestinalis* larvae causing horse gastric myiasis and characterization of horse immune reaction

Liselore Roelfstra[1§], Cornelia A. Deeg[3], Stefanie M. Hauck[2,3], Christina Buse[3], Mathieu Membrez[4], Bruno Betschart[1], Kurt Pfister[4,1]

[1] Institute of Biology, University of Neuchâtel, Switzerland

[2] Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Neuherberg, Germany

[3] Institute of Animal Physiology, Ludwig Maximillians University, Munich, Germany

[4] Institute of Tropical Medicine and Parasitology, Ludwig Maximillians University, Munich, Germany

[§]Corresponding author

Abstract

Background

Little information is available on the immunological aspect of parasitic *Gasterophilus intestinalis* (Diptera, Oestridae) larvae causing horse gastro-intestinal myiasis. The objectives of this research were to analyze the protein content of larval crude extracts of the migrating second and third larvae (L2 and L3) of *G. intestinalis* in order to characterize the immune response of horses.

Results

The proteomic profile of L2 and L3, investigated by using dimensional approaches, revealed a migration pattern specific to each larval stage. Furthermore, Western blots were performed with horse sera and with sera of Balb/c mice immunised with the larval crude extracts of L2 or L3, revealing a different immune reaction in naturally infected horses *vs.* artificially induced immune reaction in mice. The comparisons of the immunoblot profiles demonstrate that the stage L2 is more immunogenic than the stage L3 most likely as an effect of the highest enzymatic production of L2 while migrating into the host tissues. Fifteen proteins were identified by mass spectrometry.

Conclusions

This work provides further information into the understanding of the interaction between *G. intestinalis* and their host and by contributing novel scheme of the proteomic profile of the main larval stages.

Background

Nine species of *Gasterophilus* (Diptera, Oestridae) flies have been described causing, at larval stage, gastrointestinal myiasis in equids. While *Gasterophilus intestinalis* (De Geer, 1776) and *Gasterophilus nasalis* (Linnaeus, 1758) are distributed worldwide and are often the only species reported in many parts of the New World, the remaining species are only reported in very limited areas of Europe, Eastern Countries (Zumpt 1965) and Africa (Horak et al. 1984). Adult bot flies deposit their eggs on the hosts hair at different locations depending on the species of *Gasterophilus* (Soulsby 1982). *G. pecorum* is an exception as females lay their eggs on grasses, leaves and stems of plants (Zumpt 1965). Infection occurs when eggs are introduced into horse mouth by animal licking and grooming. The first larval stage (L1) hatches, starts migrating and moulting into second larval stage (L2) in the oral cavity (Cogley and Cogley 1999). Larvae of different species of *Gasterophilus* are specifically present in one or more regions of the gastrointestinal tract where third larval stage (L3) remains attached to the mucosa for about 8-10 months (Otranto et al. 2005). The clinical signs associated with the migration and maturation stages of the larvae are difficult to diagnose, but it has been shown that different species of *Gasterophilus* can cause severe damages during their lifecycle (Shefstad 1978; Cogley et al. 1982; Principato and Tosti 1988; Cogley 1989).

In the past years studies concerning the immunology and immunopathology of many oestrid myiasis causing larvae have increased because of their important implications in diagnostics and in immunisation programmes (Otranto 2001). While immunological studies were mainly focused on *Hypoderma* cattle grub infection (Boldbaatar et al. 2001), and sheep nasal oestrosis by *Oestrus ovis* (Alcaide et al. 2005), the immunology of *Gasterophilus* spp. caused myiasis received little attention. This is also due to the inherent difficulties in studying immunological host-parasite interactions at the gastrointestinal mucosa interface. As a consequence, so far no major immunogens have been reported (Colwell 2006d). A single study discussed the development of antibodies for the diagnosis of myiasis by *G. intestinalis* larvae although the specificity of immune reaction was not tested in the occurrence of concomitant horse parasitic infection (Escartin-Pena and Bautista-Garfias 1993). More recently, many proteomics-based analyses, combined with two-dimensional gel-electrophoresis, have offered a comprehensive approach to better understand biological and immunological processes of pathogens and diseases (Jungblut et al. 1999; Dea-Ayuela and Bolas-Fernandez 2005; Shin et al. 2005). The aim of this study was to characterize L2 and L3 proteins of *G. intestinalis* and to analyze the immune response of horses and immunized mice against larval antigens.

Results

1-D analysis of the larval crude extract (LCE) of L2 and L3

Migration of the LCE2 on the 1-D silver-stained gel showed a specific pattern (fig. 1A) with 14 bands that were isolated from the gel for further identification by mass spectrometry (MS) (Table 1). The selection of the bands was based upon the intensity of the band on the silver stained gel, as well as the immuno-reactivity observed after immunoblotting with horse serum (fig. 1B) or L2-mice serum (fig. 1C). Three proteins in 4 out of the 14 selected bands gave a significant score ($p<0.05$) and were identified as actin (fig. 1A, band 8), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (fig. 1A, band 9) and hemoglobin (fig. 1A, bands 13 and 14). A different migration pattern was observed for the LCE3 on the 1-D silver-stained gel (fig. 1D). Analogously, immunoblots were performed with horse sera (fig. 1E) and sera of L3-mice (fig. 1F). The selection of 13 bands, indicated with arrows (fig. 1D), was based upon the same criteria as above. They were isolated for further identification by MS (Table 2). Ten proteins out of the 13 selected bands gave a significant score ($p<0.05$) and were identified as the alpha chain of larval serum protein (fig. 1D, bands 1-4), arylphorin (fig. 1D, band 6), beta chain of larval serum protein (fig. 1D, band 7), hemoglobin (fig. 1D, bands 10-12) and murein lipoprotein (fig. 1D, band 13).

2-D analysis of the LCE of L2

The protein migration of the LCE of L2 on a 2-D gel (fig. 2A) showed the presence of proteins all along the pH spectrum. Western-blots were performed with L2-mice serum (fig. 2B) and with horse serum (fig. 2C). The silver stained gel was used to align detected protein spots on both immunoblot profiles. A larger range of immuno-reactive proteins was observed on the horse immunoblot profile than on the L2-mice immunoblot. Circles indicate 12 spots that were considered as immuno-reactive with L2-mice serum (fig. 2A, spots: 1, 2, 6, 8, 14-20, 25) and arrows indicate 24 spots that were considered as immune reactive with horse serum (fig. 2A, spots: 1-13 and 16-26). A total of 26 spots were selected for MS analysis (Table 3). Among the 26 selected spots, 7 proteins were successfully identified ($p<0.05$) by MS: paramyosin (fig. 2A, spot 1), serum albumin (fig. 2A, spot 5), tubulin (fig. 2A, spot 9), enolase (fig. 2A, spot 11), tropomyosin (fig. 2A, spot 14), GAPDH (fig. 2A, spot 19) and hemoglobin (fig. 2A, spot 20).

2-D analysis of the LCE of L3

The proteomic profile of the LCE of L3 presented on figure 3A shows that most of the proteins are located in a basidic range between pH 7-11. Western-blots were performed with L3-mice serum (fig. 3B) and with horse serum (fig. 3C). The intensity of the immune reaction differs when the LCE of L3 is exposed with L3-mice serum or with horse serum but the immunoblot profiles were similar. After comparison of the immunoblots with the silver-stained gel, 39 spots, indicated by circles were selected for further MS identification (Table 4). 19 out of the 39 isolated spots were successfully identified ($p<0.05$), corresponding to 8 different proteins: filamin (fig. 3A, spot 1), heat shock protein (HSP-70) (fig. 3A, spot 2), serum albumin precursor (fig. 3A, spot 3,18-20), phosphoenolpyruvate carboxykinase (PEPCK) (fig. 3A, spot 8), enolase (fig. 3A, spot 22,23), fumarase (fig. 3A, spot 1), beta-actin (fig. 3A, spot 27), hemoglobin (fig. 3A, spot 32-39).

Discussion

The comparison of the migration patterns of the LCE2 and LCE3 in both dimensional analysis (1-D and 2-D), indicates that the larval proteinic profile is stage specific, thus suggesting a different composition in larvae metabolism and antigenic properties. The 1-D silver stained gels of the larval crude extracts indicated a very dense concentration of proteins; consequently the alignment of the immuno-reactive bands detected by horse and mice might present some differences. The proteomic approach confirmed the specific protein profile of both larval stages and allowed better identification of the different spots.

Mice were artificially immunised against a crude proteins extract from whole larvae. A large number of proteins that are normally not directly in contact with horses were presented to the immune system of the mice. Furthermore, unlike natural infection eliciting a horse immune reaction, subcutaneous injection in mice induces a systemic immune reaction shifted to a Th1 response by the adjuvant. This difference in immune reaction explains the fact that most of the L2 and L3 proteins that reacted with mice sera showed a more intense signal when compared to the reaction with horse sera. Since the lifecycle of *G. intestinalis* occurs in the gastro-intestinal tract of horses, the mucosal immune system is in contact with the larvae and thus exposed to excreted or secreted substances during larval migration and development (Baron and Colwell 1991). Although L2 migration patterns from mouth to stomach remains unclear, the immune reaction detected in horses and in experimentally immunized mice might suggest that the larval stage L2 possesses more antigenic proteins than the larval stage L3, probably useful for larval enzymatic migration (Brocard and Pfister 1991). Accordingly, enolase was identified by MS and has previously been reported as an important enzyme localized on the surface of several pathogens when invading tissue (Bernal et al. 2004). L2 is a stage inducing a strong host immune response so that its development into L3 in the stomach might be a defence mechanism of the larvae to bypass the horse immune defences. Conversely, the fact that L3 remains attached for 8-10 months to the stomach wall suggest a hypometabolic status, or reduced immunogenic properties. This can explain the weak immune reaction observed in the presence of horse serum against the L3.

Two important larval proteins, arylphorin and LSP-2 (larval serum protein), respectively homologous to *Calliphora vicina* and *Drosophila melanogaster,* were identified in the L3. In holometabolous insects the construction of adult tissues during metamorphosis requires a large amount of energy. It is known that before formation of the puparium, the fat body cells reabsorb proteins and other macromolecules that have accumulated in the haemolymph during the larval feeding period (Hansen et al. 2002). The major fraction of incorporated proteins consists of arylphorins and LSP-2 (Telfer and Kunkel 1991). In addition, hemoglobin was identified in both larval stages of *G. intestinalis.* This abundant and circulating molecule is present in highly tracheated cells forming the posterior spiracular plate and allows the larvae to make better use of intermittent contact when air is swallowed with food (Dewilde et al. 1998).

Most of the other identified proteins in L2 (paramyosin, tubulin, tropomyosin, GAPDH and a protein similar to actin) or in L3 (filamin, fumarase, PEPCK, HSP-70, enolase) are shared with those of *Drosophila* spp. suggesting that structural or metabolic homologies do exist between these species.

During this research, different intestinal parasites (*Anoplocephala perfoliata*, *Parascaris equorum*, Cyathostominae) were simultaneously present with *G. intestinalis* in the gastro-intestinal tract of the slaughtered horses. The risk of cross-reactivity has still to be evaluated.

But unlike some immunological studies about intestinal helminths in equids (Hoglund et al. 1995; Proudman and Trees 1996; Dowdall et al. 2004), the cross-reactivity between *G. intestinalis* and gastro-intestinal parasites has not yet been studied.

This work provides further information into the understanding of the interaction between *G. intestinalis* and their host and by contributing novel scheme of the proteomic profile of the main larval stages. Thus our results further demonstrate the complexity of this host-parasite interaction. Indeed, this study reveals the necessity to develop a reliable serological tool to detect infested horses, particularly because the only means to detect a *G. intestinalis* infestation is by necropsy.

The identification of most of the proteins will be the next step to define their role, their cellular or tissue localisation and their potential antigenic properties.

Methods

Larval collection and antigen preparation

Gasterophilus spp. L2 and L3 were collected from the pyloric portion of the stomach of horses originating from two farms located in the District of the Swiss Jura; Delémont: N 47°21’; E 7°20’, Switzerland. Simultaneously, the gastro-intestinal tract of each animal was examined and the presence of *Parascaris equorum*, *Anoplocephala perfoliata* and Cyathostominae was observed in any case.

All the larvae collected were washed in a sterile phosphate saline buffer (PBS 0.1M, pH 7.2), identified as *G. intestinalis* on the basis of morphological keys (Zumpt 1965) and frozen at -20°C.

For preparation of the larval crude extracts, 10 L2 larvae harvested on two different horses of a same herd, and seven L3 larvae harvested on one horse originating from the second herd, were sonicated and homogenised on ice under sterile conditions. The homogenate was extracted overnight in a 0.1M pH 9.6 carbonate buffer containing 1mM phenylmethylsulfonyl fluorid (PMSF) and 5mM ethylenediamin tetraacetic acid (EDTA) by further adding 1ml/gr of a protease inhibitor (Sigma-Aldrich, Buchs, Switzerland). The extracts were centrifuged at 20’000 *x g* for 30 minutes (4°C). The supernatant containing the antigens was collected and the final protein content determined by spectrometry (Bradford method, BioRad). LCE was lyophilized and stored at -20°C.

Horse serum samples

Blood samples were taken on a group of twenty horses originating from the two farms in the above described area. Although blood samples and larval collection could not be made on the same animals our observations made during a simultaneously performed epidemiological survey (Roelfstra et al, in prep.), including field observations on live animals and in slaughterhouses, confirmed the high level of endemicity of *G. intestinalis* previously described by Brocard (Brocard and Pfister 1991) in the same area and allow us to presume that all horses used in this study are infested by *G. intestinalis*. Foetal horse serum was used as a negative control for the Western blots. The blood was centrifuged at 3500 *x g* for 15 minutes at room temperature and the sera were stored at -20°C.

Immunisation of mice and serum samples

Balb/c mice were immunised with LCE of L2 (L2 mice) or with LCE of L3 (L3 mice) from *G. intestinalis.* Two grams of L3 and two grams of L2 were sonicated, homogenised in a sterile PBS buffer pH 7.2 and centrifuged at 20’000 *x g*. The supernatant was then emulsified in equal volume of Freund’s Incomplete Adjuvant (Sigma-Aldrich, Buchs, Switzerland).

A dose of 100µg protein/mouse, in a volume of 200 µl, was injected by intramuscular route every 2 weeks through 6 weeks. Animals were bled seven weeks after the first immunisation. Control mice were inoculated with a combination of PBS and Freund’s Incomplete Adjuvant every time above. Sampled blood was centrifuged at 3500 *x g* for 15 minutes at room temperature and sera stored at -20°C. All procedures were approved by the cantonal commission on animal experimentations.

One dimensional electrophoresis (1-D)

The proteins of L2 (5 µg/well) and L3 (5 µg/well) were separated on gradient SDS-PAGE gels (4-20%) under reducing conditions (2-β-mercaptoethanol, 95°C for 5 min). Electrophoresis was performed at 80/100 V for 30 min/2hrs. One set of the gels was stained with silver for mass spectrometry, and the second was transferred onto nitrocellulose membranes (GE Healthcare, Uppsala, Sweden) for Western blot analysis.

Two dimensional electrophoresis (2-D)

Lyophilized LCE samples were solubilised in 2-DE lysis buffer (9 M urea, 2 M thiourea, 1% dithioerythriol, 4% CHAPS, 2.5 µM EGTA, and 2.5 µM EDTA). Immobiline dry strips pH 3-11 non linear, 11 cm (GE Healthcare, Uppsala, Sweden) were immersed overnight in lysis buffer containing 75 µg protein sample, additional 1% Pharmalyte pH 3-10 (GE Healthcare, Uppsala, Sweden), and 0.5% bromphenol blue. IEF on a Multiphor (GE Healthcare) for 15 kV·h at 20°C was followed by separation on gradient SDS-PAGE gels (9-15%) at constant 45 V per gel. One set of gels was stained with silver for MS and the second was transferred onto nitrocellulose membranes (GE Healthcare, Uppsala, Sweden) for Western blot analysis.

Western blot analysis (WB)

Non-specific binding was blocked with 1% polyvinylpyrrolidone in PBS-Tween for 1 hour. Blots were subsequently incubated with primary antibody in PBS-Tween (overnight at 4°C; horse sera or mice sera 1:1000) and washed. The immunoreactive spots were detected using a goat anti-mouse IgG (H+L) (1:20000, Nordic Immunology Laboratories, Tilburg, The

Netherlands) or an anti-horse IgG (1:10000, Sigma-Aldrich, Buchs, Switzerland) antibody conjugated with horseradish peroxidase. Signals were detected with ECL (enhanced chemiluminescence) on Hyperfilm ECL (GE Healthcare, Uppsala, Sweden) (Buse et al. 2008). After ECL detection, the blots were subsequently stained with colloidal gold in order to match the visible spots to overall pattern on silver-stained gels.

Mass spectrometry (MS)

Selected spots were excised from 2-D gels, destained, processed by proteolysis with trypsin (Deeg et al. 2006) and analyzed by MALDI-TOF and MS/MS on a MALDI-TOF/TOF tandem mass spectrometer (ABI 4700 Proteomics Analyzer, Applied Biosystems). Combined PMF (peptide mass fingerprint) and MS/MS queries were done with MASCOT$^{©}$ Database search engine v1.9 (Perkins et al. 1999) (Matrix Science) embedded into GPS-Explorer Software (version 3.6, Applied Biosystem) on the Swiss-Prot database (version 20051206; 201594 sequences; 73123101 residues) or MSDB (version 20040703; 1501893 sequences; 480537664 residues). Protein identification was considered positive (Tables 1, 2 3 and 4) if (i) the probability-based MOWSE score (Pappin 1997) obtained from both MS and MS/MS analysis was significant (i.e. scores >66 were significant at $p<0.05$ for Expasy database, and scores >74 significant at $p<0.05$ for MSDB database; confidence interval >99% as given by GPS explorer, version 3.6); (ii) the matched peptide masses were abundant in the spectrum; and (iii) the theoretical molecular weights (MW) of the significant hits fit the experimental observed values.

Competing interests

The authors declare that they have no competing interests

Authors' contributions

LR and KP conceived and designed the study. LR carried out the acquisition of the material and the morphological data as well as the immunoassays and drafted the manuscript. KP supervised the work and helped to write the manuscript. CD outlined and supervised all the proteomic features and helped to draft the manuscript. SMH carried out the mass spectrometry and analysed the data. CB has supported LR in all the laboratory analysis. MM conceived and carried out the immunological study with mice. BB participated in the design and coordination of the study and helped to analyse the results. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by SFB 571 grant A5 Deeg. The authors are grateful to Prof. D. Otranto and Prof. D.D. Colwell for their critical comments.

References

1. **Zumpt F: *Myasis in man and animals in the old world.* Butterworths, London, United Kingdom; 1965.**
2. **Horak IG, De Vos V, De Klerk BD: Parasites of domestic and wild animals in South Africa. XVII. Arthropod parasites of Burchell's zebra, Equus burchelli, in the eastern Transvaal Lowveld. *Onderstepoort J Vet Res* 1984, 51:145-154.**
3. **Soulsby EJL: *Helminths, arthropods and protozoa of domestical animals.* 7th ed. edn. Eastbourne, United Kingdom: Baillière, Tindall & Cassell; 1982.**
4. **Cogley TP, Cogley MC: Inter-relationship between Gasterophilus larvae and the horse's gastric and duodenal wall with special reference to penetration. *Vet Parasitol* 1999, 86:127-142.**
5. **Otranto D, Milillo P, Capelli G, Colwell DD: Species composition of Gasterophilus spp. (Diptera, Oestridae) causing equine gastric myiasis in southern Italy: parasite biodiversity and risks for extinction. *Vet Parasitol* 2005, 133:111-118.**
6. **Cogley TP: Effects of migrating Gasterophilus intestinalis larvae (Diptera: Gasterophilidae) on the mouth of the horse. *Vet Parasitol* 1989, 31:317-331.**

7. **Cogley TP, Anderson JR, Cogley LJ: Migration of Gasterophilus intestinalis larvae (Diptera:Gasterophilidae) in the equine oral cavity. *Int J Parasitol* 1982, 12:473-480.**
8. **Principato M, Tosti M: Scanning electron microscope observations on the anterior thoracic and post-abdominal spiracles of Gasterophilus larvae (Diptera: Gasterophilidae). *Int J Parasitol* 1988, 18:191-196.**
9. **Shefstad DK: Scanning electron microscopy of Gasterophilus intestinalis lesions of the equine stomach. *J Am Vet Med Assoc* 1978, 172:310-313.**
10. **Otranto D: The immunology of myiasis: parasite survival and host defense strategies. *Trends Parasitol* 2001, 17:176-182.**
11. **Boldbaatar D, Xuan X, Kimbita E, Huang X, Igarashi I, Byambaa B, Battsetseg B, Battur B, Battsetseg G, Batsukh Z, et al: Detection of antibodies to Hypoderma lineatum in cattle by Western blotting with recombinant hypodermin C antigen. *Vet Parasitol* 2001, 99:147-154.**
12. **Alcaide M, Reina D, Frontera E, Navarrete I: Analysis of larval antigens of Oestrus ovis for the diagnosis of oestrosis by enzyme-linked immunosorbent assay. *Med Vet Entomol* 2005, 19:151-157.**
13. **Colwell DD: Part C: Gasterophilinae Host-Parasite Interactions. In *The Oestrid Flies Biology, Host-parasite relationships, impact and management.* Edited by D.D. Colwell, Hall M.J.R., Scholl P.J. Oxfordshire, United Kingdom: CABI Publishing; 2006: 189-191**
14. **Escartin-Pena M, Bautista-Garfias CR: Comparison of five tests for the serologic diagnosis of myiasis by Gasterophilus spp. larvae (Diptera: Gasterophilidae) in horses and donkeys: a preliminary study. *Med Vet Entomol* 1993, 7:233-237.**
15. **Dea-Ayuela MA, Bolas-Fernandez F: Two-dimensional electrophoresis and mass spectrometry for the identification of species-specific Trichinella antigens. *Vet Parasitol* 2005, 132:43-49.**
16. **Jungblut PR, Zimny-Arndt U, Zeindl-Eberhart E, Stulik J, Koupilova K, Pleissner KP, Otto A, Muller EC, Sokolowska-Kohler W, Grabher G, Stoffler G: Proteomics in human disease: cancer, heart and infectious diseases. *Electrophoresis* 1999, 20:2100-2110.**
17. **Shin YS, Shin GW, Kim YR, Lee EY, Yang HH, Palaksha KJ, Youn HJ, Kim JH, Kim DY, Marsh AE, et al: Comparison of proteome and antigenic proteome between two Neospora caninum isolates. *Vet Parasitol* 2005, 134:41-52.**
18. **Baron RW, Colwell DD: Mammalian immune responses to myiasis. *Parasitol Today* 1991, 7:353-355.**
19. **Brocard P, Pfister K: [The epidemiology of gasterophilosis of horses in Switzerland]. *Schweiz Arch Tierheilkd* 1991, 133:409-416.**
20. **Bernal D, de la Rubia JE, Carrasco-Abad AM, Toledo R, Mas-Coma S, Marcilla A: Identification of enolase as a plasminogen-binding protein in excretory-secretory products of Fasciola hepatica. *FEBS Lett* 2004, 563:203-206.**
21. **Hansen IA, Meyer SR, Schafer I, Scheller K: Interaction of the anterior fat body protein with the hexamerin receptor in the blowfly Calliphora vicina. *Eur J Biochem* 2002, 269:954-960.**
22. **Telfer WH, Kunkel JG: The function and evolution of insect storage hexamers. *Annu Rev Entomol* 1991, 36:205-228.**
23. **Dewilde S, Blaxter M, Van Hauwaert ML, Van Houte K, Pesce A, Griffon N, Kiger L, Marden MC, Vermeire S, Vanfleteren J, et al: Structural, functional, and genetic characterization of Gastrophilus hemoglobin. *J Biol Chem* 1998, 273:32467-32474.**

24. **Dowdall SM, Proudman CJ, Klei TR, Mair T, Matthews JB: Characterisation of IgG(T) serum antibody responses to two larval antigen complexes in horses naturally- or experimentally-infected with cyathostomins. *Int J Parasitol* 2004, 34:101-108.**
25. **Hoglund J, Ljungstrom BL, Nilsson O, Uggla A: Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to Anoplocephala perfoliata in horse sera. *Vet Parasitol* 1995, 59:97-106.**
26. **Proudman CJ, Trees AJ: Use of excretory/secretory antigens for the serodiagnosis of Anoplocephala perfoliata cestodosis. *Vet Parasitol* 1996, 61:239-247.**
27. **Buse C, Altmann F, Amann B, Hauck SM, Poulsen Nautrup C, Ueffing M, Stangassinger M, Deeg CA: Discovering novel targets for autoantibodies in dilated cardiomyopathy. *Electrophoresis* 2008, 29:1325-1332.**
28. **Deeg CA, Pompetzki D, Raith AJ, Hauck SM, Amann B, Suppmann S, Goebel TW, Olazabal U, Gerhards H, Reese S, et al: Identification and functional validation of novel autoantigens in equine uveitis. *Mol Cell Proteomics* 2006, 5:1462-1470.**
29. **Perkins DN, Pappin DJ, Creasy DM, Cottrell JS: Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999, 20:3551-3567.**
30. **Pappin DJ: Peptide mass fingerprinting using MALDI-TOF mass spectrometry. *Methods Mol Biol* 1997, 64:165-173.**

Figure legends

Figure 1 – 1-D analysis of the LCE of L2 and L3

Representative 1-D silver-stained gel of LCE of L2 (A) and L3 (D). The arrows indicate the bands that were selected for mass spectrometry. Western blot analysis of L2 incubated with horse serum (B) and mouse serum (C). Western blot analysis of L3 incubated with horse serum (E) and mouse serum (F). The protein identification by MS is presented in Table 1 and 2.

Figure 2 – 2-D analysis of the LCE of L2

Silver-stained representative 2-D protein map of the LCE of L2 comprising pH gradient from 3 to 11 with the MWs ranking from 10 to 250 KDa (A). The silver stained gel was used to align detected protein spots on the Western blots performed with serum of mice immunised with the LCE of L2 (B) and with horse serum (C). The circles indicate the spots that were immunolabelled with mice sera (B; spots 1,2,6,8,14-19,25,26) and the arrows indicate the spots that were immunolabelled with horse sera (C; spots 1-13 and 16-26). A total of 26 spots were isolated for further MS identification. The protein identification by MS is presented in Table 3.

Figure 3 – 2-D analysis of the LCE of L3

Silver-stained representative 2-D protein map of the LCE of L3 comprising pH gradient from 3 to 11 with the MWs ranking from 10 to 250 KDa (A). The silver stained gel was used to align detected protein spots on the Western blots performed with serum of mice immunised with the LCE of L3 (B) and with horse serum (C). 39 spots immunolabelled with both sera were isolated for further MS identification. The protein identification by MS is presented in Table 4.

Table 1 – Mass spectrometry identification of proteins identified from the LCE of L2.

Spots assignments refer to Fig.1. Proteins listed have been identified with a significant probability score at $p<0.05$ in MSDB.

Band ID	Protein name	Species	Accession number	MW (Da)	p*I*	Protein score
8	Protein similar to Actin-87E isoform 2	*Drosophila melanogaster*	AAM29410	37816	5.36	223
9	Glyceraldehyde-3-phosphate dehydrogenase	*Drosophila hydei*	S24630	35369	8.2	224
13	Hemoglobin	*Gasterophilus intestinalis*	O96457	17912	8.44	440
14	Hemoglobin	*Gasterophilus intestinalis*	O96457	17912	8.44	144

Table 2 – Mass spectrometry identification of proteins identified from the LCE of L3.

Spots assignments refer to Fig.1. Proteins listed have been identified with a significant probability score at $p<0.05$ (1, 2, 3, 4, 6, 7, 13: Expasy database; 10, 11, 12: MSDB).

Band ID	Protein name	Species	Accession number	MW (Da)	p*I*	Protein score
1	Larval serum protein 1 alpha chain precursor	*Drosophila melanogaster*	LSP1A_DROME	98802	5.72	92
2	Larval serum protein 1 alpha chain precursor	*Drosophila melanogaster*	LSP1A_DROME	98802	5.72	89
3	Larval serum protein 1 alpha chain precursor	*Drosophila melanogaster*	LSP1A_DROME	98802	5.72	98
4	Larval serum protein 1 alpha chain precursor	*Drosophila melanogaster*	LSP1A_DROME	98802	5.72	102
6	Arylphorin subunit A4 precursor	*Calliphora vicina*	ARY1_CALVI	92282	5.59	71
7	Larval serum protein 1 beta chain precursor	*Drosophila melanogaster*	LSP1B_DROME	95849	5.41	69
10	Hemoglobin	*Gasterophilus intestinalis*	O96457	17912	8.44	247
11	Hemoglobin	*Gasterophilus intestinalis*	O96457	17912	8.44	132
12	Hemoglobin	*Gasterophilus intestinalis*	LPEBWM	17912	8.44	112
13	Major outer membrane lipoprotein precursor (Murein-lipoprotein)	*Pectobacterium atrosepticum*	LPP_ERWCT	8396	9.36	69

Table 3 – Mass spectrometric identifications of proteins identified from the LCE of L2.

Spots assignments refer to Fig.2. Proteins listed have been identified with a significant probability score at $p<0.05$ in MSDB.

Spot ID	Protein name	Species	Accession number	MW (Da)	p*I*	Protein score
1	Paramyosin	*Drosophila melanogaster*	S22028	102277	5.5	97
5	Serum albumin precursor	*Bos taurus*	ABBOS	69225	5.8	99
9	Tubulin alpha-1 chain	*Drosophila melanogaster*	A26488	49876	5.0	260
11	Enolase	*Oryza sativa*	Q7XBE4	47942	5.4	80
14	Tropomyosin	*Drosophila melanogaster*	C25242	32740	4.7	78
19	Glyceraldehyde-3-phosphate dehydrogenase	*Drosophila hydei*	S24630	35369	8.2	100
20	Hemoglobin	*Gasterophilus intestinalis*	O96457	17912	8.4	209

Table 4 – Mass spectrometric identifications of proteins identified from the LCE of L3. Spots assignments refer to Fig.3. Proteins listed have been identified with a significant probability score at $p<0.05$ (2, 8, 18, 19, 20, 23, 27: Expasy database; 1, 3, 22, 24, 32, 33, 34, 38, 39: MSDB).

Spot ID	Protein name	Species	Accession number	MW (Da)	p*I*	Protein score
1	Filamin 1	*Drosophila melanogaster*	Q8T3K7	151931	5.72	76
2	Heat shock 70 kDa protein 70C	*Drosophila melanogaster*	HSP7A_DROME	70871	5.34	70
3	serum albumin	*Bos taurus*	AAN17824	71274	5.82	171
8	Phosphoenolpyruvate carboxykinase	*Drosophila melanogaster*	PPCK_DROME	71882	6.07	79
18	Serum albumin precursor	*Bos taurus*	ALBU_BOVIN	71244	5.82	108
19	Serum albumin precursor	*Bos taurus*	ALBU_BOVIN	71244	5.82	117
20	Serum albumin precursor	*Bos taurus*	ALBU_BOVIN	71244	5.82	165
22	Enolase (Fragment)	*Drosophila subobscura*	O44101	44548	5.92	142
23	Enolase	*Schistosoma mansoni*	ENO_SCHMA	47421	6.18	163
24	Fumarase	*Drosophila melanogaster*	Q9VTI5	51239	8.47	111
27	Beta-actin	*Danio rerio*	ACTB1_BRARE	42082	5.3	184
32	Hemoglobin	*Gasterophilus intestinalis*	O96457	18026	8.44	95
33	Hemoglobin	*Gasterophilus intestinalis*	O96457	18026	8.44	113
34	Hemoglobin	*Gasterophilus intestinalis*	O96457	18026	8.44	131
38	Hemoglobin	*Gasterophilus intestinalis*	O96457	18026	8.44	415
39	Hemoglobin	*Gasterophilus intestinalis*	O96457	18026	8.44	410

Figure 1

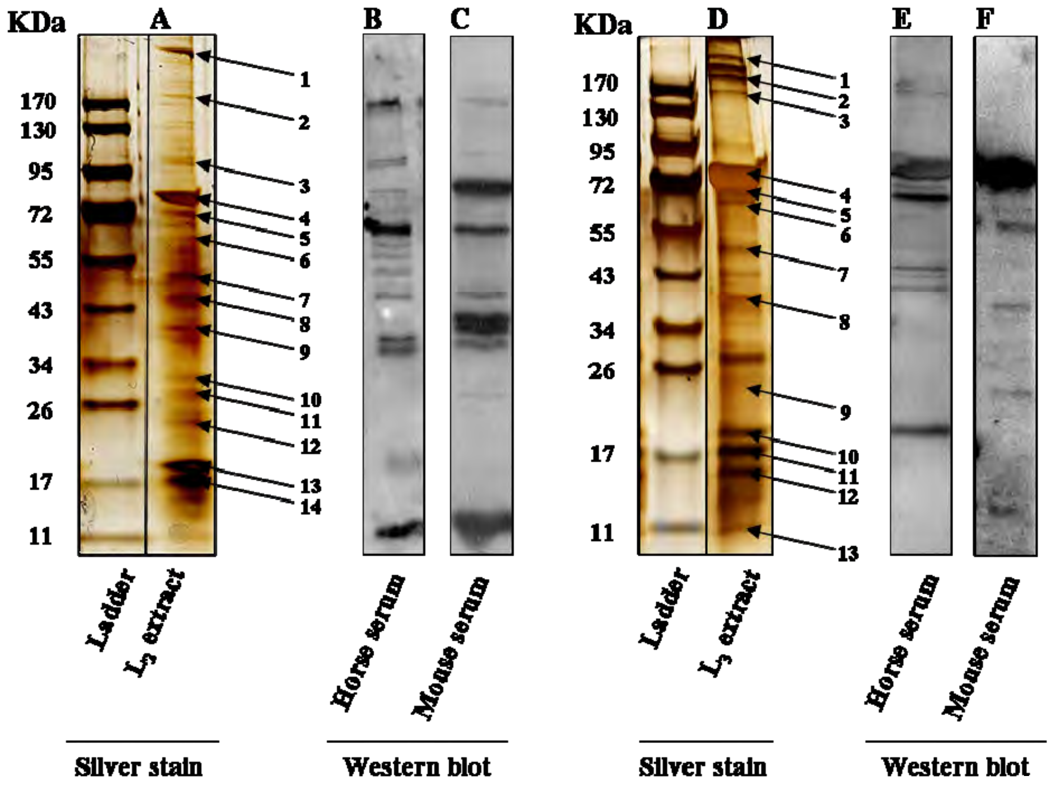

KDa
A
170
130
95
72
55
43
34
26
17
11
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Ladder
L_3 extract
Silver stain
B
C
Horse serum
Mouse serum
Western blot
KDa
D
170
130
95
72
55
43
34
26
17
11
1
2
3
4
5
6
7
8
9
10
11
12
13
Ladder
L_3 extract
Silver stain
E
F
Horse serum
Mouse serum
Western blot

Figure 2

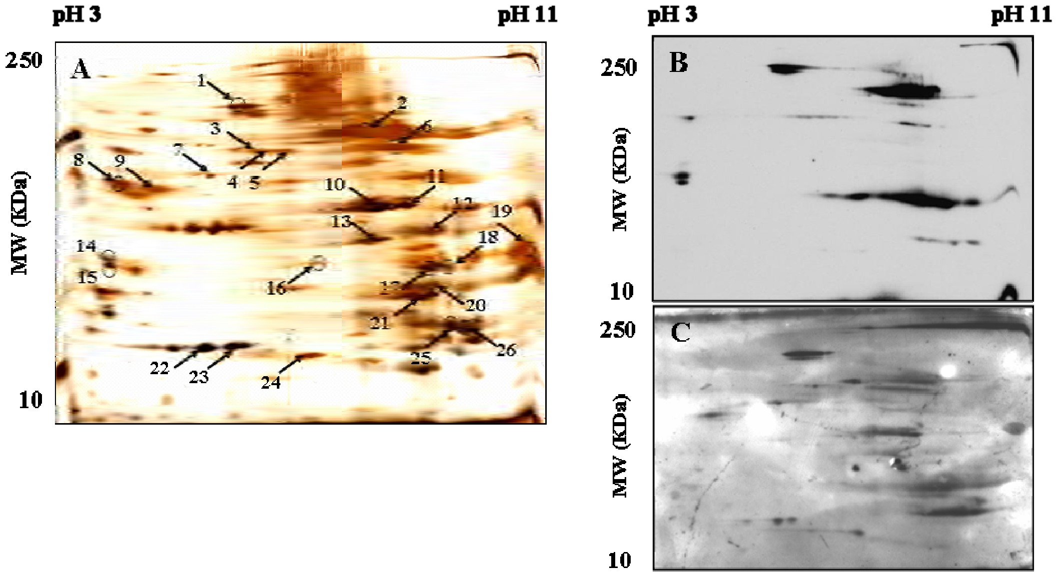
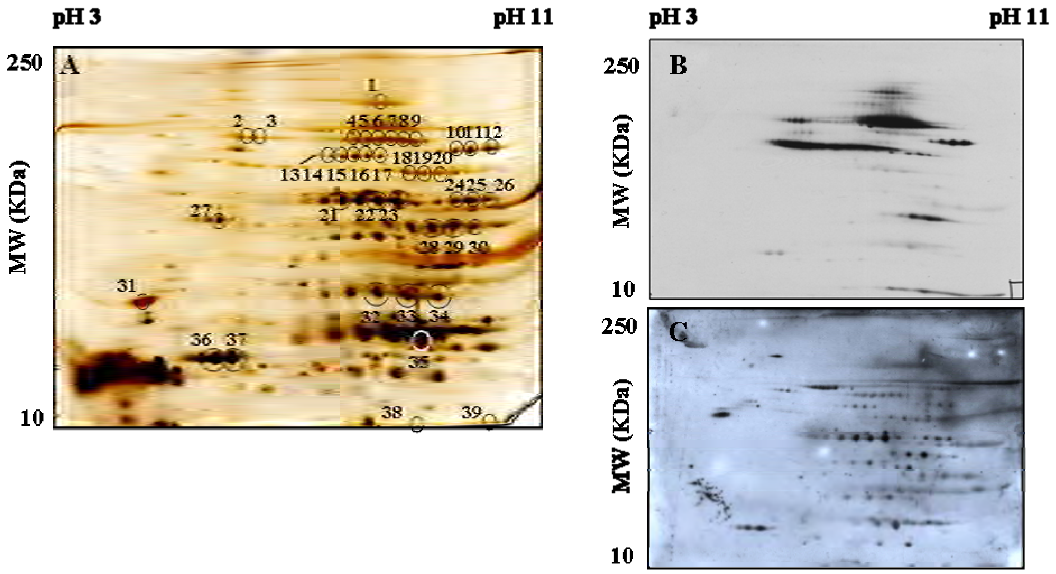

pH 3
pH 11
250
10
MW (KDa)
A
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
pH 3
pH 11
250
10
MW (KDa)
B
250
10
MW (KDa)
C

Figure 3


pH 3
pH 11
250
10
MW (KDa)
A
1
2
3
4 5 6 7 8 9
10 11 12
13 14 15 16 17
18 19 20
21
22 23
24 25 26
27
28 29 30
31
32
33
34
35
36
37
38
39
pH 3
pH 11
250
10
MW (KDa)
B
250
10
MW (KDa)
C

4.1.2 Publication 2

Optical and ultrastructural studies of the midgut and salivary glands of second and third-instar larvae of *Gasterophilus intestinalis*

L. Roelfstra[*1] and M. Vlimant[1], B. Betschart[1], Kurt Pfister[4,1], P.-A. Diehl[1]

Medical and Veterinary Entomology

Submitted: May 7th 2009

[1] Institute of Biology, University of Neuchâtel, Switzerland
[2] Institute of Tropical Medicine and Parasitology, Ludwig Maximilians University, Munich, Germany
[*] Corresponding author

Roelfstra et al.: Midgut and salivary gland structure

Medical and Veterinary Entomology

Liselore Roelfstra
Institute of Biology
University of Neuchâtel
Rue Emile-Argand 11
CH-2000 Neuchâtel
Switzerland
Tel.: +41 32 718 30 00
Fax.: +41 32 718 30 01
E-mail: Liselore.Roelfstra@unine.ch

Optical and ultrastructural studies of the midgut and salivary glands of second and third-instar larvae of *Gasterophilus intestinalis*

L. Roelfstra[*1] and M. Vlimant[1], B. Betschart[1], Kurt Pfister[4,1], P.-A. Diehl[1]

[1] Institute of Biology, University of Neuchâtel, Switzerland

[2] Institute of Tropical Medicine and Parasitology, Ludwig Maximilians University, Munich, Germany

[*] Corresponding author

Abstract

A morphological study of the midgut and salivary glands of second and third instar larvae of *Gasterophilus intestinalis* (DeGeer) (Diptera: Gasterophilidae) was conducted by light, scanning and transmission electron microscopy.

The midgut is anteriorly delimited by a proventriculus, without caeca, composed of posterior foregut and anterior midgut tissue from which a double-layered peritrophic membrane type II is produced. The larval midgut is formed by a monolayer of epithelial cells on a non-cellular basal lamina surrounded by connective tissue containing muscles and tracheae. The midgut can be divided into anterior, median and posterior regions on the basis of the structural and physiological variations of the columnar cells which appear along its length. Two other types of cells were identified; regenerative cells scattered throughout the columnar cells and more rarely, endocrine cells in two various shapes (close-type and open-type). Different secretion mechanisms (merocrine, apocrine and microapocrine) occur along the midgut epithelium. Abundant microorganisms are observed in the lumen of the anterior midgut, moving through the endoperitrophic space. The origin and nature of these microorganisms remain unknown. No structural differences are observed between the second and third instar larvae midgut.

The salivary structure of *G. intestinalis* second and third instar larvae consist of pairs of elongated tubular glands connected to efferent ducts which unite to form a single deferent duct linked dorsally to the pharynx. The salivary ducts comprised a monolayer of epithelial cells, internally lined by a cuticle. Several intermediate cells, without cuticle, make the junction with the salivary gland epithelium layer. The salivary system is surrounded by a thick basal lamina. Cytological characteristics of the glands epithelial cells demonstrate a high cellular activity and some structural variations are noticed between the two larval stages. A tracheolar network penetrates the salivary glands and numerous tracheae insert in the intercellular space.

Key words. Equids, *Gasterophilus intestinalis*, larvae, midgut, salivary glands, peritrophic membrane, microorganisms.

Introduction

Larvae of the horse bot fly *Gasterophilus intestinalis* cause gastrointestinal myiasis in equids throughout the world (Zumpt 1965). Adult bot flies deposit their eggs on the host's hair and parasitic migration begins when eggs are introduced in the oral cavity of the horse. The first stage larvae (L1) hatch and after a first moult, second stage larvae (L2) enter the gastrointestinal tract, migrate to host stomach and undergo another molt. Third stage larvae (L3) remain attached for about 8-10 months to the mucosa of the non-glandular portion of host stomach (Cogley and Cogley 1999). As adult flies do not feed, their nutritional requirements for basic metabolisms, dispersal and reproduction have to be ingested during the obligatory parasitic larval stage (Hall and Wall 1995). The digestive system responsible for all the steps of food processing in the larvae comprises an alimentary canal (gut) and salivary glands.

Scanning electron microscopy has been used to determinate the pathologies associated to the different instars of *Gasterophilus* spp (Diptera: Gasterophilidae) larvae (Shefstad 1978; Cogley 1989) and to analyse the morphological and sensorial properties necessary to the larvae to complete their life cycle inside their host (Principato and Tosti 1988; Cogley 1999; Leite and Scott 1999; Leite et al. 1999; Colwell et al. 2007). However, the description of the structure of the digestive system of *Gasterophilus* species is lacking.

In this paper, we studied the fine structure of the epithelium of the midgut and salivary glands of second and third instar larvae of *G. intestinalis* under light, scanning and transmission electron microscopy. Morphological studies on the midgut and salivary glands may be useful for further investigations about the physiology of the larvae and their interaction with their host.

Materials and Methods

Larval collection

Second and third-instar larvae of *G. intestinalis* were recovered at necropsy of the stomach of three horses originating from the District of the Swiss Jura; Delémont: N 47°21'; E 7°20', Switzerland. All larvae collected were identified on the basis of morphological keys (Zumpt 1965).

Specimen preparation for light and transmission electron microscopy (TEM)

Eight L2 and seven L3 were killed in fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.4) (Karnowsky 1965) and partially dissected. Digestive tube and the salivary glands were left in original posture in larvae's body. The specimens remained overnight in a fresh fixative solution at 4°C. Specimens were washed in 0.1 M cacodylate buffer (pH 7.4) and postfixation was performed using 1% osmium tetroxide in same buffer for 60 minutes at room temperature. After 3 washes in the 0.1 M cacodylate buffer (pH 7.4), the specimens were dehydrated in ascending acetone series (30 – 100 %) and embedded in Spurr's resin (Polysciences, Inc). Polymerisation occurred for 24 hours at 60°C. The specimens were cut with a diamond knife (Diatome, Switzerland) using a Reichert Ultracut S microtome (Wien, Austria).

Histological sections

Serial semi-thin sections (500nm) were collected each 10 µm; from the proventricle to the hindgut of the eight L2 and the seven L3, on albumined slides and stained with toluidine blue (Fluka). Observations were made on an Olympus BX50 (Olympus Optical, Switzerland). Images were captured with a CC-12 digital camera (Olympus Optical, Switzerland) and treated by analySIS 3.2 image analytic software (Gloor Instruments AG, Switzerland).

Ultra-thin sections (60-100nm) were mounted on Formvar-carbonated copper grids, contrasted with uranyl acetate and Reynold's lead citrate. Observations were made on a Philips CM 100 transmission electron microscope (Philips Electron Optics, The Netherlands) at 60kV. Microphotographs were taken on 35mm films (Copyline HDU 1p, Agfa) and scanned on Epson 1640.

Specimen preparation for scanning electron microscopy (SEM)

Fixation of the larvae for SEM was processed as above (Karnowsky 1965). Specimens were dehydrated in ascending acetone series (up to100 %) and desiccated by critical point drying, using carbon dioxide (Baltec CPD 030, Balzers Liechtenstein). Dried specimens were mounted on aluminium stubs with Leit-tabs (Plano, Germany) and coated with a gold layer (23nm) in a Sputter Baltec SCD 005 (Balzers Liechtenstein). Observations and images were made on a scanning electron microscope PHILIPS XL 30 (Philips Electron Optics, The Netherlands) at 10kv.

Results

The digestive tube of *G. intestinalis* L2 and L3 is composed of a single tube of varying diameter with convoluted regions, running from mouth to anus. Three main regions can be recognized: foregut (pharynx, oesophagus), midgut (anterior, median, posterior) and hindgut (Fig.1). The proventriculus, without caeca, represents the junction between the foregut and the anterior midgut (Fig.2A, 3), while the Malpighian tubules delimited the posterior midgut. The digestive tube is formed by a single-layered epithelium resting on a continuous basal lamina surrounded by connective tissues containing muscles and trachea (Fig.6A, B, E, 7A, 8A, E).

Proventriculus, peritrophic matrix and microorganisms

The oesophagus passes through the cephalic ganglia and enters the proventriculus (Fig.1, 2A, 3A). Epithelial oesophageal cells internally lined with a thin cuticle (Fig.3C) join the proventricular cells at the posterior end of the proventriculus, forming an oesophageal invagination (Fig.3A). The thin cuticle lines continuously the proventricular cells and a stretched basal lamina separates the basal surfaces of the proventricular cells and the oesophageal cells (Fig.3C, D). Junction between the two gut regions occurs when the proventricular cells curve around the bulb-like structure and meet the anterior midgut cells, or columnar cells (Fig.2B). In both larval stages, a type II peritrophic membrane (PM) is synthesized where the apical surfaces of the proventricular cells and the columnar cells meet. First represented as a fibrous substance (Fig.3B), a double-layered PM is formed while passing through the proventriculus (Fig.3E). The PM was observed along the entire midgut, until within the hindgut (Fig.9A).

The presence of microorganisms was noticed in the lumen of the anterior midgut of each analyzed larvae, just below the proventriculus, where the PM is highly abundant (Fig.4B). Microorganisms were observed until the first half of the anterior midgut, moving through the endoperitrophic space (ENPT) (Fig.4C, D).

The midgut

The proventricular epithelium is continuous with the midgut epithelium, formed predominantly by columnar cells (also called digestive cells). The histological aspect of the columnar cells varies along the midgut delimiting three distinct regions: anterior (Fig.6), median (Fig.7) and posterior (Fig.8) midgut. Regenerative cells were found singly, scattered along the entire midgut (Fig.5A). These undifferentiated cells are typically characterized by a large nucleus and small peripheral cytoplasm, containing few differentiated organelles. More seldom, endocrine cells in two various shapes were observed along the midgut: the “close-type” (Fig.5B) which does not extent through the epithelial layer, and the “open-type” (Fig.5C) which is in contact with midgut lumen. These cells show at their basal end electron-dense vesicles surrounded by a bright halo called haloed vesicles (Fig.5D).

The columnar cells of the anterior midgut bear long microvilli, around 3.7 µm, extending into the lumen (Fig.6B, C). The basal plasma membrane is deeply enfolded forming a basal labyrinth associated with vesicles and mitochondria (Fig.6B, E). Small openings close to the basal lamina allow communication between the extracellular space and the channels of the basal labyrinth. The nucleus is centrally located in a vast cytoplasmic area (Fig.6A, B). Rough reticulum endoplasmic is highly abundant, in particular at the cell apex (Fig.6D) and near the nucleus. Polymorphous mitochondria, present in whole cytoplasm, are more concentrated in the basal and apical part of the cell. Many secretory vesicles and large vacuoles are visible near the base of the microvilli (Fig.6D). Other small secretory structures are present within or at the apex of the microvilli (Fig.6C) and remain in clusters, close to the peritrophic membrane.

Some digestive cells of the anterior midgut show an apical protuberance, called protuberant bud or bleb, extending in the lumen (Fig.6A). These large cytoplasmic projections are formed in area devoid of microvilli and contain no organelles. The formation of similar protuberant buds has been observed in epithelium of the posterior midgut digestive cells (not shown).

The median midgut epithelium is characterized by columnar cells with short microvilli, around 1.4 µm, that extent into the lumen (Fig.7A, B) and the absence of a basal labyrinth. The basal cytoplasm contains numerous polymorphous mitochondria (Fig.7C). Some mitochondria seem to be swollen; they appear oval or rounded (Fig.7C). The prominent nucleolus typical of nucleus of the midgut digestive cells was observed (Fig.7A).

In the posterior midgut, the structure of the columnar cells resemble to those of the anterior midgut. The cells bear at their apical region long microvilli, around 5 µm (Fig.8A, B, C) and the basal plasma membrane presents the infoldings forming a basal labyrinth (Fig.8A, E), associated with mitochondria and microtubules. Small secretory vesicles are released in the lumen from the cell apex or microvilli (Fig.8C). Numerous swollen mitochondria, Golgian vesicles, multivesicular bodies and large autolytic vacuoles containing granular and lamellar material were observed in the cytoplasm (Fig.8A, B, D). A higher concentration of glycogen was noticed in the posterior midgut (Fig.8B, D).

The larval midgut is posteriorly delimited by the insertion of two Malpighian tubules, or trunk, which diverge into two, totalling four long Malpighian tubules. Only the first region of the hindgut, was investigated and comprised of a monolayer of epithelial cells with a large nucleus and a cuticular layer that line the lumen region, containing fragments of PM (Fig.9A, B).

Salivary glands

The salivary system of *G. intestinalis* L2 and L3 consist of pairs of long tubules which lie ventrally in the anterior body cavity. A single deferent duct, inserted dorsally to the cephalopharingeal skeleton separate into two efferent ducts which are connected to the tubular glands. The glands bath in hemolymph and are invaded by numerous tracheae (Fig.10).

The fine structure of the salivary system shows a monolayer of epithelial cells surrounded by a basal lamina.

The epithelial cells apices of the efferent and deferent ducts are lined by a cuticle and the basement membrane forms some infoldings (Fig.11A). These cells contain a small nucleus (about 5 µm Ø), widely dispersed mitochondria, free ribosomes and microtubules. At the junction between the ductal and the salivary gland epithelial cells, the presence of another type of cell was detected, which was called intermediate cell (Fig.11B). These cells were not lined by a cuticle nor bear microvilli at the apical region. They show a highly enfolded lateral plasma membrane, a small nucleus and mitochondria.

The tubular salivary glands comprised closely packed epithelial cells. The cells are tightly linked to adjacent cells by deeply folded septet junctions (Fig.12B). Numerous tracheae insertions are found at the basement of the cells and in the intercellular space (Fig.12A, C). Irregular microvillar-like projections (microvilli linked with apical lamellar membrane) extend into the lumen (Fig.12D). The basement membrane does not form a basal labyrinth (Fig.12A). The cytoplasm presents an abundant rough endoplasmic reticulum, free ribosomes, lipidic droplets, Golgi complexes and polymorphous mitochondria, generally swollen, are widespread throughout the cytoplasm (Fig.12A, B).

Structural differences observed between L2 and L3 salivary glands

The dense vesicles produced by the Golgi apparatus in the L2 are less common, or more difficult to observe, in the L3.
Numerous lysosomes (residual bodies) appear in the L3
Swollen mitochondria also present in the L2, became more abundant and bigger in the L3. The swollen mitochondria were seen closely associated, forming sort of vacuolization, and the matrix seemed to be empty.

Discussion

The nutrition phase of *G. intestinalis* larvae is crucial for the accumulation of the energetic resources necessary for the survival of the adult flies which have to complete their lifecycle without feeding. The gut consists of three different regions involved in the digestion, absorption and elimination procedures: the foregut, midgut and hindgut. The salivary glands, connected to the foregut, usually have a limited or no action in digestion (Terra and Ferreira 2005). The gut epithelium of *G. intestinalis* L2 and L3 is similar to those described in most insects and is composed of a simple epithelium resting on a basal lamina surrounded by connective tissue and the contraction of muscles cause peristalsis that propel the food along the gut (Terra and Ferreira 2005).

The midgut is considered as the most important region of the digestive system responsible for digestion and absorption (Dow 1986). The caeca generally present in Diptera larvae are absent in several bot fly larvae like *Dermatobia hominis* (L. Jr) (Evangelista and Leite 2003), *Hypoderma bovis* (L.) (Boulard 1969) or *G. intestinalis* (Keilin 1944). Unlike the preliminary study which described no regional and cellular differentiation in the midgut of *D. hominis* late instar (Evangelista and Leite 2003), this ultrastructural study demonstrates that the midgut of *G. intestinalis* L2 and L3 can be divided into three distinct regions termed anterior, median and posterior midgut as previously described for *Lucilia* spp (Robineau-Desvoidy) larvae

(Hobson 1931) and three different type of cells were observed. No histological differences were observed in the midgut between the two larval stages and only the number of circumvolution rise with age. Therefore the comments concerning the midgut always refer to L2 and L3.

The PM compartmentalizes the midgut lumen in two compartments: the ectoperitrophic space (between the epithelium and the PM) and the endoperitrophic space (between the PM and the lumen). This semi-permeable chitinous membrane that lines the gut of most insects is thought to be associated to many digestive processes and to a protective function (Tellam et al. 1999). A peritrophic membrane of type II, produced by the specialized epithelial cells of the proventriculus, has been identified in *G. intestinalis* L2 and L3 (Fig.3B, E, 4B, C). Further investigations to characterize the function and composition of the PM in these larvae will help to understand the digestion processes and maybe allow the isolation of molecules that could serve as pests control agents as demonstrated for *Lucilia cuprina* (Wiedemann) (East and Eisemann 1993; East et al. 1993; Tellam et al. 2000). An intriguing feature in this study was the presence of microorganisms in each analyzed larvae. They were always observed in the anterior region of the midgut, generally highly abundant at the junction between the proventriculus and the anterior midgut (Fig.4B). If usually they were localized in the endoperitrophic space (Fig.4C), several microorganisms were observed in the ectoperitrophic space or moving between the microvilli (not shown). If the presence of microorganisms in the intestinal tract of insects is common and widespread, little is known about their existence in the myiasis-causing species. They might be symbiotic or fortuitous contaminants that gain access from the external environment (Douglas and Beard 1996). Symbionts are rarely associated to digestion but sometimes thought to provide nutrient factors like essential amino acids, B vitamins or prevent the colonization of the gut by other species (Dillon and Dillon 2004). Therefore further studies are required to identify and understand the origin and function of these microorganisms.

The distinction between the different midgut regions was based upon the morphological comparison of the columnar cells. The anterior and posterior segments demonstrate morphological similarities. The columnar cells constituting the epithelium of the anterior and posterior midgut bear long microvilli at the apical plasma membrane (Fig.6B, C, 8A, C) thus the available membrane for secretion or absorption is enlarged. The basal plasma membrane has numerous infoldings that form a complex labyrinth of channels associated to numerous mitochondria and microtubules (Fig.6E, 8E), sometimes stretching into the apical third of the cell (Fig.6B). These membrane infoldings are generally related to the active transport of water and ions (Martoja and Ballan-Dufrançais 1984; Terra et al. 1988) and might be involved in midgut fluxes important for the translocation of enzymes and products of digestion (Billingsley and Lehane 1996; Terra and Ferreira 2005).

The columnar cells of the anterior and posterior midgut regions in both larval stages exhibit an intense secretory activity and their cytoplasm contain a high concentration of organelles related to secretion like rough endoplasmic reticulum, Golgi complexes and secretory vesicles (Rothman and Orci 1992). The continuous synthesis and secretion of digestive enzymes has been described in continuous feeders (e.g. Diptera larvae and Lepidoptera) (Baker et al. 1984). Three secretory mechanisms were observed in these two midgut regions (Terra and Ferreira 2005): (i) apocrine secretions; which involve the loss of part of apical cytoplasm. Indeed apical extrusions called protuberant buds, or blebs, projecting into the lumen were frequently noted (Fig.6A). According to some authors these blebs can be associated to healthy cells as normal cell renewal process (Anderson and Harvey 1966; Baker et al. 1984) (ii) microapocrine secretions; when the loss of cytoplasm is small. Numerous pinched off vesicles were observed at the top of the long microvilli (Fig.6C, 8C) and it has been suggested that the content of these vesicles might be incorporated into the PM (Jordao et al. 1999). (iii) merocrine secretions, or exocytosis; which consist of the fusion of the secretory vesicles with the apical cell membrane without cytoplasm loss, and content is emptied in lumen (not shown).

The columnar cells of the anterior midgut present some large vacuoles (Fig.6D), while the posterior midgut cells appeared more vacuolated showing large autolytic vacuoles containing various lamellar and granular residues (Fig.8D). The presence of numerous autolytic vacuoles and multivesicular bodies can be associated to autolytic activity (Anton-Erxleben et al. 1983).

The morphology of the columnar cells of the median midgut differs from those of the two other regions. The surface of basal membrane exposed to hemolymph is not increased by a basal labyrinth (Fig.7C) and only short microvilli are visible at the apical region of the cells (Fig.7B). Microapocrine secretion consisting in the release of budding secretory vesicles was the only secretion mechanism noticed in these columnar cells. Blebs have never been observed in this region. These cells seem to contain a much higher concentration of mitochondria compared to the other columnar cells. If the anterior and posterior midgut regions probably possess the dual function of absorption and secretion, it is laboured to attribute clearly a function to the median midgut.

A specific feature observed in the midgut columnar cells and salivary gland cells was the numerous swollen mitochondria (Fig.7C, 8B, 12A, B). The ultrastructure of mitochondria is known to vary between tissue, organisms and physiological status of cells (Zick et al. 2009). Swollen mitochondria are often described as a manifestation of ageing process, cellular disorder or apoptosis in all kind of organisms (Anton-Erxleben et al. 1983; Charles 1987; Yasuda et al. 2006). These structures might also be a consequence of *G. intestinalis* larvae living in microaerobic conditions and swollen mitochondria could allow optimizing the production of energy.

Aside the columnar cells, regenerative cells were found scattered throughout the epithelium (Fig.5A, 7A, C) indicating that epithelium may be renewed regularly or under certain conditions. Regenerative cells were found in *D. hominis* (Evangelista and Leite 2003) larvae but appear to be absent in some dipteran larvae (Terra et al. 1988). The third type of cells observed were the endocrine cells (Fig.5B, C). The role they play in the control of midgut events is yet unclear but as in vertebrates, they are likely to have a function in the regulation of intestinal activities (Lehane et al. 1996).

The tubular form of the salivary glands of *G. intestinalis* L2 and L3 can be considered similar to that of other Diptera like *D. hominis* (Evangelista and Leite 2007) or *Hypoderma bovis* (Boulard 1969). The group of cells which make the transition between the salivary canal cells and the glandular cells, termed intermediate cells, were also identified in the salivary system of *H. bovis* (Boulard 1969). They might only have a transitional function between the canal and gland structures as their cytoplasm contains few organelles and a small nucleus.

Unlike the glandular cells which exhibit a narrow basement membrane (Fig.12 A, B), the plasma membrane of the salivary canal cells form sort of basal labyrinth (Fig.11A) exposing a larger membrane surface to hemolymph that might have an active transport function.

In the glandular cell, the septet junctions that link the adjacent cells tightly to each other show numerous infoldings (Fig.12B), allowing the cells to increase in volume when necessary, maybe to store synthesised products. Indeed these cells demonstrate an intense synthesis activity; the RER, free ribosomes and Golgi complexes seem to completely fill the apical region of some cells (Fig.12B). Additionally, the numerous tracheae insertions which supply the cells in oxygen (Fig.12A, C), the highly abundant mitochondria (Fig.12A) and the large nucleus exhibiting several nucleolus (not shown) support the hypothesis of important cellular synthesis and storage processes. The microvillar-like projections associated to the microvilli

(Fig.12D) at the apical portion of the glandular cell might serve to reinforce the structure of the gland, or resist to osmotic pressure.
The differences noted between the two larval stages only concerned the glandular cell content. However these results should be reconfirmed as they were only based on few observations. Further investigations are needed to understand the contribution of the salivary glands to digestion processes and the impact of salivary enzymes on host tissue during migration and maturation phases.

This work presents a first step toward understanding the functional morphology of the midgut and salivary glands of *G. intestinalis* second and third instars larvae. It is known that in insects the organization of the digestive processes depend on compartmentalization of digestive enzymes and on midgut fluxes (Terra et al. 1996). The localization in each midgut luminal compartment and corresponding tissue of various digestive enzymes described in *G. intestinalis* larvae (Tatchell 1958; El-Ebiarie et al. 2005), will bring useful information to further study the midgut functions.

References

Anderson, E. and W. R. Harvey (1966). "Active transport by the cecropia midgut. II. Fine structure of the midgut epithelium." Journal of Cell Biology **31**(1): 107-34.

Anton-Erxleben, F., J. Miquel and D. E. Philpott (1983). "Fine-structural changes in the midgut of old Drosophila melanogaster." Mechanisms of Ageing and Development **23**(3-4): 265-76.

Baker, J. E., S. M. Woo and R. V. Byrd (1984). "Ultrastructural features of the gut of Sitophilus granarius (L.) (Coleoptera: Curculionidae) with notes on distribution of proteinases and amylases in crop and midgut. ." Canadian Journal of Zoology **62**: 1251-1259.

Billingsley, P. F. and M. J. Lehane (1996). Structure and ultrastructure of the insect midgut. Biology of the Insect Midgut. M. J. Lehane and P. F. Billingsley. London, Chapman & Hall**:** 3-30.

Boulard, C. (1969). "Anatomie et histologie du tube digestif de la larve d'*Hypoderma bovis* (Diptera: Oestriforme)." Annales de la Société Entomologique de France **5**: 371-387.

Charles, J. F. (1987). "Ultrastructural midgut events in Culicidae larvae fed with Bacillus sphaericus 2297 spore/crystal complex." Annales de l'Institut Pasteur Microbiology **138**(4): 471-84.

Cogley, T. P. (1989). "Effects of migrating Gasterophilus intestinalis larvae (Diptera: Gasterophilidae) on the mouth of the horse." Veterinary Parasitology **31**(3-4): 317-31.

Cogley, T. P. (1999). "Morphology of a newly discovered sensory array on the mouthhooks of Gasterophilus larvae." Medical and Veterinary Entomology **13**(4): 439-46.

Cogley, T. P. and M. C. Cogley (1999). "Inter-relationship between Gasterophilus larvae and the horse's gastric and duodenal wall with special reference to penetration." Veterinary Parasitology **86**(2): 127-42.

Colwell, D. D., D. Otranto and I. G. Horak (2007). "Comparative scanning electron microscopy of Gasterophilus third instars." Medical and Veterinary Entomology **21**(3): 255-64.

Dillon, R. J. and V. M. Dillon (2004). "The gut bacteria of insects: nonpathogenic interactions." Annual Review of Entomology **49**: 71-92.

Douglas, A. E. and C. B. Beard (1996). Microbial symbioses in the midgut of insects. Biology of the Insect Midgut. M. J. Lehane and P. F. Billingsley. London, Chapman & Hall**:** 419-431.

Dow, J. A. T. (1986). "Insect midgut function." Advances in Insect Physiology **19**: 187-328.

East, I. J. and C. H. Eisemann (1993). "Vaccination against Lucilia cuprina: the causative agent of sheep blowfly strike." Immunology and Cell Biology **71 (Pt 5)**: 453-62.

East, I. J., C. J. Fitzgerald, R. D. Pearson, R. A. Donaldson, T. Vuocolo, L. C. Cadogan, R. L. Tellam and C. H. Eisemann (1993). "Lucilia cuprina: inhibition of larval growth induced by immunization of host sheep with extracts of larval peritrophic membrane." International Journal for Parasitology **23**(2): 221-9.

El-Ebiarie, A. S., N. Taha, A. A. El-Meguid and M. A. Mustafa (2005). "A preliminary characterization of digestive protease in the mid-gut of the third larval instar of *Gasterophilus intestinalis*." Veterinary Medical Journal, Giza **53**(1): 5-23.

Evangelista, L. G. and A. C. Leite (2003). "Midgut ultrastructure of the third instar of Dermatobia hominis (Diptera: Cuterebridae) based on transmission electron microscopy." Journal of Medical Entomology **40**(2): 133-40.

Evangelista, L. G. and A. C. Leite (2007). "Salivary glands of second and third instars of Dermatobia hominis (Diptera: Oestridae)." Journal of Medical Entomology **44**(3): 398-404.

Hall, M. J. R. and R. Wall (1995). "Myiasis of humans and domestic animals." Advances in Parasitology **35**: 257-334.

Hobson, R. P. (1931). "Studies on the nutrition of blow-fly larvae." Journal of Experimental Biology **8**(2): 109-123.

Jordao, B. P., A. N. Capella, W. R. Terra, A. F. Ribeiro and C. Ferreira (1999). "Nature of the anchors of membrane-bound aminopeptidase, amylase, and trypsin and secretory mechanisms in Spodoptera frugiperda (Lepidoptera) midgut cells." Journal of Insect Physiology **45**(1): 29-37.

Karnowsky, M. J. (1965). "A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy." Journal of Cell Biology **27**(137A).

Keilin, D. (1944). "Respiratory system and respiratory adaptations in larvae and pupae of Diptera." Parasitology **36**: 1-66.

Lehane, M. J., H. M. Müller and A. Crisanti (1996). Mechanisms controlling the synthesis and secretion of digestive enzymes in insects. Biology of the Insect Midgut. M. J. Lehane and P. F. Billingsley. London, Chapman & Hall**:** 195-205.

Leite, A. C. and F. B. Scott (1999). "Scanning electron microscopy of the second-instar larva of Gasterophilus nasalis." Medical and Veterinary Entomology **13**(3): 288-94.

Leite, A. C., F. B. Scott and L. G. Evangelista (1999). "Scanning electron microscope observations on third-instar Gasterophilus nasalis (Diptera: Oestridae)." Journal of Medical Entomology **36**(6): 643-8.

Martoja, M. and C. Ballan-Dufrançais (1984). The Ultrastructure of the digestive and excretory organs. Insect Ultrastructure. R. C. King, Akai, H. New York, Plenum Press. **2:** 199-268.

Principato, M. and M. Tosti (1988). "Scanning electron microscope observations on the anterior thoracic and post-abdominal spiracles of Gasterophilus larvae (Diptera: Gasterophilidae)." International Journal for Parasitology **18**(2): 191-6.

Rothman, J. E. and L. Orci (1992). "Molecular dissection of the secretory pathway." Nature **355**(6359): 409-15.

Shefstad, D. K. (1978). "Scanning electron microscopy of Gasterophilus intestinalis lesions of the equine stomach." Journal of the American Veterinary Medical Association **172**(3): 310-3.

Tatchell, R. J. (1958). "The physiology of digestion in the larvae of the horse bot-fly, Gasterophilus intestinalis (De Geer)." Parasitology **48**(3-4): 448-58.

Tellam, R. L., C. Eisemann, R. Casu and R. Pearson (2000). "The intrinsic peritrophic matrix protein peritrophin-95 from larvae of Lucilia cuprina is synthesised in the cardia and regurgitated or excreted as a highly immunogenic protein." Insect Biochemistry and Molecular Biology **30**(1): 9-17.

Tellam, R. L., G. Wijffels and P. Willadsen (1999). "Peritrophic matrix proteins." Insect Biochemistry and Molecular Biology **29**(2): 87-101.

Terra, W. R., F. P. Espinoza-Fuentes, A. F. Ribeiro and C. Ferreira (1988). "The larval midgut of the housefly (*Musca domestica*): ultrastructure, fluid fluxes and ion secretion in relation to the organization of digestion." Journal of Insect Physiology **34**: 463-472.

Terra, W. R. and C. Ferreira (2005). Biochemistry of digestion. Comprehensive Molecular Insect Science. L. I. Gilbert, Iatrou, K., Gill, S.S. Oxford, Elsevier **4:** 171-224.

Terra, W. R., C. Ferreira and J. E. Baker (1996). Compartmentalization of digestion. Biology of the Insect Midgut. M. J. Lehane and P. F. Billingsley. London, Chapman & Hall**:** 206-235.

Yasuda, K., T. Ishii, H. Suda, A. Akatsuka, P. S. Hartman, S. Goto, M. Miyazawa and N. Ishii (2006). "Age-related changes of mitochondrial structure and function in Caenorhabditis elegans." Mechanisms of Ageing and Development **127**(10): 763-70.

Zick, M., R. Rabl and A. S. Reichert (2009). "Cristae formation-linking ultrastructure and function of mitochondria." Biochimica and Biophysica Acta **1793**(1): 5-19.

Zumpt, F. (1965). Myasis in man and animals in the old world, Butterworths, London, United Kingdom.

Figure legends.

Fig.1. Schematic drawing of the digestive system of *G. intestinalis* L2 and L3 adapted from McFarlane (1985). Proportions are not respected. The three gut regions are represented: foregut (FG), midgut (MG) and hindgut (HG). The oesophagus (OE) passes through the cephalic ganglia (CG) and enters the proventriculus (PROV) (see Fig. 2,3,4). The midgut is separated in three different regions: (I) anterior midgut (see Fig.6); (II) median midgut (see Fig.7) and (III) posterior midgut (see Fig.8). The posterior end of midgut is delimited by the Malpighian tubules (MT), after which the hindgut (IV) (see Fig. 9) continues to the rectum. Columnar cells (CC), endocrine cells (ENC) and regenerative cells (RGC) compose the midgut epithelium (here only represented in the anterior midgut). The FG and HG have an epithelium composed of epithelial cells (EC) lined by a cuticle (C). Salivary glands (SG), efferent ducts (Ed), deferent ducts (Dd).

Fig.2. SEM of the proventriculus of *G. intestinalis* L3. (A) The oesophagus (OE) enters the proventriculus (PROV) and the anterior midgut (MGant) begins at the posterior end of the PROV. Numerous tracheae (T) insert in the PROV. (Scale bar = 200µm). (B) Transverse section of the proventriculus showing the junction between the proventricular cells (PVc) and the columnar cells (CC). The oesophagus has been removed showing the underlying stretched basal lamina (sbl). (Scale bar = 100µm).

Fig.3. (A) Schematic drawing of the proventriculus of *G. intestinalis* L2 and L3 adapted from Spenve (1991). Oesophageal cells (OEc), cuticle (C), proventricular cells (PVc), stretched basal lamina (sbl), columnar cells (CC), peritrophic membrane (PM). B-E: Ultrastructural details of the regions encircled on the drawing of *G. intestinalis* L3. (B) Apical region of CC and PVc at the top of the proventricular bulb-like structure, where the synthesis of the PM begins. Vesicle (v), microvilli (mv). (Scale bar = 1 µm). (C) OEc lined at the apical region by a cuticle (C) and the basal surface is surrounded by sbl. Tracheae (T), mitochondria (mi), lumen (LU). (Scale bar = 2 µm). (D) Basal region of PVc, surrounded by the thick layer of sbl. Microtubules (mt). (Scale bar = 2 µm). (E) Apical region of CC and PVc at the base of the proventriculus, showing the double-layered PM. Ectoperitrophic space (EPT). (Scale bar = 200 nm).

Fig.4. (A) Schematic drawing of the proventriculus of *G. intestinalis* L2 and L3 adapted from Spenve (1991). (B) TEM of the section encircled in (A) showing a transverse section of the posterior proventriculus of *G. intestinalis* L2. Proventricular cells (PVc), cuticle (C), columnar cells (CC), peritrophic membrane (PM), lumen (LU), microorganisms (Morg), basal lamina (bl). (Scale bar = 10 µm). (C) TEM of the apical section of a CC showing Morg in the endoperitrophic space (ENPT) of the anterior midgut of *G. intestinalis* L2. Ectoperitrophic space (EPT), long microvilli (lmv). (Scale bar = 1 µm). (D) TEM of Morg in the ENPT of *G. intestinalis* L2. (Scale bar = 1 µm).

Fig.5. A-D: Ultrastructural view of the regenerative and endocrine cells in *G. intestinalis* L3. (A) Regenerative cell (RGC) in an undifferentiated state located at the basement of a columnar cell (CC), on the basal lamina (bl). Nucleus (N), mitochondria (mi), basal labyrinth (LB). (Scale bar = 1 µm). (B) Endocrine cell (ENC) in a “close-type” shape (not extending until the midgut lumen). Rough endoplasmic reticulum (RER), tracheae (T) (bar = 2 µm). (C) ENC in an “open-type” shape (in contact with midgut lumen). Short microvilli (smv) (bar = 1 µm). (D) Detail of the haloed vesicles (H) characteristic of the endocrine cells. Microtubules (mt). (Scale bar = 500 nm).

Fig.6. A-E: Ultrastructural detail of *G. intestinalis* L3 anterior midgut epithelium. (A) Light electron micrograph showing a well developed apical protuberant bud (PB) projecting into the midgut lumen (LU). Columnar cell (CC), nucleus (Nu), basal labyrinth (LB), long microvilli (lmv), basal lamina (bl), muscle (mu). (Scale bar = 20 µm). (B) CC showing the deeply enfolded basal membrane forming the LB. (Scale bar = 5 µm). (C) Apical region of a CC showing pinching off vesicles (star). Rough endoplasmic reticulum (RER), peritrophic membrane (PM). (Scale bar = 2 µm). (D) Details of a CC apical cytoplasm showing numerous small vesicles (v), large vacuole (Va) and RER. (Scale bar = 1 µm). (E) Details of the basal region of a columnar cell representing the channels of the LB. Mitochondria (mi), glycogen (gly). (Scale bar = 500 nm).

Fig.7. A-C: Ultrastructural detail of *G. intestinalis* L3 median midgut epithelium. (A) View of a columnar cell (CC) showing a thin basal membrane (bm) and short microvilli (smv). A regenerative cell (RGC) rests on the basal lamina (bl) at the basement of the CC. Nucleus (N), peritrophic membrane (PM), septate junction (se), muscle (mu). (Scale bar = 5 µm). (B) Detail of the smv and concentration of dense materiel at the basement of the smv. Secretory

or digestive materiel (star) is highly abundant in the ectoperitrophic space (EPT). (Scale bar = 1 µm). (C) Basal cytoplasm of a CC showing numerous mitochondria (mi) and swollen mitochondria (smi). The RGC contains lipidic droplets (li). (Scale bar = 1 µm).

Fig.8. A-E: Ultrastructural detail of *G. intestinalis* L3 posterior midgut epithelium. (A) View of a columnar cell (CC) with the folded plasma membrane forming the basal labyrinth (LB). Long microvilli (lmv) extent into midgut lumen. Cytoplasm contains large autolytic vacuoles (Va). Basal lamina (bl), muscle (mu). (Scale bar = 5 µm). (B) Detail of the apical cytoplasm showing mitochondria (mi) swollen mitochondria (smi) and glycogen (gly). (Scale bar = 1 µm). (C) Detail of pinching off vesicles (pov). (Scale bar = 1 µm). (D) Detail of a Va containing granular and lamellar material and surrounded by a vacuolar membrane (Vam). Multivesicular body (mvb). (Scale bar = 500 nm). (E) Basal region of a columnar cell showing the channels of the LB. Microtubules (mt). (Scale bar = 1 µm).

Fig.9. (A) Light electron micrograph of the transition between the posterior midgut columnar cells (CC) and hindgut epithelial cells (EC) of *G. intestinalis* L3. EC are lined by a cuticle (C). Fractions of peritrophic membrane (PM) are present in hindgut lumen (LU). Basal lamina (bl), long microvilli (lmv). (Scale bar = 20 µm). (B) TEM of the hindgut epithelial cells (EC) of *G. intestinalis* L3. Nucleus (N), lipidic droplet (li), septate junction (se). (Scale bar = 1 µm).

Fig.10. Scanning electron micrograph *G. intestinalis* L2 salivary gland (sg). The gland is connected to an efferent duct (Ed) and numerous tracheae (T) insert in the gland. (scale bar = 200 µm).

Fig.11. (A) TEM of salivary gland efferent duct cells (Dct) of *G. intestinalis* L2 showing the cuticle (C) that lines the cells at the apical region, the infoldings of the basement membrane (bm) and of the lateral plasma membrane (lpm). Basal lamina (bl), nucleus (N). (Scale bar = 1 µm). (B) TEM of the intermediate cells (Inc) at the junction of the Dct and the glandular cells (SGc) of *G. intestinalis* L2. The nucleus of the INc are indicated N1 and N2. Microvilli (mv), lumen (LU), mitochondria (mi). (Scale bar = 1 µm).

Fig.12. A-F: Ultrastructural details of the salivary gland cells of *G. intestinalis* L3. (A) View of salivary glandular cells, showing the high concentration of mitochondria and swollen mitochondria (smi). Tracheae (T), residual bodies (rb), basal lamina (bl), lumen (LU). (Scale bar = 5 µm). (B) Apical cytoplasm of SGc showing the abundant rough endoplasmic reticulum (RER) and the folded septate junction (se). Microvilli (mv) linked with apical lamellar membrane (alm) extent into the LU. (Scale bar = 500 nm). (C) Detail of the tracheae “clusters” that penetrate the glandular cells. (Scale bar = 1 µm). (D) Detail of the apical projection; mv and alm. Small coated vesicles (vc). (Scale bar = 500 nm).

Fig.1.

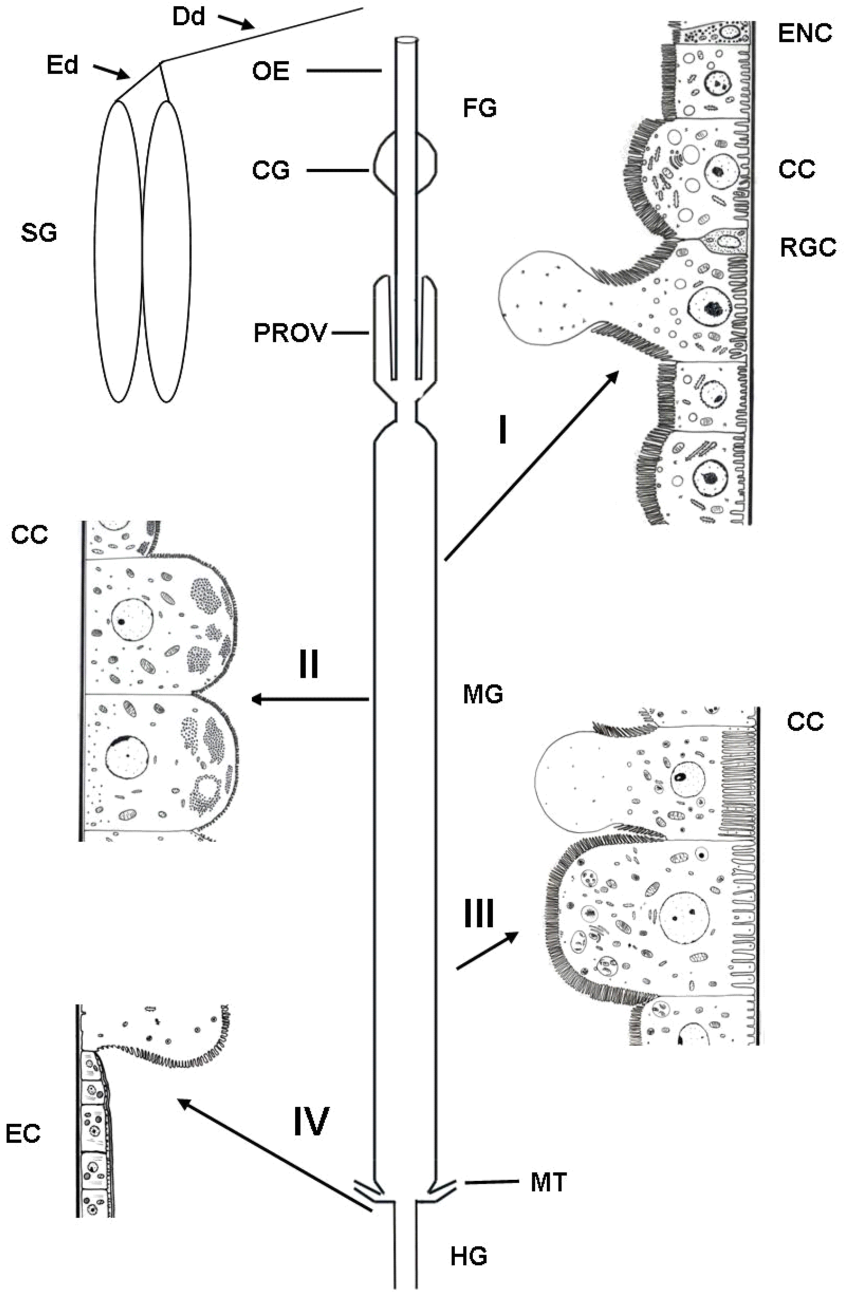


Fig.2.

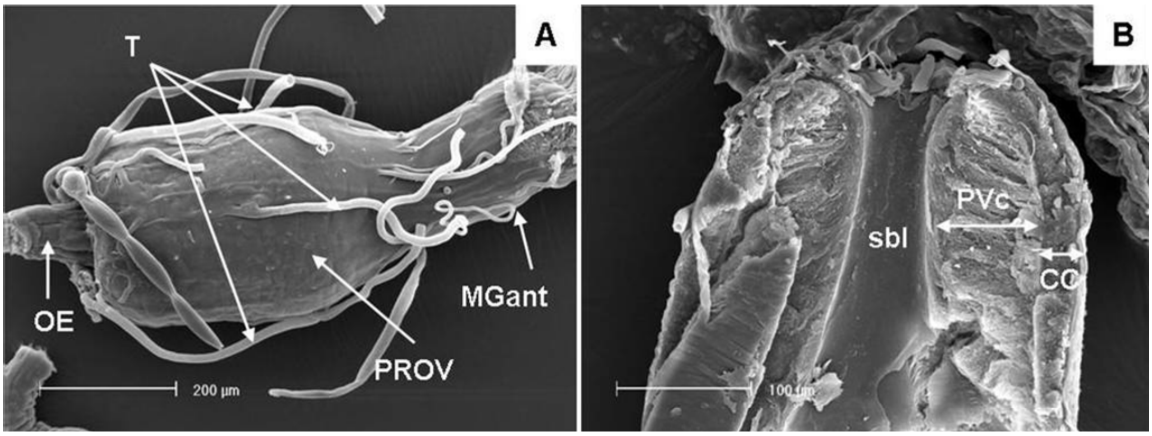


Fig.3.

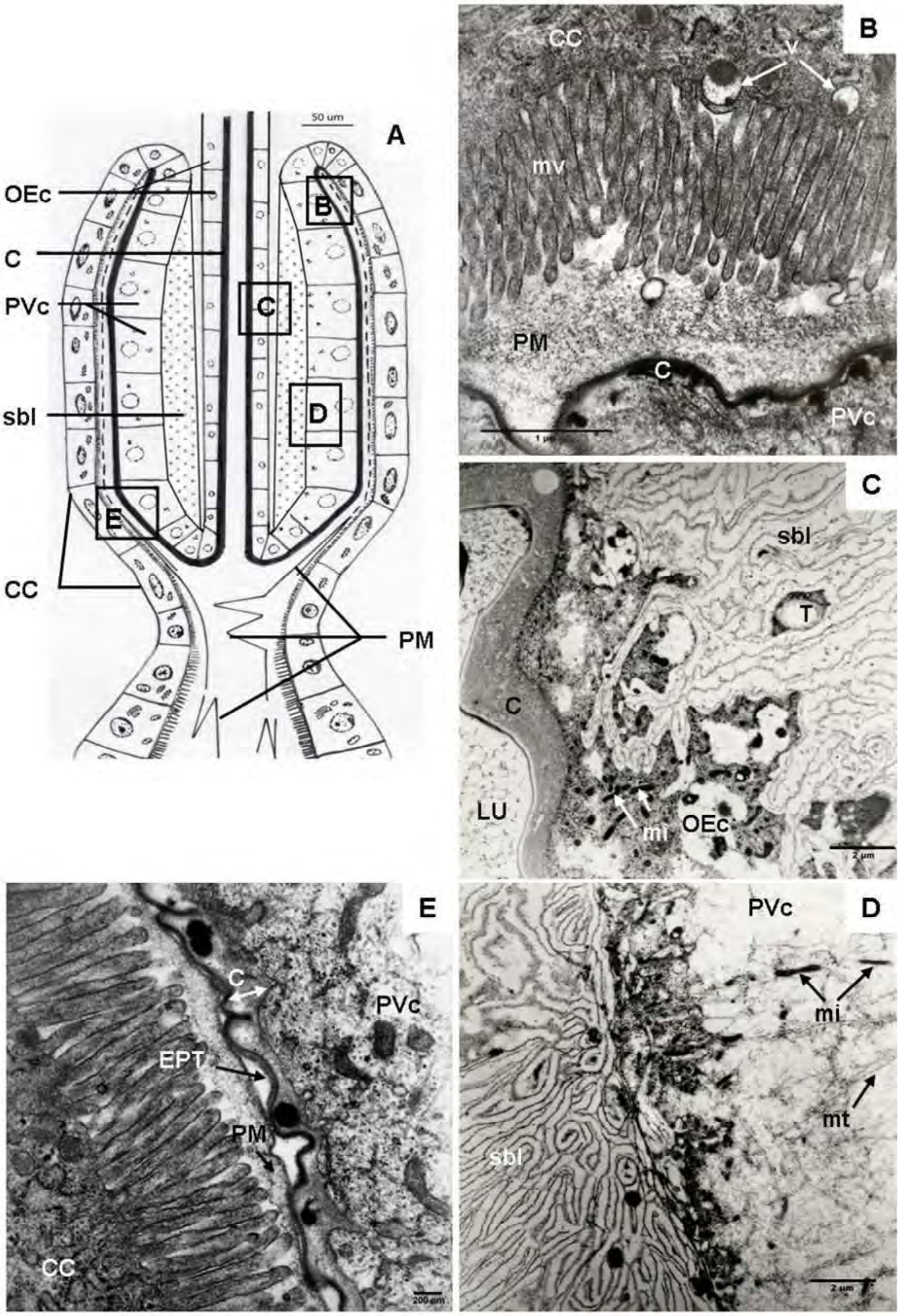

A
50 um
OEc
C
PVc
sbl
CC
PM
B
C
D
E
B
CC
V
mv
PM
C
PVc
1 µm
C
sbl
T
C
LU
mi
OEc
2 µm
E
C
PVc
EPT
PM
CC
200 nm
PVc
D
mi
mt
sbl
2 µm

Fig.4.

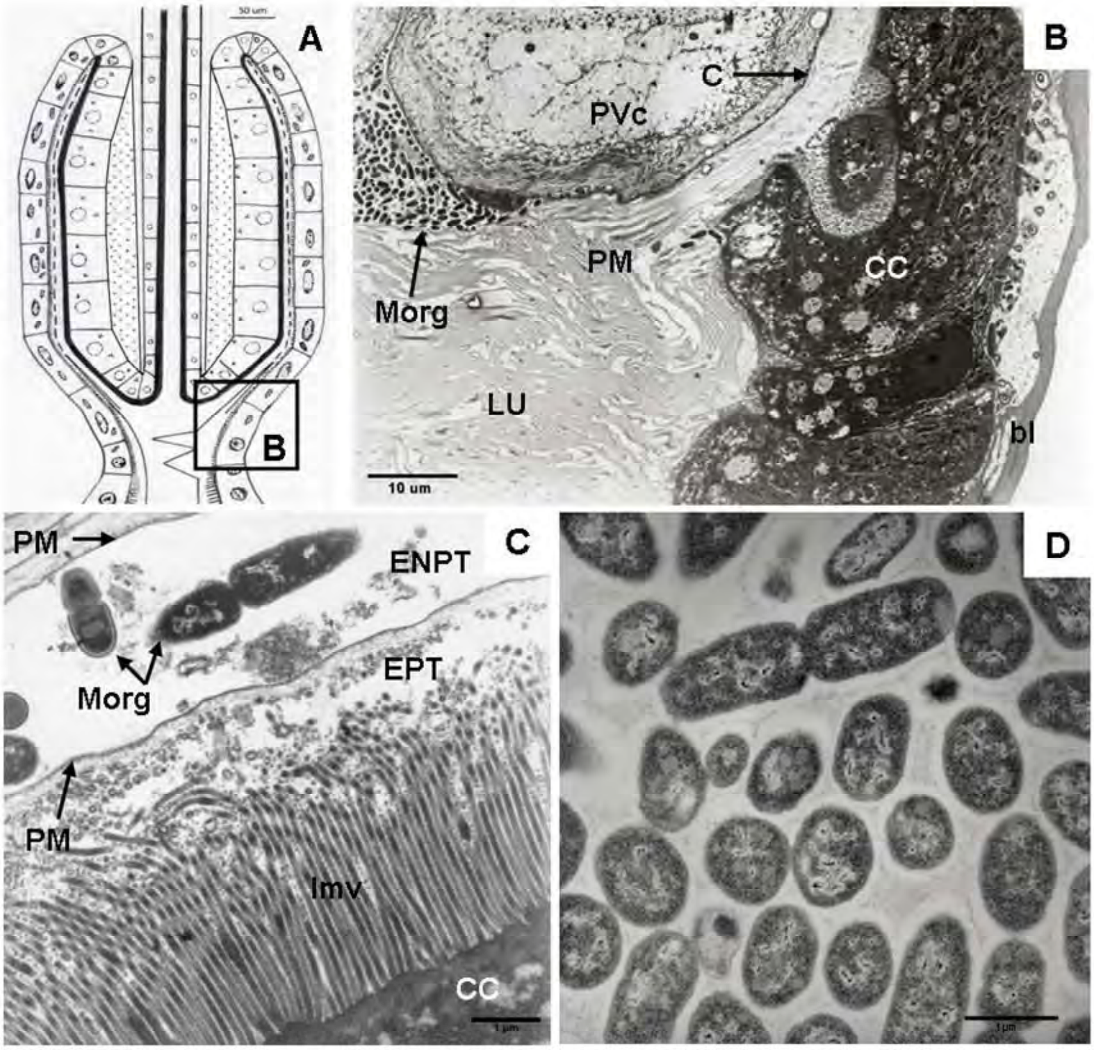

50 um
A
B
B
C
PVc
PM
Morg
CC
LU
bl
10 um
PM
C
ENPT
Morg
EPT
PM
lmv
CC
1 µm
D
1 µm

Fig.5.

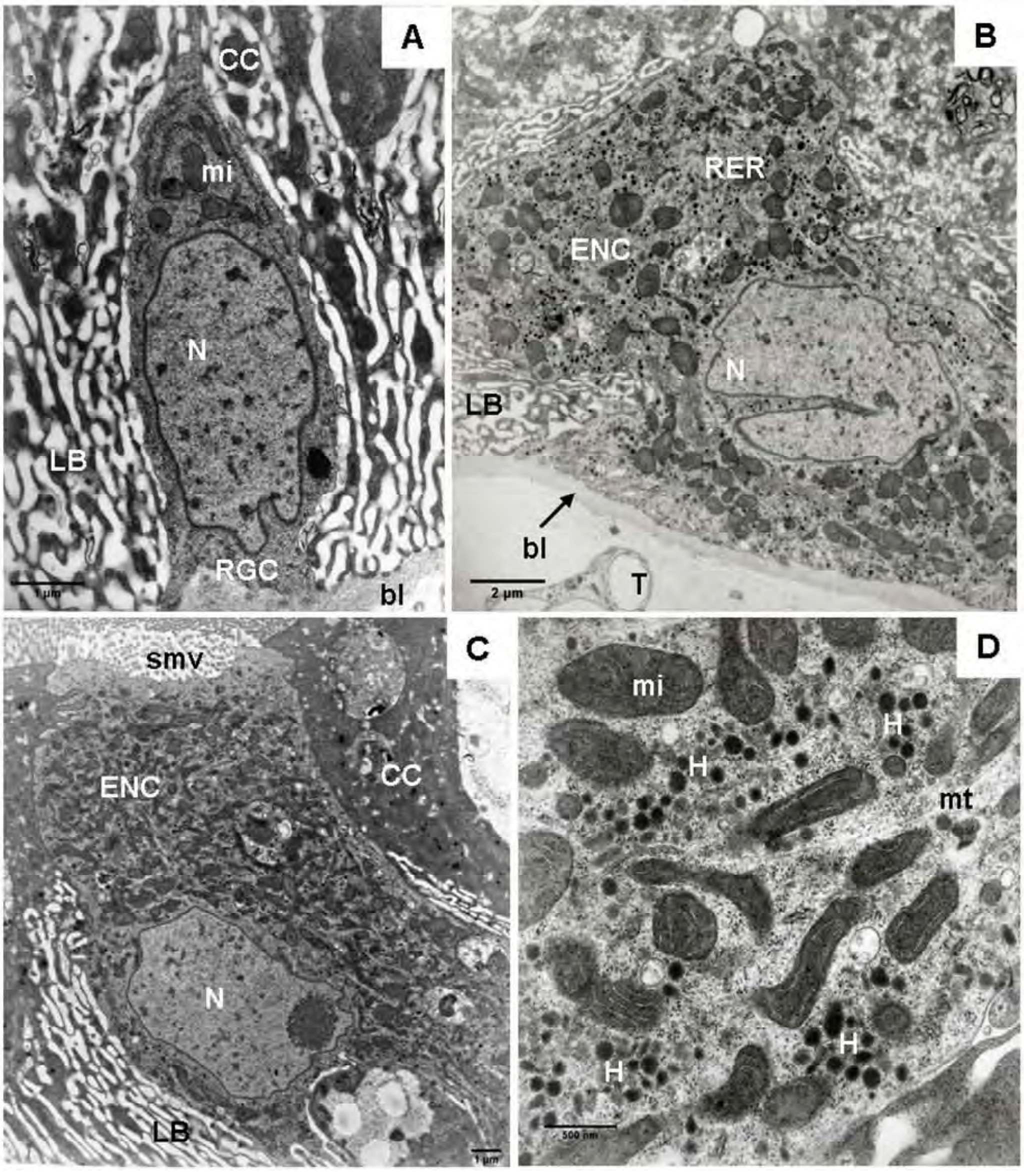

A
CC
mi
N
LB
RGC
bl
1 µm
B
RER
ENC
N
LB
bl
T
2 µm
C
smv
ENC
CC
N
LB
1 µm
D
mi
H
H
mt
H
H
500 nm

Fig.6.

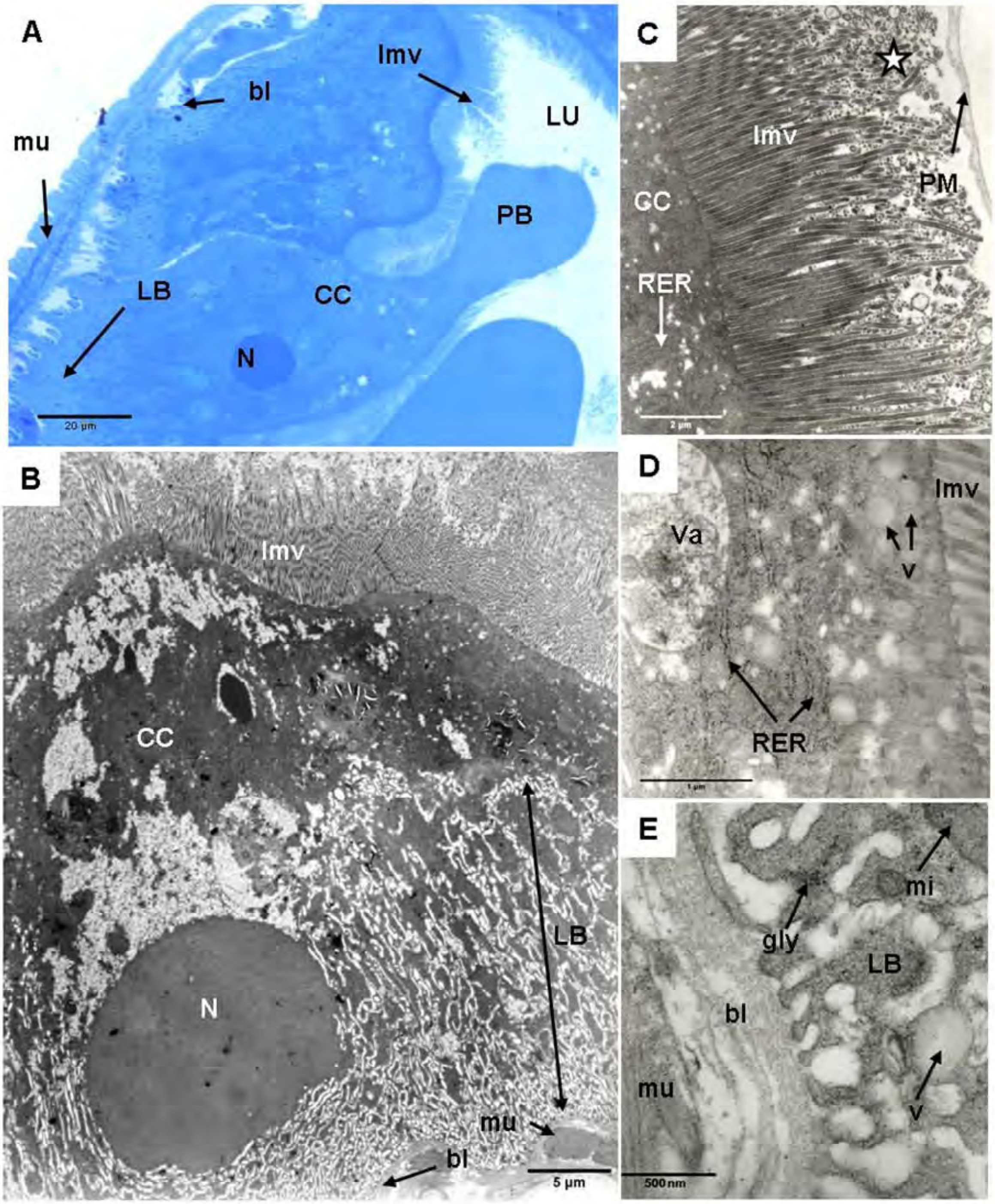

A
mu
bl
lmv
LU
PB
LB
CC
N
20 µm
B
lmv
CC
N
LB
mu
bl
5 µm
C
lmv
PM
CC
RER
2 µm
D
Va
lmv
v
RER
1 µm
E
mi
gly
LB
bl
mu
v
500 nm

Fig.7.

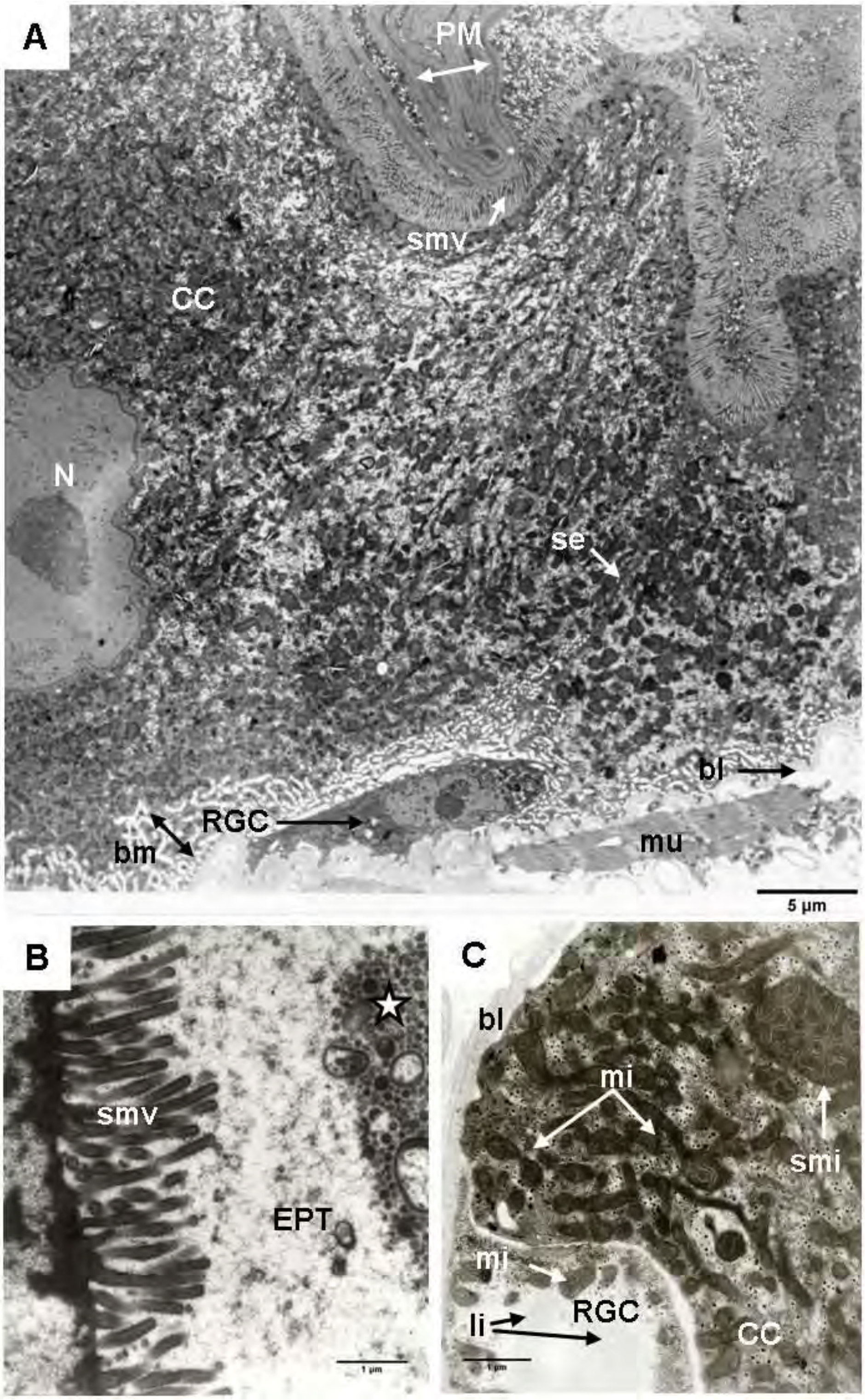

A
PM
smv
CC
N
se
bl
RGC
bm
mu
5 µm
B
smv
EPT
1 µm
C
bl
mi
smi
mi
RGC
li
CC
1 µm

Fig.8.

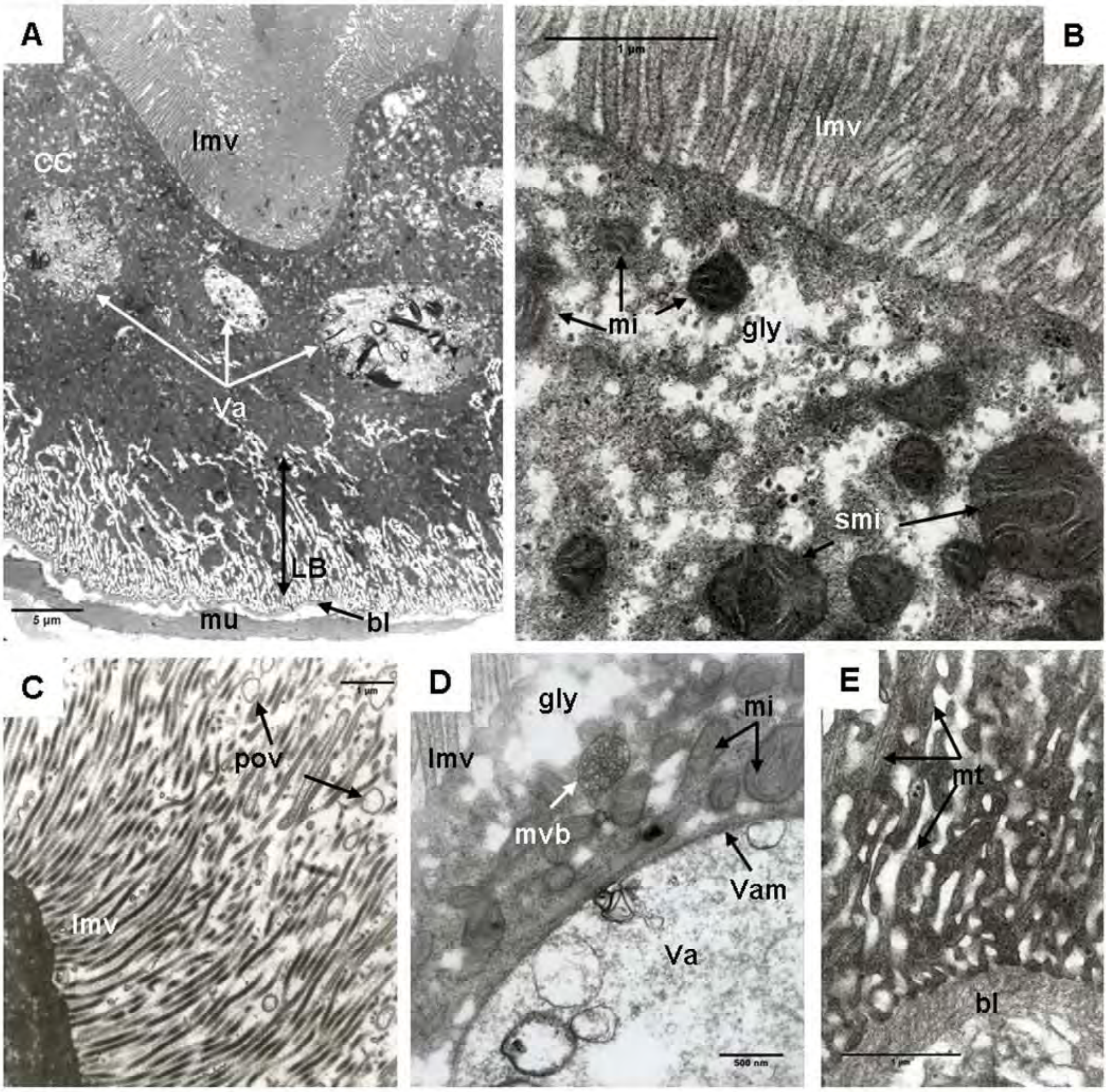

A
lmv
CC
Va
LB
mu
bl
5 µm
B
1 µm
lmv
mi
gly
smi
C
1 µm
pov
lmv
D
gly
lmv
mi
mvb
Vam
Va
500 nm
E
mt
bl
1 µm

Fig.9.

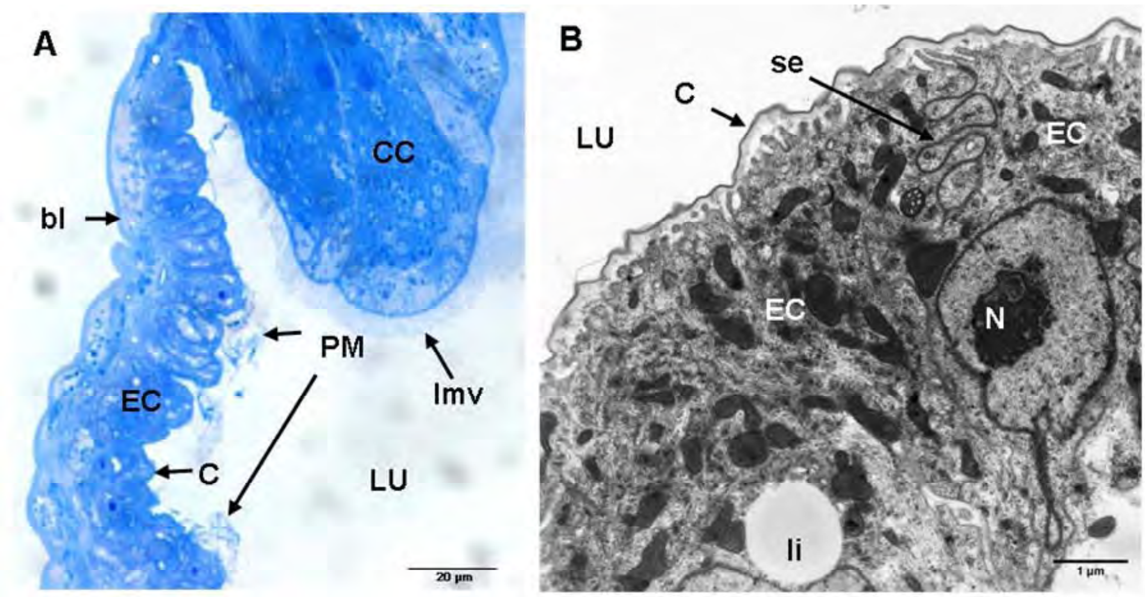


Fig.10.

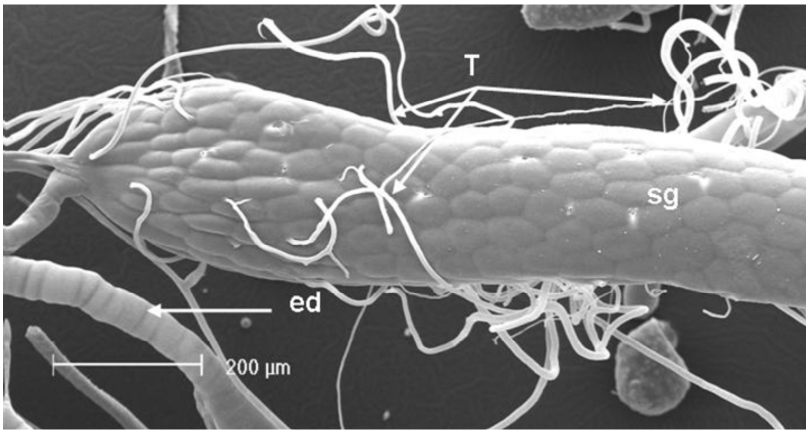


Fig.11.

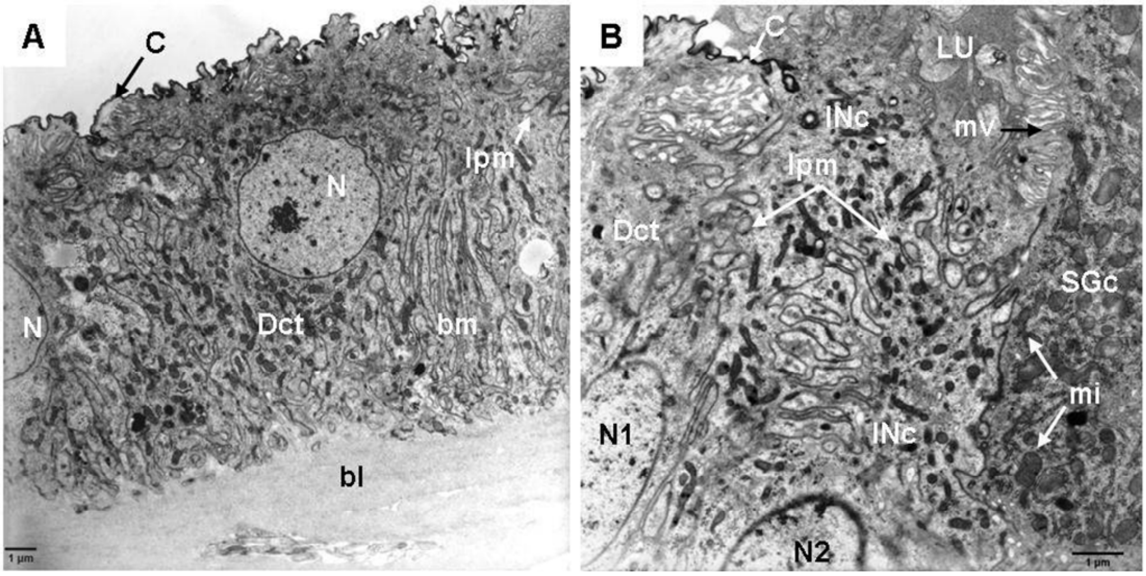
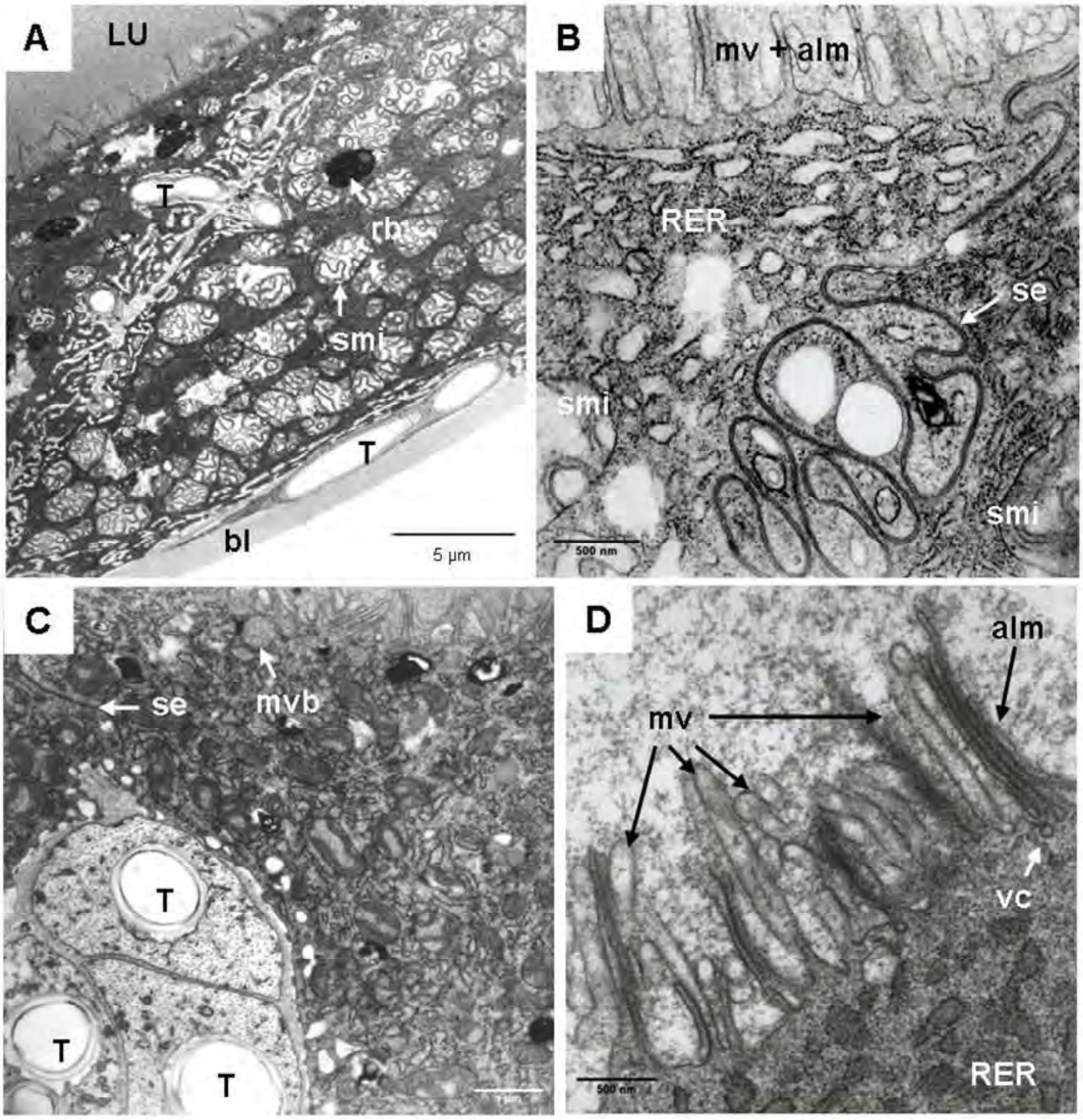


Fig.12.


A
LU
T
rb
smi
T
bl
5 µm
B
mv + alm
RER
se
smi
smi
500 nm
C
se
mvb
T
T
T
1 µm
D
alm
mv
vc
RER
500 nm

4.2 Three different approaches to the analysis of *G. intestinalis* larvae

4.2.1 Fast performance liquid chromatography, FPLC

The protein composition of *G. intestinalis* larvae is unknown; therefore a first trial was made to separate L3 crude larval extract into fractions by fast performance liquid chromatography (FPLC).

4.2.1.1 Superose 6 column

First separations of L3 crude larval extracts were performed on a Superose 6 column. The two runs gave similar profiles (Fig.38).

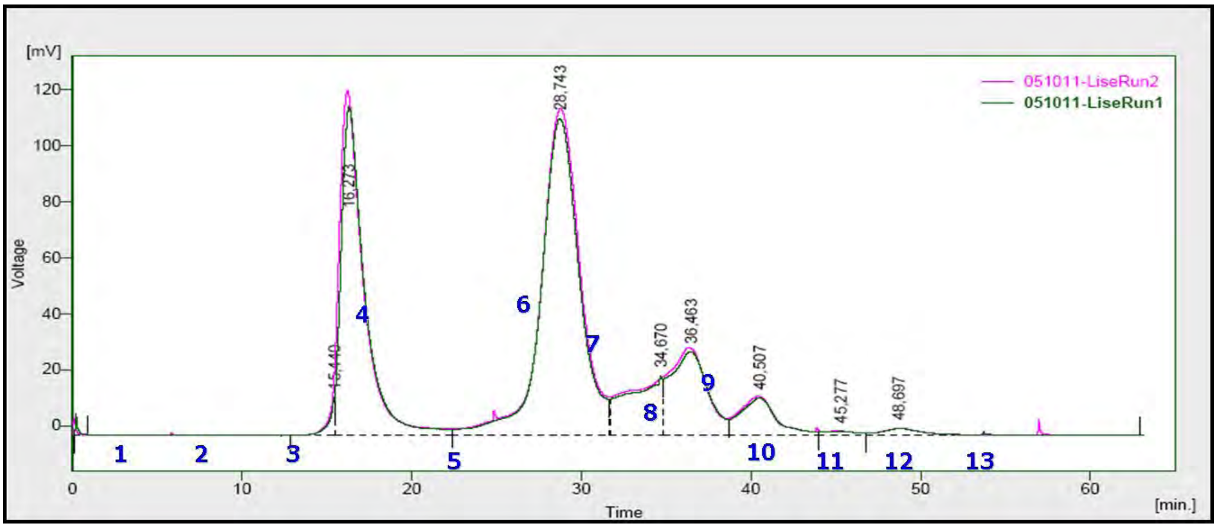


Fig.38. Elution profile of Superose 6 gel filtration chromatography.

The different fractions collected were separated on SDS electrophoresis gel (Fig.39) and transferred onto nitrocellulose membrane for Western-blot.

Western-blots were performed with horse sera (originating from the Swiss Jura) but almost no signal could be detected, except for a very weak signal emerging from fractions 8 and 9, at approximately 17 KDa.

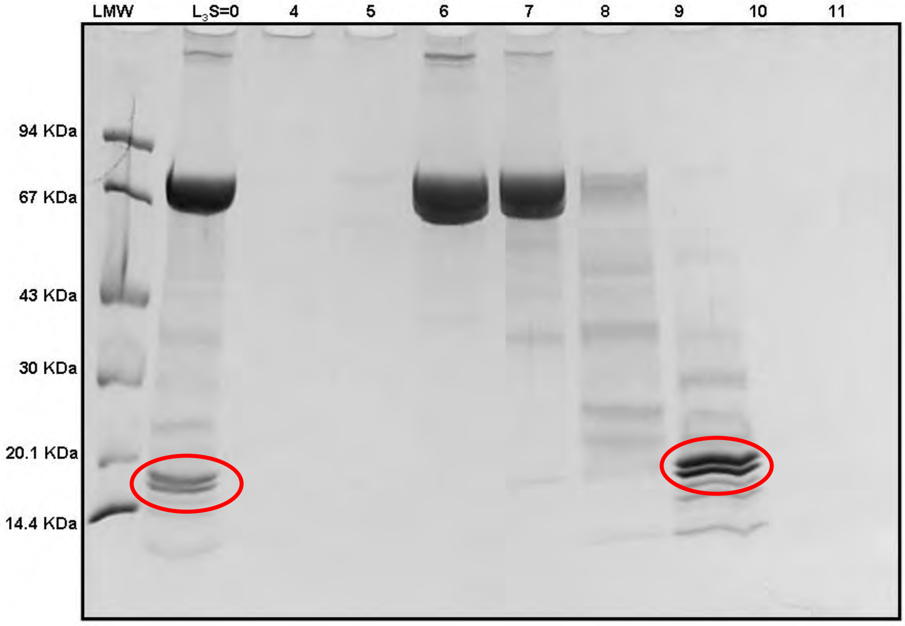


Fig.39. Analysis by SDS gel electrophoresis of fractions eluted from the FPLC Superose 6 column. Line 1 = LMW (low molecular weight-SDS marker). Line 2 = L_3S (crude larval extract). Lines 3-10 = fractions 4-11 eluted from Superose 6 column. The bands which were immune reactive are encircled.

4.2.1.2 MonoQ column

Fractions 8 and 9 collected from previous separation on superose 6 column were applied to a MonoQ ion-exchange chromatography (Fig.40).

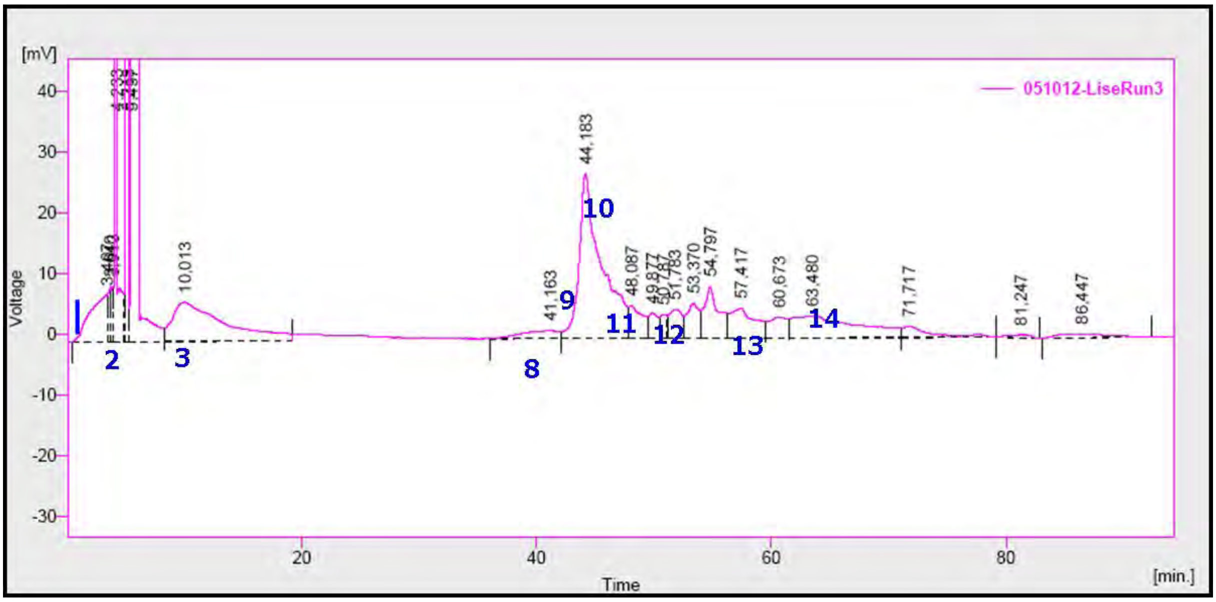


Fig.40. Elution profile of fraction 8 and 9 (lane 7 and 8 of Fig.2) chromatographed on Mono Q 5/5.

The fractions collected were separated by SDS gel electrophoresis (Fig.41) and transferred onto nitrocellulose membrane for Western-blot. Western blots were performed with sera of horses originating from the Swiss Jura.

Similarly to the Western blot performed with the fractions collected from the superose 6 column, no immune reactive band could be detected. In addition to this lack of immune reactivity, the results obtained with the MonoQ column were difficult to evaluate as the elution profile was weird and the results could not be reproduced.

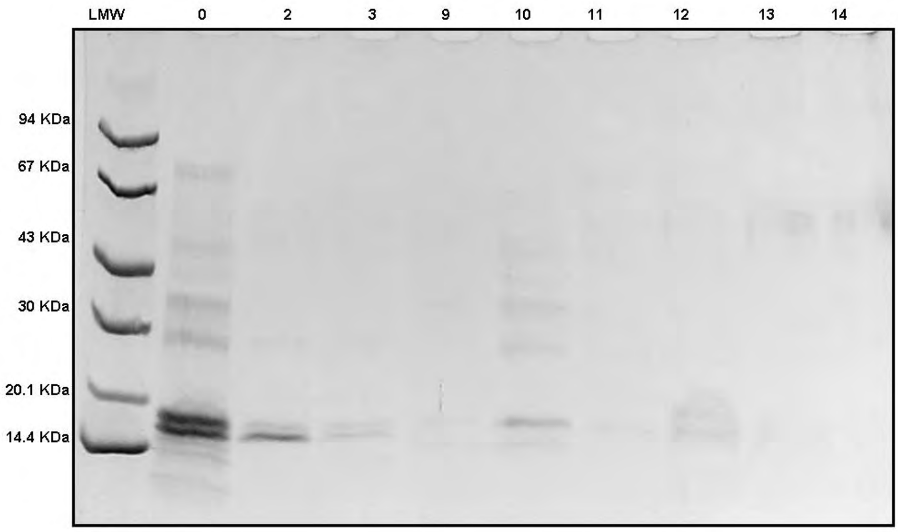


Fig.41. Analysis by SDS gel electrophoresis of fractions eluted from the Mono Q column. Line 1 = LMW (low molecular weight-SDS marker). Line 2 (called 0) = sample 8 and 9 injected into the column. Lines 3-10 = fractions 2, 3, 9-14 eluted from Mono Q column.

4.2.2 Enzyme-linked immunosorbent assay (ELISA)

4.2.2.1 Sera from Greenlandic and Swiss horses *vs. G. intestinalis* extracts

The sera of four Greenlandic horses were exposed to *G. intestinalis* crude larval extracts (L2 and L3) and L3 fractions (salivary glands, fatbody, tracheal cells and midgut) and compared to the reaction of four horses coming from Swiss Jura known to be infested by *G. intestinalis* larvae (Fig.42).

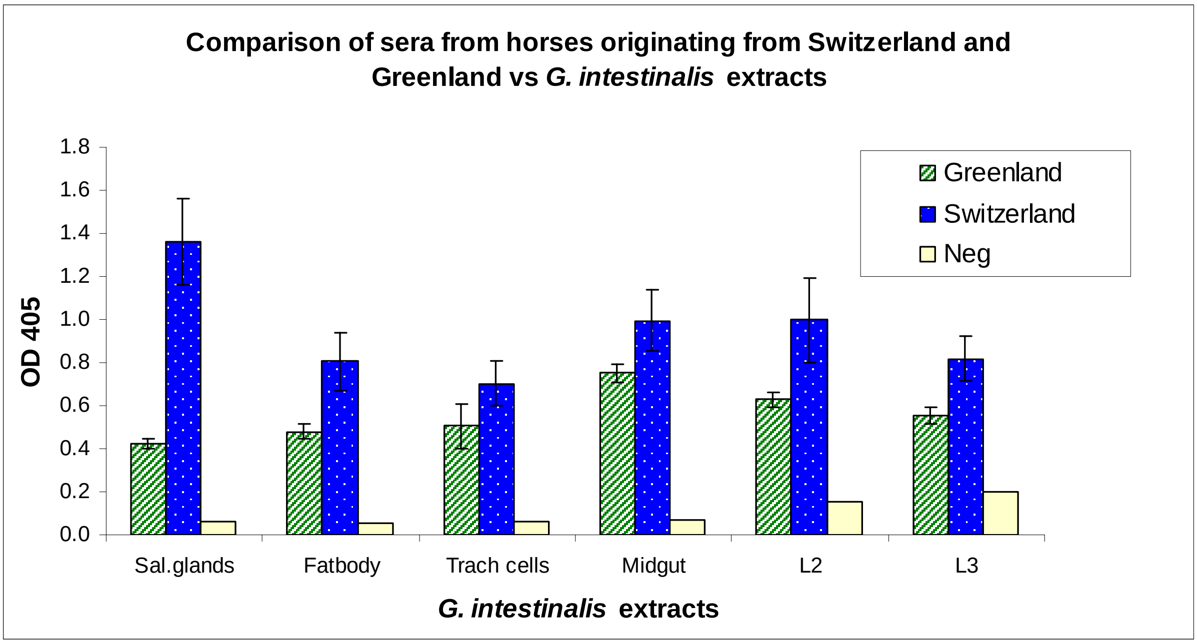


Fig.42. Optical density (OD) measurements of indirect ELISA tests performed with sera of horses originating from Greenland (N=4) and the Swiss Jura (N=4). Secondary antibodies (anti horse) were used as negative control. The larval antigens are L3 salivary glands, fatbody, tracheal cells, midgut and crude larval extracts of L2 and L3. The values are expressed as mean +- SEM (standard error of the mean).

Unexpectedly, the sera of Greenlandic horses also have antibodies against every *G. intestinalis* extract. Even if the OD values of the reactions are lower for the Greenlandic than for the Swiss horses, there is an antibody reaction of sera from Greenlandic horses to each extract of *G. intestinalis*.

4.2.2.2 Horse and immunised-mice sera *vs. G. intestinalis* extracts

Sera samples from 9 horses originating from the Swiss Jura, 4 mice immunised with L3 crude larval extracts (mice-L3) and 4 mice immunised with a *G. intestinalis* hemoglobin recombinant molecule (mice-Hb) were examined for antibodies to crude antigens of *G. intestinalis* second instar (L2 hemolymph) and third instar (salivary glands, midgut, fatbody, tracheal cells and L3 hemolymph) using an ELISA-test (Fig. 43, 44).

The evolution of the immune reaction in immunised mice was checked by ELISA-tests and Western-blots. For each immunised animal, the immune reaction developed was optimal after 4 immunisations.

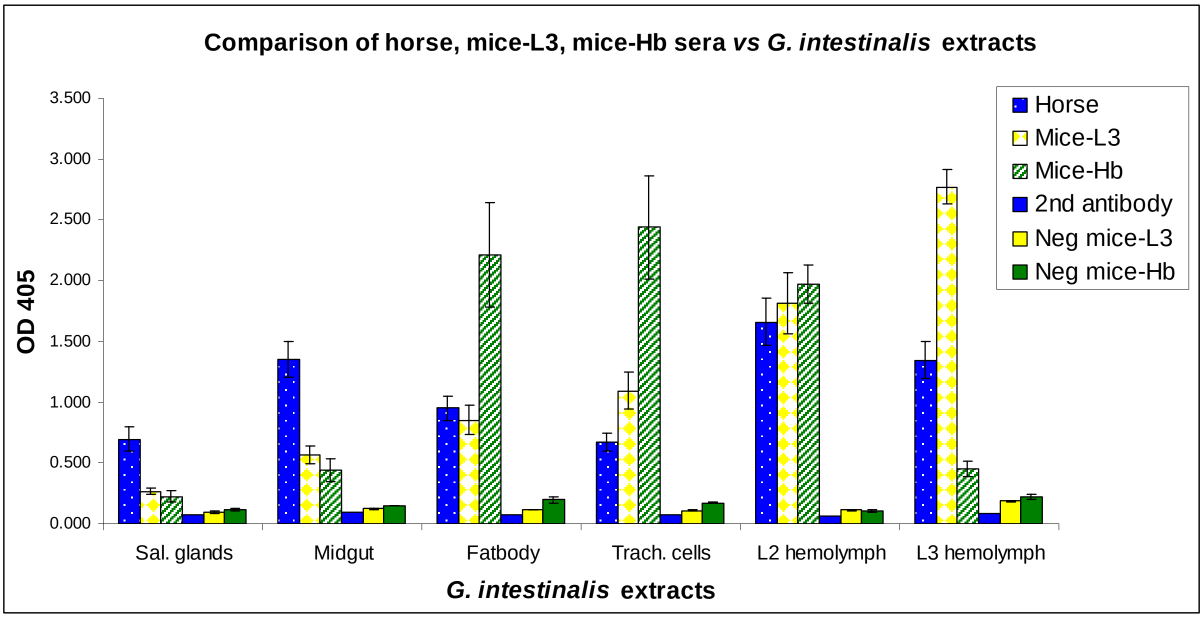


Fig.43: OD measurements of indirect ELISA tests performed with sera of horses originating from the Swiss Jura, naturally infested by *G. intestinalis* (N=9), mice-L3 (N=4) and mice-Hb (N=4). Secondary antibodies (anti horse) (N=1), negative mice-L3 (N=2) and negative mice-Hb (N=2) were used as negative controls. The values are expressed as mean +- SEM (standard error of the mean).

The mice immunised with the recombinant Hb molecule show an immune reaction against the larval extracts that contain hemoglobin molecules (fatbody, tracheal cells and L2 hemolymph) (Fig.43,44). The horse show a stronger immune reaction against the midgut extract and salivary glands, compared to the immunised mice. Mice-L3 show a stronger immune reaction against the L3 hemolymph compared to horse sera and to mice-Hb. No clear difference can be observed between horse and mice-L3 sera against the salivary glands, fatbody, tracheal cells and L2 hemolymph.

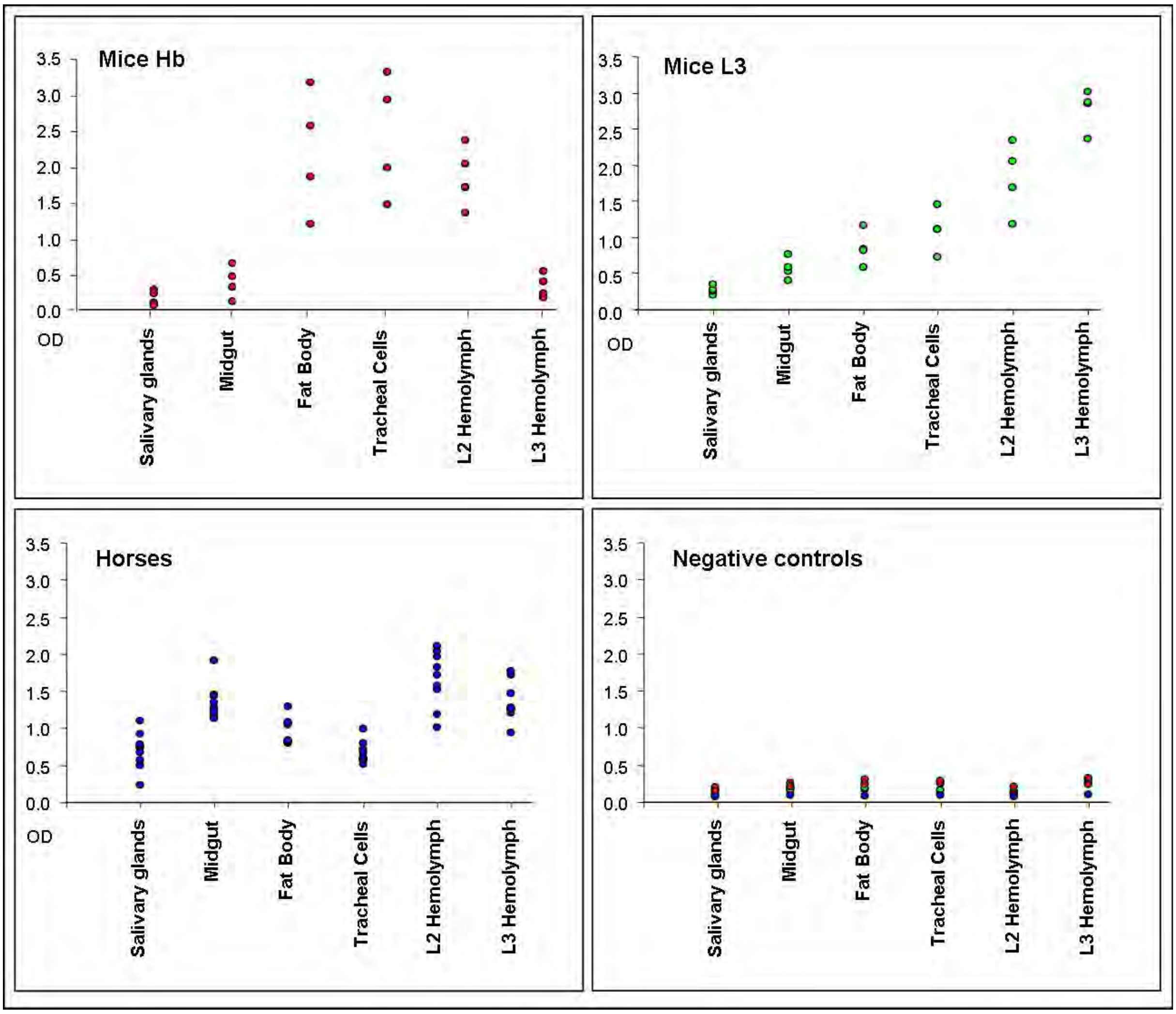


Fig.44. OD measurements of the antibody reaction of the sera of each individual against the different *G. intestinalis* larval extracts (salivary glands, midgut, fatbody, tracheal cells, L2 and L3 hemolymph). Mice-Hb (N = 4), mice-L3 (N = 4) horses (N = 9), negative controls: negative mice-L3 (N=2), negative mice-Hb (N=2), anti-horse secondary antibody (N=1).

4.2.2.3 Sera from mares, foals, umbilical blood and colostrum originating from France *vs. G. intestinalis* extracts

The sera of mares (N=8), new born foals prior to colostrum uptake (N=8) and umbilical blood (N=8) were examined for antibodies to crude larval extracts of *G. intestinalis* (L2 and L3) (Fig.45). The sera of horses (N=4) from the Swiss Jura were used for comparison and secondary antibody (anti horse) was used as negative control.

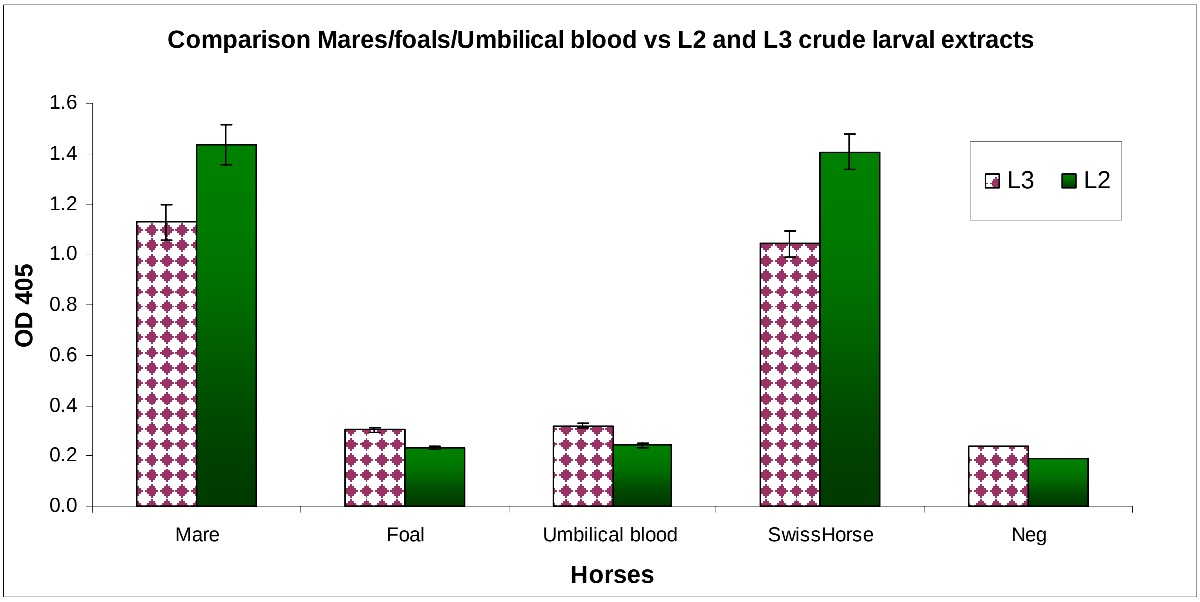


Fig.45. OD measurements of indirect ELISA tests performed with sera of mares originating from Nantes (N=8), their foals (N=8) and the associated umbilical blood (N=8) and the sera of horses originating from the Swiss Jura (N=4) against the L2 and L3 LCE. Secondary antibody (anti horse) was used as negative control. The values are expressed as mean +- SEM (standard error of the mean).

The sera of the mares from France and the sera of the horses coming from the Swiss Jura show similar antibody reaction when exposed to L2 and L3 crude larval extracts. In both cases the value of the OD is higher for the L2 then for the L3. The immune reactivity of umbilical blood and foals is similar to the OD value obtained with only using secondary antibodies and can be consequently considered as negative.

Mares, new born foal’s sera and colostrum were examined for antibodies against different *G. intestinalis* extracts (Fig.46).

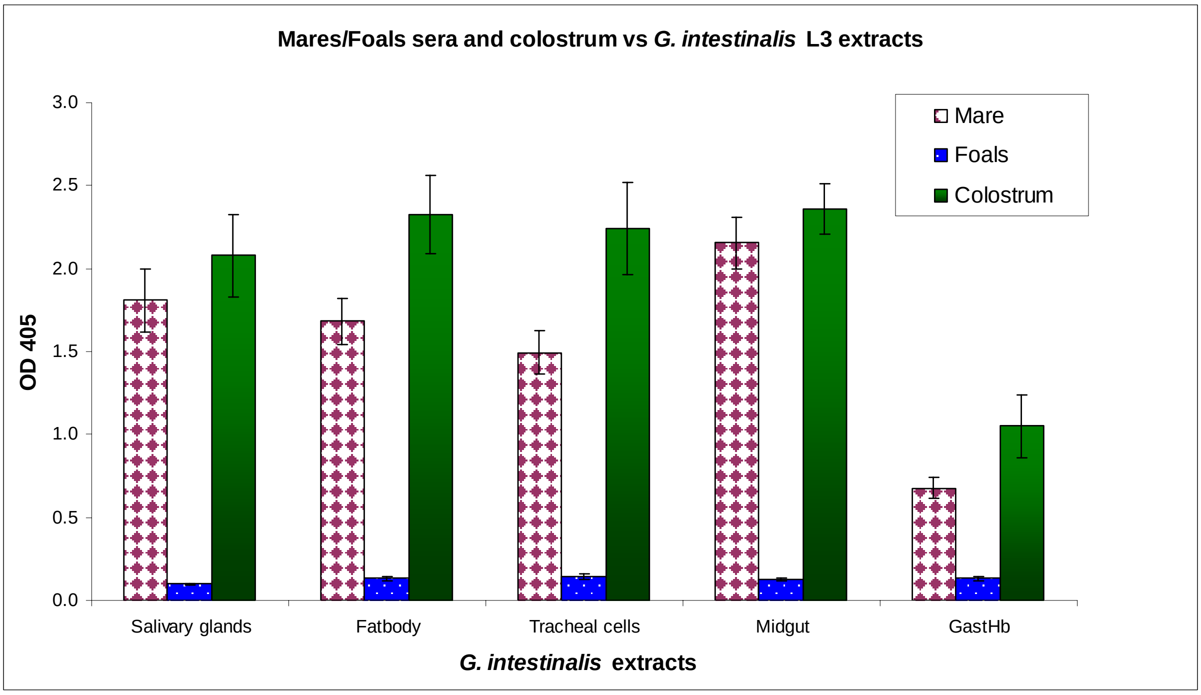


Fig.46. OD measurements of indirect ELISA tests performed with the sera of mares originating from Nantes (N=8), their foals (N=8), and the mare's colostrum (N=8) against third instar larvae extracts, including the salivary glands, fatbody, tracheal cells midgut and *Gasterophilus* hemoglobin recombinant molecule (Gast Hb). The values are expressed as mean +- SEM (standard error of the mean).

As previously observed, the foals did not show any antibody reaction when exposed to the different larval extracts and can be considered as negative. The mare's sera did react against each larval extract, but the reaction is more moderated against the pure molecule of *Gasterophilus* hemoglobin. The colostrum show similar reaction then observed with mare sera, with also a less strong reaction against the *Gasterophilus* hemoglobin.

4.2.3 Analysis of *G. intestinalis* first instar larvae

4.2.3.1 1-D analysis of L1, L2 and L3 crude larval extracts

The migration patterns of the larval crude extract (LCE) of the L1, L2 and L3 are compared on the 1-D silver-stained gel (Fig.47).

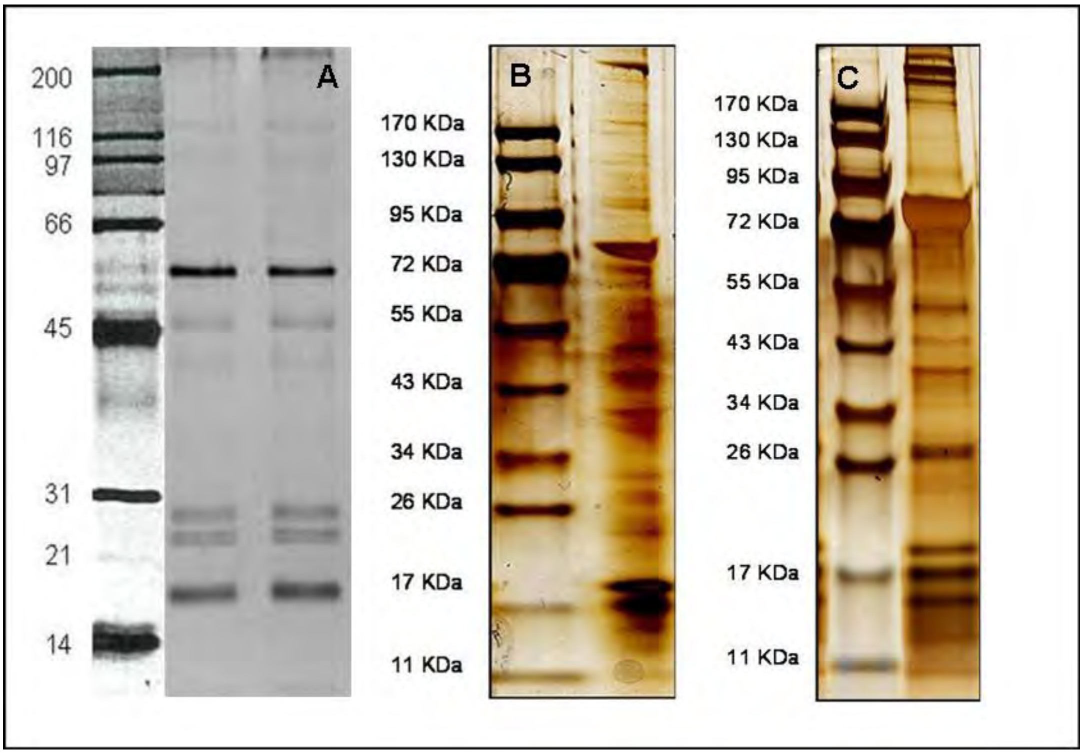


Fig.47. Representative 1-D silver-stained gel of larval crude extract of L1 (A), L2 (B) and L3 (C). The marker used for the L1 is not the same than the one used for the L2 and L3.

L1, L2 and L3 LCE were separated on SDS-PAGE and transferred onto nitrocellulose membranes for further Western blotting. Western blots were performed with sera of mice immunised against the three larval stages: mice-L1 (Fig.48A), mice-L2 (Fig.48B), mice-L3 (Fig.48C) and with horse serum (Fig.48D).

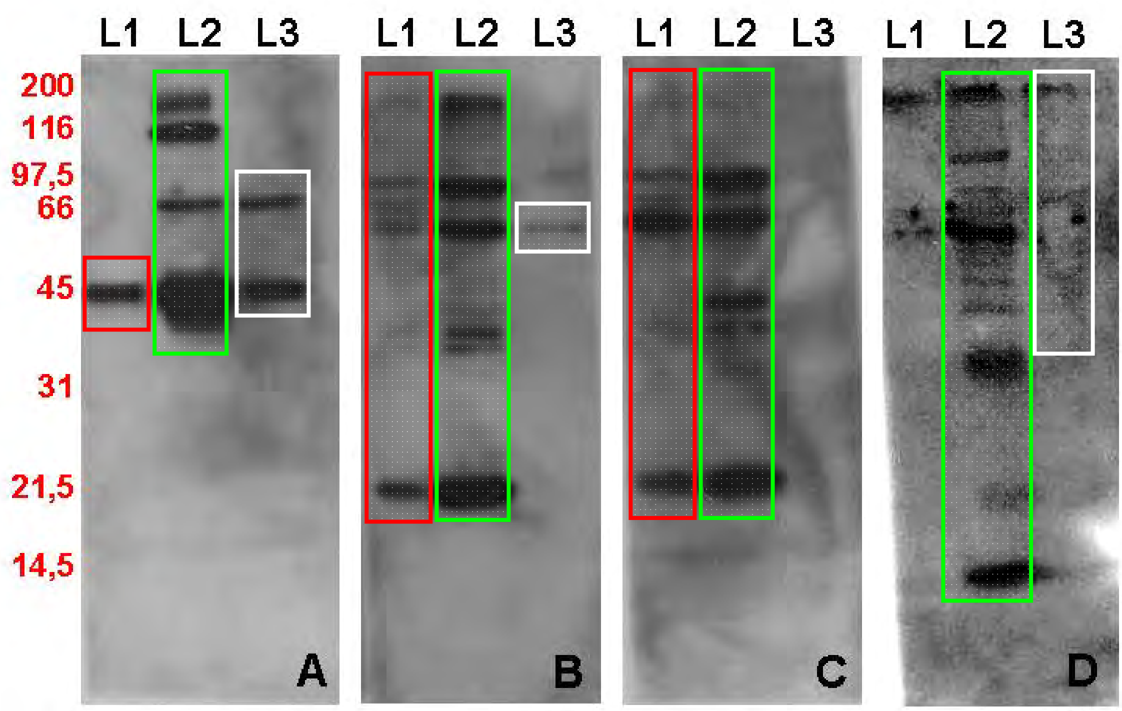


Fig.48. Western blot analysis of the three larval stages (L1, L2 and L3) exposed to sera from mice-L1 (A), mice-L2 (B), mice-L3 (C) and horse (D).

Several immune reactive bands are clearly visible when L1 LCE extract is exposed to L2-mice (Fig.48B, red frame) and L3-mice (Fig.48C, red frame), and one band appears when exposed to L1-mice (Fig.48A, red frame). The L2 LCE showed strong immune reactions when exposed to any sera (Fig. 48A,B,C, green frame), mostly in horses (Fig.48D, green frame), whereas the L3 LCE induced a weak immune reaction with only few immune reactive bands in each case (Fig.48A,B,D, white frame). Some bands were so weak they were almost impossible to detect (Fig.48C). The immunoblot profiles of L2-mice and L3 mice show many similarities (Fig 48B,C). Horse immunoblot profile (Fig.48D) is unclear and except for the L2 extract, it is difficult to identify the presence of immune reactive bands.

4.2.3.2 Sera from Swiss horses *vs.* L1 and L3 LCE of *G. intestinalis*

The sera of 12 horses originating from the Swiss Jura were examined for antibodies to L1 and L3 LCE at different concentrations (2.5, 5 and 10 µg/ml) (Fig.49).

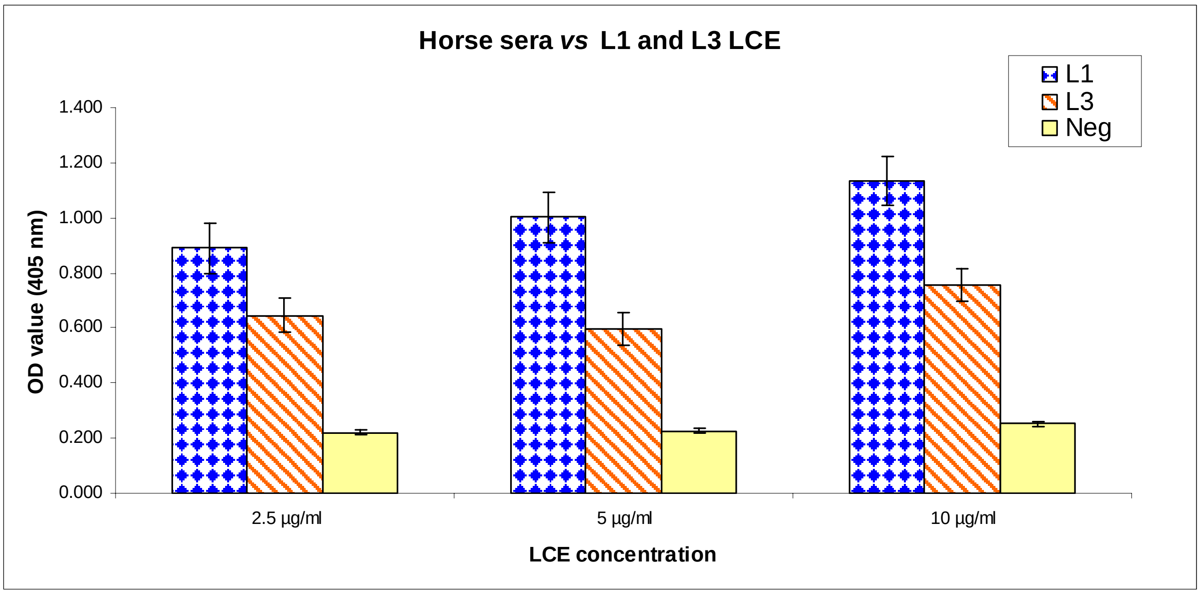


Fig.49. OD measurements of indirect ELISA tests performed with sera of horses originating from the Swiss Jura (N = 12), against the LCE of L1 and L3. Umbilical blood was used as negative control. The values are expressed as mean +- SEM (standard error of the mean).

These results demonstrate that the L1 LCE extract might induce a stronger immune reaction then L3 LCE when exposed to horse sera.

5 Discussion

The primary experiments performed to analyse the protein composition and the antigenic properties of *G. intestinalis* L3 crude larval extract, used chromatography. It was assumed that the separated fractions would exhibit different antigenic properties, which would be detected by immunoblotting with horse sera. If the separation of the L3 larval crude extract on the Superose 6 column allowed having a clear elution profile, the Western blots performed with the collected fractions did not give any significant signal, excepting for two bands. Those two fractions were further separated on a MonoQ column, best suited for chromatography where a higher level of resolution is needed. Unfortunately, the elution profile gave weird results that could not be reproduced. The Western blots with the fractions collected from the MonoQ column did not exhibit any signal. The weakness or the absence of signals might be associated to the chromogenic reaction, as the 3.3-Diaminobenzidine (DAB) was probably not best suited for immunoblotting staining these samples. Nowadays, the Enhanced Chemiluminescence (ECL) detection system is the most commonly used method for protein detection in Western blots, but this system was not available at the time when the FPLC were performed.

The ELISA tests are used to identify the presence of an antibody or an antigen in a sample and are known to be valid techniques to detect antibodies as demonstrated in other myiasis causing-species; e.g. *O. ovis* (Deconninck et al. 1995) or *H. bovis* (Sinclair and Wassall 1983). Performing ELISA involves at least one antibody with specificity for a particular antigen. A major disadvantage of the indirect ELISA is that the antigen fixation is non-specific, consequently any protein in a sample can attach to the microtiter plate well. While working with crude larval extracts or organ extracts of *G. intestinalis*, it is impossible to know which protein (or antigen) will fix to the well. Another controversial aspect of this test is the determination of the cut-off point between positive and negative results. Usually, it is the sera of young animals kept indoors that are used as negative control, and the cut-off values are determined as a value of two standard deviations ($P<0.05$) above the mean optical densities from negative control serum samples (Alcaide et al. 2005). But the lack of investigations and data concerning the immunological interaction between horses and *G. intestinalis* larvae did not allow establishing a cut-off point to interpret the ELISA results, and an important question remained: how to evaluate that a positive is a positive and a negative is a negative?

To overcome these problems two options were taken: (i) the localisation of equids that had not been in contact with *Gasterophilus* spp and whose sera will serve as negative controls (ii) the immunisation of mice with *G. intestinali*s L1, L2 and L3 crude larval extracts. The sera of these mice, in which the immune reaction had been artificially induced, were used to compare with the sera of horses, naturally infested by the parasite.

The probability that botflies would survive in Greenland seemed limited, not only because of the rough subarctic climate, but also from an epidemiological point of view, as only approx. 200 horses are distributed on the whole territory. Therefore, it was assumed that Greenlandic horses would be perfect negative controls; however, the sera of Greenlandic horses showed an immune reaction when exposed to *G. intestinalis* extracts. This might be a consequence of cross-reactions with other parasites present in horses and/or in sheep. Indeed the close proximity of the horses and sheep that remain in the same stable during the long winter period implicate also the sharing of some parasites. According to these results, it was impossible to use the sera of Greenlandic horses as negative controls and only the sera of immunized mice were used for further comparisons with horse sera.

Undoubtedly the identification of specific antigens involves the determination of the larval tissue that elicits the strongest host immune response (Tabouret et al. 2001). It has been demonstrated on several myiasis models that the content of secretory organs might provide target antigens (Pruett et al. 1988; Innocenti et al. 1995; Casu et al. 1996). Accordingly, different organs were collected from the L3 (salivary glands, midgut, fatbody and tracheal cells) and hemolymph was collected from both L2 and L3 and used as target antigens in the ELISA tests. The results do not allow to confirm that any organ induce a drastic stronger immune reaction with horse sera, even if the OD values of the midgut are higher compared to the salivary glands, the fatbody and the tracheal cells OD values. The mice immunized with the L3 LCE show a stronger immune reaction against the extracts that are the most abundant in larval body, i.e. the fatbody, tracheal cells and both hemolymph. However, a specific antigen-antibody reaction was generated in mice-Hb by using the specific *G. intestinalis* hemoglobin molecule. The mice immunised with the recombinant molecule of *G. intestinalis* hemoglobin show an immune reaction exclusively against the larval organs that naturally contain hemoglobin. This molecule is very specific to *G. intestinalis* (Pesce et al. 2005) and the existence of horse specific antibodies against this hemoglobin should be further evaluated.

The sera from two groups of horses originating from two different areas endemic to *G. intestinalis*, in Nantes and in the Swiss Jura, demonstrated a similar immune reaction when exposed to L2 and L3 LCE. The results allow to suppose that the L2 LCE is most likely to induce a stronger immune reaction than the L3 LCE. The umbilical and foal blood examined for antibodies to L2 and L3 LCE did not show any immune reaction. This can be explained by the fact that the transfer of circulating antibodies from mare's blood to the foal is prevented by placental barrier and only low levels of antibodies are present in foal's blood at birth. The foal's immune system can produce antibodies from 2-3 weeks of age and a full scale response can only be observed after 10-12 weeks. The colostrum provides the antibodies that are concentrated into mare's milk 10-14 days prior to foaling and is essential to foals survival during the first weeks of life. A foaled mare produces colostrum for only 2-3 days but the antibody concentration present in it decreases rapidly. The colostrum tested against the different *G. intestinalis* extracts demonstrated the presence of antibodies against all the extracts. Thus, it would be interesting to test if the foals develop, at least for a certain period after colostrum uptake, an immune reaction against *G. intestinalis* larval extracts without being infested.

The analysis of the L1 by SDS-PAGE, Western blot and ELISA allowed bringing into light the importance of this larval stage. Indeed, these larvae are the first to make a physical contact with their host by entering the tongue epithelium and consequently they are the most likely to stimulate the horse immune system. But the general lack of enthusiasm to study the L1 can be explained by the difficulties to collect and prepare enough quantities of material for the experimental procedures. The comparison of the 1-D migration pattern of the L1, L2 and L3 LCE demonstrate that they are all stage specific, thus the hypothesis that the L2 and L3 possess their own structural, metabolic and antigenic properties (publication 1), should also encompass the L1. The Western blot analysis demonstrates that the L2 LCE possesses the most antigenic properties when exposed to any sera (immunised mice or horse sera) and the L1 LCE has more antigenic properties than the L3 LCE. The immunoblot profiles of the L1-mice, L2-mice and L3-mice might indicate that there are proteins that are synthesized in the L1 (recognized by both mice-L2 and mice-L3), that are fully expressed in the L2, for instance during migration phase, and that are no longer present in the L3 (absence or few immune reactive from almost each sera).

The ELISA analyses support the hypothesis that the L1 LCE has more antigenic properties compared to the L3. However, the results obtained with the L1 must be interpreted with cautious because the larvae might not have entirely finished their embryonic period inside the egg when collected and thus they might not possess their full range of proteins.

According to the difficulties that were encountered during the ELISA analyses, especially with regard to the identification of specific antigens, attention focused on the proteomic-based analyses (publication 1) that have recently demonstrated their effectiveness (Jungblut et al. 1999; Dea-Ayuela and Bolas-Fernandez 2005; Shin et al. 2005). The comparison of the migration patterns of the L2 and L3 LCE in both dimensional analysis (1-D and 2-D), indicated that the larval proteinic profile is stage specific, thus suggesting a different composition in larvae metabolism and antigenic properties. The Western blots that were performed with horse and immunised mice sera did reveal a different immune reaction in naturally infected horses *vs.* artificially induced immune reaction in mice. Indeed, the mice were exposed by subcutaneous injections to the crude protein extracts from the whole larvae, and consequently to proteins that are normally not directly in contact with horses, whereas the mucosal immune system of horses is in direct contact with the larvae and thus exposed to the excreted and secreted substances. This difference can explain the fact that most of the L2 and L3 proteins that reacted with mice sera showed a more intense signal when compared to the reaction with horse sera. The comparisons of the immunoblot profiles demonstrated that the stage L2 is more immunogenic than the stage L3, most likely as an effect of the highest enzymatic production of L2 while migrating into the host tissues. Conversely, the fact that L3 remains attached for 8-10 months to the stomach wall suggest a hypometabolic status, or reduced immunogenic properties induced by immunosuppressive agents. This can explain the weak immune reaction observed in the presence of horse serum against the L3. A total of 27 bands and 65 spots were selected for the identification by mass spectrometry. Two important larval proteins, arylphorin and LSP-2 (larval serum protein), respectively homologous to *C. vicina* and *D. melanogaster*, were identified in the L3. Most of the other identified proteins in L2 (paramyosin, tubulin, tropomyosin, GAPDH and a protein similar to actin) or in L3 (filamin, fumarase, PEPCK, HSP-70, enolase) are shared with those of *Drosophila* spp. suggesting that structural or metabolic homologies do exist between these species. The only protein specific to *G. intestinalis* that could be identified was the hemoglobin molecule. That only fifteen proteins could be identified among the 92 proteins selected during the whole proteomic analyses might be a consequence of the limited number of higher Dipterans species represented by DNA sequences (Nirmala et al. 2001).

During this research, different intestinal parasites (*Anoplocephala perfoliata*, *Parascaris equorum*, Cyathostominae) were simultaneously present with *G. intestinalis* in the gastro-intestinal tract of the slaughtered horses, thus the risk of cross-reactivity has still to be evaluated. This work provided further information into the understanding of the interaction between *G. intestinalis* and their host. The identification of most of the proteins will be the next step to define their role, their cellular or tissue localisation and their potential antigenic properties.

The description of the structure of the digestive system of *Gasterophilus* species was lacking, and this morphological study (publication 2) presented a first step toward the understanding of the functional morphology of the midgut and salivary glands of *G. intestinalis* second and third instars larvae. Because of the involvement of the peritrophic membrane in the organization of the digestive processes and since antigenic proteins were isolated from this structure in other myiasis-causing species (East and Eisemann 1993; East et al. 1993; Tellam et al. 2000), further research to characterize the function and composition of the PM in *Gasterophilus* larvae is of major importance. The occurrence of microorganisms in the anterior regions of the midgut should also receive specific attention, as their identification could bring useful information about *G. intestinalis* biology.

6 Conclusion

This work is a first step toward the comprehension of the immunological mechanisms and the functional morphology that link the *G. intestinalis* larvae to horses. The results demonstrate that it is during the migration phases, L1 and L2, that the larvae are the most likely to stimulate the horse immune system, while the L3 have to be more discreet during their long maturation period inside the host body. This project demonstrates the importance and the difficulty to identify specific antigen-antibody reactions.

Consequently, the new research projects should focus particularly on the following points:

- Determination of the stage specific antigens, by using proteomics

- Evaluation of the cross-reaction(s) with other parasites

- Analysis of the evolution of the immune system of horses in case of primo-infestation by *G. intestinalis* larvae

- Evaluation of the development of a defensive immune reaction from the host after several successive larval infestations

The understanding of the complex interaction between the myiasis-causing species and their host will enables the development of immunological tools, and the identification of antigenic proteins might lead to the isolation of a vaccine candidate for use in horses.

7 Summary

Nowadays, the immunological status of horses against *Gasterophilus intestinalis* remains unknown. The present study offered several approaches to better understand the interaction of *G. intestinalis* larvae with horses. A preliminary chromatography study of the third instar crude larval extracts (LCE) allowed to obtain a clear elution profile but no antigenic properties could be attributed to any collected fraction. Indirect ELISA tests were performed to analyse the antigenic properties of different *G. intestinalis* larval extracts (second and third instar LCE, salivary glands, midgut, fatbody, and tracheal cells) when exposed to horse sera. Since the sera of horses originating from Greenland could not be used as reliable negative controls, as they showed an immune reaction against the *G. intestinalis* larval extracts, an alternative was found by using experimental animals and several groups of Balb/C mice were immunised against *G. intestinalis* larval extracts. The results demonstrated that it could be assumed that the L2 LCE induce a stronger immune reaction in horses than the L3 LCE, but it was not clear if any specific larval organ was more immunogenic than another. The first instar larvae were investigated, using Western blots and ELISA tests, and their strong antigenic potential was demonstrated.

The proteomic profile of second and third instar larvae, investigated by using dimensional approaches, revealed a migration pattern specific to each larval stage. Furthermore, the Western blots performed with horse sera and with immunised mice sera, revealed a different immune reaction in naturally infected horses *vs.* artificially induced immune reaction in mice. The comparisons of the immunoblot profiles demonstrated that the stage L2 is more immunogenic than the stage L3 most likely as an effect of the highest enzymatic production of L2 while migrating into the host tissues. Fifteen proteins were identified by mass spectrometry.

The microscopic analyses focused on the description of the midgut and salivary glands of *G. intestinalis* second and third instar larvae. The presence of a peritrophic membrane type II was identified in the midgut and unidentified microorganisms were found in the anterior midgut. No major differences were observed in the structure between the two larval stages.

8 Résumé

De nos jours, le statut immunologique des chevaux contre *Gasterophilus intestinalis* est inconnu. Dans la présente étude, plusieurs approches sont proposées pour améliorer les connaissances sur l'interaction des larves de *G. intestinalis* avec les chevaux. Une étude préliminaire de l'extrait total du troisième stade larvaire par chromatographie a permis d'établir un profil d'élution mais aucune propriété antigénique n'a pu être attribuée aux fractions collectées. Des tests ELISA indirects ont été réalisés pour analyser les propriétés antigéniques de différents extraits larvaires de *G. intestinalis* (extraits larvaires totaux des stades 2 et 3, glandes salivaires, intestin, corps gras et cellules trachéales) lorsqu'ils sont exposés aux sérums de chevaux. Les sérums de chevaux provenant du Groenland n'ont pas pu être utilisés comme contrôles négatifs étant donné leur réactivité contre les extraits de *G. intestinalis*. Une alternative a été trouvée dans l'utilisation d'animaux de laboratoire et plusieurs groupes de souris Balb/C ont été immunisés contre différents extraits larvaires de *G. intestinalis*. Les résultats laissent à supposer que les extraits totaux de L2 induisent une plus forte réaction immunitaire chez les chevaux que les extraits totaux de L3. Cependant, aucun organe spécifique n'a pu être défini comme plus immunogénique qu'un autre. Les larves du premier stade larvaires ont été analysées par Western blot et test ELISA et leur fort potentiel antigénique a pu être mis en évidence.

Les profils protéomiques des deuxièmes et troisièmes stades larvaires ont été établis en utilisant une approche dimensionnelle et les profils de migration sont propres à chacun des stades. De plus, les analyses par Western blot réalisées avec les sérums de chevaux et de souris immunisées ont démontrés une réaction immunitaire différente entre les chevaux qui sont naturellement infestés par le parasite et les souris ou la réaction a été artificiellement induite. La comparaison des profils des immunoblots démontre que les L2 sont plus immunogéniques que les L3, très probablement suite à une plus forte production enzymatique des L2 lors de leur phase migratoire à travers les tissus de l'hôte. La spectrométrie de masse a permis d'identifier quinze protéines.

L'analyse microscopique s'est concentrée sur la description de l'intestin et des glandes salivaires des deuxièmes et troisièmes stades larvaires de *G. intestinalis*. Une membrane péritrophique de type II a été identifiée dans l'intestin ainsi que la présence de microorganismes. Aucune différence majeure n'a été observée entre les structures des deux stades larvaires.

9 References

Alcaide, M., D. Reina, E. Frontera and I. Navarrete (2005). "Analysis of larval antigens of *Oestrus ovis* for the diagnosis of oestrosis by enzyme-linked immunosorbent assay." Medical and Veterinary Entomology **19**(2): 151-7.

Anderson, E. and W. R. Harvey (1966). "Active transport by the cecropia midgut. II. Fine structure of the midgut epithelium." Journal of Cell Biology **31**(1): 107-34.

Anderson, G. S. (2000). "Minimum and maximum development rates of some forensically important Calliphoridae (Diptera)." Journal of Forensic Sciences **45**(4): 824-32.

Anderson, J. R. (2006a). Adult Biology. The Oestrid Flies. Biology, Host-parasite relationships, impact and management. D.D. Colwell, Hall M.J.R. and Scholl P.J. Oxfordshire, United-Kingdom, CABI Publishing**:** 140-166.

Anderson, J. R. (2006b). Oestrid Myiasis of Humans. The Oestrid Flies. Biology, Host-parasite relationships, impact and management. D.D. Colwell, Hall M.J.R. and Scholl P.J. Oxfordshire, United-Kingdom, CABI Publishing**:** 201-209.

Angulo-Valadez, C. E., R. Cepeda-Palacios, F. Ascencio, P. Jacquiet, P. Dorchies, M. J. Romero and R. M. Khelifa (2007). "Proteolytic activity in salivary gland products of sheep bot fly (*Oestrus ovis*) larvae." Veterinary Parasitology **149**(1-2): 117-25.

Angulo-Valadez, C. E., R. Cepeda-Palacios, P. Jacquiet, P. Dorchies, F. Prevot, F. Ascencio-Valle, J. M. Ramirez-Orduna and F. Torres (2007). "Effects of immunization of Pelibuey lambs with *Oestrus ovis* digestive tract protein extracts on larval establishment and development." Veterinary Parasitology **143**(2): 140-6.

Anton-Erxleben, F., J. Miquel and D. E. Philpott (1983). "Fine-structural changes in the midgut of old *Drosophila melanogaster*." Mechanisms of Ageing and Development **23**(3-4): 265-76.

Araujo Jorge, T. C., J. L. Charbon and K. Pfister (1994). Influence of hypodermosis on the incidence of other diseases in cattle. Improvements in the control methods for warble-fly in cattle and goats. K. Edited by Pfister, J. L. Charbon, D. W. Tarry and K. Pithan. COST 811. European Commission, Brussels**:** 121-126.

Aurstad, K. and A. H. Skeie (1973). "Proteolytic and lipolytic enzymes from larvae of *Oedemagena tarandi* (L.)." Acta Veterinaria Scandinavica **14**(1): 168-75.

Baird, J. K., C. R. Baird and C. W. Sabrosky (1989). "North American cuterebrid myiasis. Report of seventeen new infections of human beings and review of the disease." Journal of the American Academy of Dermatology **21**(4 Pt 1): 763-72.

Baker, J. E., S. M. Woo and R. V. Byrd (1984). "Ultrastructural features of the gut of *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) with notes on distribution of proteinases and amylases in crop and midgut. ." Canadian Journal of Zoology **62**: 1251-1259.

Baron, R. W. and D. D. Colwell (1991). "Mammalian immune responses to myiasis." Parasitology Today **7**(12): 353-5.

Baron, R. W. and J. Weintraub (1986). "Immunization of cattle against hypodermatosis (*Hypoderma lineatum* (deVill.) and *H. bovis* (L.)) using *H. lineatum* antigens." Veterinary Parasitology **21**(1): 43-50.

Baron, R. W. and J. Weintraub (1987a). "Immunological responses to parasitic arthropods." Parasitology Today **3**(3): 77-82.

Baron, R. W. and J. Weintraub (1987b). "Lymphocyte responsiveness in cattle previously infested and uninfested with *Hypoderma lineatum* (de Vill.) and *H. bovis* (L.) (Diptera: Oestridae)." Veterinary Parasitology **24**(3-4): 285-96.

Barraclough, D. A. (2006). "Bushels of bots: Africa's largest fly is getting a reprieve from extinction." Natural History Retrieved 31.10.2008 from http://findarticles.com/p/articles/mi_m1134/is_5_115/ai_n16519922

Battisti, A., G. Panfili, G. Di Guarda and M. Principato (1997). "*Gasterophilus pecorum* larvae in an aged lion." The Veterinary Record **140**(25): 664.

Bautista-Garfias, C. R., R. M. Angulo-Contreras and E. Garay-Garzon (1988). "Serologic diagnosis of *Oestrus ovis* (Diptera:Oestridae) in naturally infested sheep." Medical and Veterinary Entomology **2**(4): 351-5.

Benakhla, A., J. F. Lonneux, A. Mekroud, B. Losson and C. Boulard (1999). "[Bovine hypodermosis in north-eastern Algeria: prevalence and intensity of infestation]." Veterinary Research **30**(5): 539-45.

Bernal, D., J. E. de la Rubia, A. M. Carrasco-Abad, R. Toledo, S. Mas-Coma and A. Marcilla (2004). "Identification of enolase as a plasminogen-binding protein in excretory-secretory products of *Fasciola hepatica*." FEBS Letters **563**(1-3): 203-6.

Bernard, N., C. Collombert, G. Tariel and C. Lamidey (1994). Epidemiological survey of bot infestations in horses at necropsy in Normandy from April 1990 to March 1992. Recueil de Médecine Vétérinaire de l'Ecole d'Alfort 170**:** 231-235.

Billingsley, P. F. and M. J. Lehane (1996). Structure and ultrastructure of the insect midgut. Biology of the Insect Midgut. M. J. Lehane and P. F. Billingsley. London, Chapman & Hall**:** 3-30.

Blum, H., H. Beier and J. Hans, Gross (1987). "Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels." Electrophoresis **8**(2): 93-99.

Boldbaatar, D., X. Xuan, E. Kimbita, X. Huang, I. Igarashi, B. Byambaa, B. Battsetseg, B. Battur, G. Battsetseg, Z. Batsukh, H. Nagasawa, K. Fujisaki and T. Mikami (2001). "Detection of antibodies to *Hypoderma lineatum* in cattle by Western blotting with recombinant hypodermin C antigen." Veterinary Parasitology **99**(2): 147-54.

Borgsteede, F. H. and G. van Beek (1998). "Parasites of stomach and small intestine of 70 horses slaughtered in The Netherlands." Veterinary Quarterly **20**(1): 31-4.

Boulard, C. (1969). "Anatomie et histologie du tube digestif de la larve d'*Hypoderma bovis* (Diptera: Oestriforme)." Annales de la Société Entomologique de France **5**: 371-387.

Boulard, C. (1970). "[Preliminary study of crude collagenase extracted from the 1st stage larva of *Hypoderma lineatum* (de Villers)]." C R Acad Sci Hebd Seances Acad Sci D **270**(10): 1349-51.

Boulard, C. (1975). "[Comparative evolution of blood antibodies in cattle infested with hypodermosis and treated with different insecticides (author's transl)]." Annales de Recherches Vétérinaires **6**(2): 143-54.

Boulard, C. (1975). "[Histological tissue study of cattly harboring the *Hypoderma lineatum* parasite (De Vill.) (Diptera-Oestriform) (author's transl)]." Annales de Recherches Vétérinaires **6**(2): 131-42.

Boulard, C. (1989). "Degradation of bovine C3 by serine proteases from parasites *Hypoderma lineatum* (Diptera, Oestridae)." Veterinary Immunology and Immunopathology **20**(4): 387-98.

Boulard, C. (2002). "Durably controlling bovine hypodermosis." Veterinary Research **33**(5): 455-64.

Boulard, C. (2006). Part A: Hypodermatinae Host-Parasite Interactions. The Oestrid Flies. Biology, Host-parasite relationships, impact and management. D.D. Colwell, Hall M.J.R. and Scholl P.J. Oxfordshire, United Kingdom, CABI Publishing**:** 167-179.

Boulard, C., Argente G. and H. E. (1988). "Hypodermose bovine. 1ere partie: description et incidence économique." Le Point Vétérinaire **20 (111)**: 17-30.

Boulard, C. and F. Bencharif (1984). "Changes in the haemolytic activity of bovine serum complement by *Hypoderma lineatum* (insect oestridae) larval proteinases in naive and immune cattle." Parasite Immunology **6**(5): 459-67.

Boulard, C. and J. Petithory (1977). "Serological diagnosis of human hypodermosis: a preliminary report." Veterinary Parasitology **3**: 259-263.

Boulard, C., C. Villejoubert and N. Moire (1996). "Cross-reactive, stage-specific antigens in the Oestridae family." Veterinary Research **27**(4-5): 535-44.

Brocard, P. (1991). Approche de la Gastérophilose en Suisse. Faculté de la Médecine Vétérinaire. Bern, University of Bern. **PhD**.

Brocard, P. and K. Pfister (1991). "[The epidemiology of gasterophilosis of horses in Switzerland]." Schweizer Archiv für Tierheilkunde **133**(9): 409-16.

Buse, C., F. Altmann, B. Amann, S. M. Hauck, C. Poulsen Nautrup, M. Ueffing, M. Stangassinger and C. A. Deeg (2008). "Discovering novel targets for autoantibodies in dilated cardiomyopathy." Electrophoresis **29**(6): 1325-32.

Casais, R., J. M. Martin Alonso, J. A. Boga and F. Parra (1998). "*Hypoderma lineatum*: expression of enzymatically active hypodermin C in Escherichia coli and its use for the immunodiagnosis of hypodermosis." Experimental Parasitology **90**(1): 14-9.

Casu, R. E., C. H. Eisemann, T. Vuocolo and R. L. Tellam (1996). "The major excretory/secretory protease from *Lucilia cuprina* larvae is also a gut digestive protease." International Journal for Parasitology **26**(6): 623-8.

Catts, E. P. (1979). "Hilltop aggregation and mating behavior by *Gasterophilus intestinalis* (Diptera: Gasterophilidae)." Journal of Medical Entomology **16**: 461-464.

Catts, E. P. (1982). "Biology of New World bot flies: Cuterebridae." Annual Review of Entomology **27**: 313-338.

Chabaudie, N. and C. Boulard (1992). "Effect of hypodermin A, an enzyme secreted by *Hypoderma lineatum* (Insect Oestridae), on the bovine immune system." Veterinary Immunology and Immunopathology **31**(1-2): 167-77.

Chamberlain, W. F., Barrett C.C. and G. O.H. (1969). "Integumental absorption versus oral ingestion of phosphate and other materials by common cattle grubs held in vitro." Annals of the Entomological Society of America **62**: 843-846.

Chapman, A. D. (2005). Numbers of Living Species in Australia and the World. Toowoomba, Australia, Australian Biodiversity Information Services. A Report for the Department of the Environment and Heritage.

Chapmann, R. F. (1982). The Insects. Structure and function. Cambridge, Harvard University Press.

Charbon, J. L., M. A. Tieche, C. Villejoubert, C. Boulard and K. Pfister (1995). "[Epidemiology of bovine hypodermyiasis in canton Vaud: comparison of two methods of mapping the infestation with a view to strategic treatment]." Schweiz Arch Tierheilkd **137**(8): 363-8.

Charles, J. F. (1987). "Ultrastructural midgut events in Culicidae larvae fed with *Bacillus sphaericus* 2297 spore/crystal complex." Annales de l'Institut Pasteur Microbiology **138**(4): 471-84.

Cirak, V. Y., C. Hermosilla and C. Bauer (1996). "Study on the gastrointestinal parasite fauna of ponies in northern Germany." Applied Parasitology **37**(4): 239-44.

Cogley, T. G. and J. R. Anderson (1983). "Ultrastructure and function of the attachment organ of *Gasterophilus* eggs (Diptera: Gasterophilidae)." International Journal of Insect Morphology and Embryology **12**(1): 13-23.

Cogley, T. P. (1989). "Effects of migrating *Gasterophilus intestinalis* larvae (Diptera: Gasterophilidae) on the mouth of the horse." Veterinary Parasitology **31**(3-4): 317-31.

Cogley, T. P. (1991). "Warble development by the rodent bot *Cuterebra fontinella* (Diptera: Cuterebridae) in the deer mouse." Veterinary Parasitology **38**(4): 275-88.

Cogley, T. P. (1999). "Morphology of a newly discovered sensory array on the mouthhooks of *Gasterophilus* larvae." Medical and Veterinary Entomology **13**(4): 439-46.

Cogley, T. P., J. R. Anderson and L. J. Cogley (1982). "Migration of *Gasterophilus intestinalis* larvae (Diptera:Gasterophilidae) in the equine oral cavity." International Journal for Parasitology **12**(5): 473-80.

Cogley, T. P. and M. C. Cogley (1999). "Inter-relationship between *Gasterophilus* larvae and the horse's gastric and duodenal wall with special reference to penetration." Veterinary Parasitology **86**(2): 127-42.

Cogley, T. P. and M. C. Cogley (2000). "Field observations of the host-parasite relationship associated with the common horse bot fly, *Gasterophilus intestinalis*." Veterinary Parasitology **88**(1-2): 93-105.

Colwell, D. D. (2006a). Life Cycle Strategies. The Oestrid Flies. Biology, Host-parasite relationships, impact and management. D.D. Colwell, Hall M.J.R. and Scholl P.J. Oxfordshire, United Kingdom, CABI Publishing**:** 67-77.

Colwell, D. D. (2006c). Larval Morphology. The Oestrid Flies. Biology, Host-parasite relationships, impact and management. D.D. Colwell, Hall M.J.R. and Scholl P.J. Oxfordshire, United Kingdom, CABI Publishing**:** 98-123.

Colwell, D. D. (2006d). Part C: Gasterophilinae Host-Parasite Interactions. The Oestrid Flies. Biology, Host-parasite relationships, impact and management. D.D. Colwell, Hall M.J.R. and Scholl P.J. Oxfordshire, United Kingdom, CABI Publishing**:** 189-191.

Colwell, D. D., Hall M.J.R. and P. J. Scholl (2006a). Introduction The Oestrid Flies. Biology, Host-parasite relationships, impact and management. D.D. Colwell, Hall M.J.R. and Scholl P.J. Oxfordshire, United Kingdom, CABI Publishing**:** 1-7.

Colwell, D. D., M. J. R. Hall and P. J. Scholl (2006b). A Synopsis of the Biology, Hosts, Distribution, Disease Significance and Management of the Genera. The Oestrid Flies. Biology, Host-parasite relationships, impact and management. D.D. Colwell, Hall M.J.R. and Scholl P.J. Oxfordshire, United Kingdom, CABI Publishing**:** 220-305.

Colwell, D. D., F. J. Martinez-Moreno, A. Martinez-Moreno, S. Hernandez-Rodriguez, C. de la Fuente-Lopez, J. M. Alunda and M. J. Hall (1998). "Comparative scanning electron microscopy of third-instar *Hypoderma* spp. (Diptera: Oestridae)." Medical and Veterinary Entomology **12**(2): 181-6.

Colwell, D. D., D. Otranto and I. G. Horak (2007). "Comparative scanning electron microscopy of *Gasterophilus* third instars." Medical and Veterinary Entomology **21**(3): 255-64.

Colwell, D. D. and P. J. Scholl (1995). "Cuticular sensilla on newly hatched larvae of *Gasterophilus intestinalis* and *Oestrus ovis*." Medical and Veterinary Entomology **9**(1): 85-93.

Cooper, J. E. and D. C. Housten (1972). "Lesions in the crop of vultures associated with bot fly larvae." Transactions of the Royal Society of Tropical Medicine and Hygiene **66**(3): 515-7.

Cope, S. E. and E. P. Catts (1991). "Parahost behavior of adult *Gasterophilus intestinalis* (Diptera: Gasterophilidae) in Delaware." Journal of Medical Entomology **28**(1): 67-73.

Costa, A. J., O. F. Barbosa, F. R. Moraes, A. H. Acuna, U. F. Rocha, V. E. Soares, A. C. Paullilo and A. Sanches (1998). "Comparative efficacy evaluation of moxidectin gel and ivermectin paste against internal parasites of equines in Brazil." Veterinary Parasitology **80**(1): 29-36.

Dart, A. J., D. R. Hutchins and A. P. Begg (1987). "Suppurative splenitis and peritonitis in a horse after gastric ulceration caused by larvae of *Gasterophilus intestinalis*." Australian Veterinary Journal **64**(5): 155-8.

Dea-Ayuela, M. A. and F. Bolas-Fernandez (2005). "Two-dimensional electrophoresis and mass spectrometry for the identification of species-specific *Trichinella* antigens." Veterinary Parasitology **132**(1-2): 43-9.

Deconninck, P., L. J. Pangui, L. Carriere and P. Dorchies (1995). "Detection of sheep nasal-bot (*Oestrus ovis*) in Senegal with specific ELISA-test." Revue de Médecine Vétérinaire **146**: 265-268.

Deeg, C. A., D. Pompetzki, A. J. Raith, S. M. Hauck, B. Amann, S. Suppmann, T. W. Goebel, U. Olazabal, H. Gerhards, S. Reese, M. Stangassinger, B. Kaspers and M. Ueffing (2006). "Identification and functional validation of novel autoantigens in equine uveitis." Molecular and Cellular Proteomics **5**(8): 1462-70.

Dewilde, S., M. Blaxter, M. L. Van Hauwaert, K. Van Houte, A. Pesce, N. Griffon, L. Kiger, M. C. Marden, S. Vermeire, J. Vanfleteren, E. Esmans and L. Moens (1998). "Structural, functional, and genetic characterization of *Gastrophilus* hemoglobin." Journal of Biological Chemistry **273**(49): 32467-74.

Dillon, R. J. and V. M. Dillon (2004). "The gut bacteria of insects: nonpathogenic interactions." Annual Review of Entomology **49**: 71-92.

Dinulescu, G. (1932). "Recherche sur la Biologie des Gastrophiles. Anatomie, physiologie, cycle évolutif." Annales des Sciences Naturelles, Zoologe, 10^{é} série: 1-183.

Dorchies, P., P. Jacquiet, J. P. Bergeaud, C. Duranton, F. Prevot, J. P. Alzieu and J. Gossellin (2001). "Efficacy of doramectin injectable against *Oestrus ovis* and gastrointestinal nematodes in sheep in the southwestern region of France." Veterinary Parasitology **96**(2): 147-54.

Dorchies, P., G. Tabouret, H. Hoste and P. Jacquiet (2006). Part D: Oestrinae Host-Parasite Interactions. The Oestrid Flies. Biology, Host-parasite relationships, impact and management. D.D. Colwell, Hall M.J.R. and Scholl P.J. Oxfordshire, United-Kingdom, CABI Publishing**:** 191-200.

Dorchies, P., J. M. Yilma and J. Savey (1993). "Lung involvement in ovine oestrosis: prevalence of lung abscesses and interstitial pneumonia." The Veterinary Record **133**: 325.

Douglas, A. E. and C. B. Beard (1996). Microbial symbioses in the midgut of insects. Biology of the Insect Midgut. M. J. Lehane and P. F. Billingsley. London, Chapman & Hall**:** 419-431.

Dow, J. A. T. (1986). "Insect midgut function." Advances in Insect Physiology **19**: 187-328.

Dowdall, S. M., C. J. Proudman, T. R. Klei, T. Mair and J. B. Matthews (2004). "Characterisation of IgG(T) serum antibody responses to two larval antigen complexes in horses naturally- or experimentally-infected with cyathostomins." International Journal for Parasitology **34**(1): 101-8.

Drudge, J. H., E. T. Lyons, Z. N. Wyant and S. C. Tolliver (1975). "Occurrence of second and third instars of *Gasterophilus intestinalis* and *Gasterophilus nasalis* in stomachs of horses in Kentucky." American Journal of Veterinary Research **36**(11): 1585-8.

Drummond, R. O. (1984). "Control of larvae of the common cattle grub (Diptera: Oestridae) with animal systemic insecticides." Journal of Economic Entomology **77**(2): 402-6.

East, I. J. and C. H. Eisemann (1993). "Vaccination against *Lucilia cuprina*: the causative agent of sheep blowfly strike." Immunology and Cell Biology **71 (Pt 5)**: 453-62.

East, I. J., C. J. Fitzgerald, R. D. Pearson, R. A. Donaldson, T. Vuocolo, L. C. Cadogan, R. L. Tellam and C. H. Eisemann (1993). "*Lucilia cuprina*: inhibition of larval growth induced by immunization of host sheep with extracts of larval peritrophic membrane." International Journal for Parasitology **23**(2): 221-9.

Ebbert, M. A., J. L. Marlowe and J. J. Burkholder (2003). "Protozoan and intracellular fungal gut endosymbionts in *Drosophila*: prevalence and fitness effects of single and dual infections." Journal of Invertebrate Pathology **83**(1): 37-45.

Edwards, G. T. (1982). "The prevalence of *Gasterophilus intestinalis* in horses in northern England and Wales." Veterinary Parasitology **11**(2-3): 215-22.

El-Ebiarie, A. S., N. Taha, A. A. El-Meguid and M. A. Mustafa (2005). "A preliminary characterization of digestive protease in the mid-gut of the third larval instar of *Gasterophilus intestinalis*." Veterinary Medical Journal, Giza **53**(1): 5-23.

Escartin-Pena, M. and C. R. Bautista-Garfias (1993). "Comparison of five tests for the serologic diagnosis of myiasis by *Gasterophilus* spp. larvae (Diptera: Gasterophilidae) in horses and donkeys: a preliminary study." Med Vet Entomol **7**(3): 233-7.

Escartin-Pena, M. and C. R. Bautista-Garfias (1993). "Comparison of five tests for the serologic diagnosis of myiasis by *Gasterophilus* spp. larvae (Diptera: Gasterophilidae) in horses and donkeys: a preliminary study." Medical and Veterinary Entomology **7**(3): 233-7.

Euzéby, J. (1998). Les parasites des viandes : Epidémiologie, Physiologie, Incidences zootechniques., Ed: Tec & Doc, Lavoisier (Paris) - Edition Médicales Internationale (Cachan).

Evangelista, L. G. and A. C. Leite (2003). "Midgut ultrastructure of the third instar of *Dermatobia hominis* (Diptera: Cuterebridae) based on transmission electron microscopy." Journal of Medical Entomology **40**(2): 133-40.

Evangelista, L. G. and A. C. Leite (2007). "Salivary glands of second and third instars of *Dermatobia hominis* (Diptera: Oestridae)." Journal of Medical Entomology **44**(3): 398-404.

Eysker, M., J. H. Boersema, G. C. Grinwis, F. N. Kooyman and J. Poot (1997). "Controlled dose confirmation study of a 2% moxidectin equine gel against equine internal parasites in The Netherlands." Veterinary Parasitology **70**(1-3): 165-73.

Fisher, W. F., J. H. Pruett, V. M. Howard and P. J. Scholl (1991). "Antigen-specific lymphocyte proliferative responses in vaccinated and *Hypoderma lineatum*-infested calves." Veterinary Parasitology **40**(1-2): 135-45.

Gawor, J. J. (1995). "The prevalence and abundance of internal parasites in working horses autopsied in Poland." Veterinary Parasitology **58**(1-2): 99-108.

Gil, R., A. Latorre and A. Moya (2004). "Bacterial endosymbionts of insects: insights from comparative genomics." Environmental Microbiology **6**(11): 1109-22.

Gingrich, R. E. (1982). "Acquired resistance to *Hypoderma lineatum*: comparative immune response of resistant and susceptible cattle." Veterinary Parasitology **9**(3-4): 233-42.

Griss, R. and H. Simhofer (2006). "[First-time endoscopic detection of larvae of *Gasterophilus* spp. in the oral cavity in 14 warmblood horses]." Berliner Munchener Tierarztliche Wochenschrift **119**(9-10): 416-20.

Groombridge, B. (1992). Global biodiversity: Status of the Earth's Living Resources. London, Chapman & Hall.

Guimarães, J. H. and N. Papavero (1999). Myiasis in Man and Animals in the Neotropical Region. E. Plêiade. São Paulo**:** p. 308.

Hall, M. J. (1995). "Trapping the flies that cause myiasis: their responses to host-stimuli." Annals of Tropical Medicine and Parasitology **89**(4): 333-57.

Hall, M. J. R. and R. Wall (1995). "Myiasis of humans and domestic animals." Advances in Parasitology **35**: 257-334.

Hansen, I. A., S. R. Meyer, I. Schafer and K. Scheller (2002). "Interaction of the anterior fat body protein with the hexamerin receptor in the blowfly *Calliphora vicina*." European Journal of Biochemistry **269**(3): 954-60.

Harcombe, W. and A. A. Hoffmann (2004). "*Wolbachia* effects in *Drosophila melanogaster*: in search of fitness benefits." Journal of Invertebrate Pathology **87**(1): 45-50.

Hilali, M., F. S. Derhalli and A. Baraka (1987). "Incidence and monthly prevalence of *Gasterophilus* spp. larvae (Diptera: Gasterophilidae) in the stomach of donkeys (Equus asinus) in Egypt." Veterinary Parasitology **23**(3-4): 297-305.

Hobson, R. P. (1931). "Studies on the nutrition of blow-fly larvae." Journal of Experimental Biology **8**(2): 109-123.

Hoglund, J., B. L. Ljungstrom, O. Nilsson, H. Lundquist, E. Osterman and A. Uggla (1997). "Occurrence of *Gasterophilus intestinalis* and some parasitic nematodes of horses in Sweden." Acta Veterinaria Scandinavica **38**(2): 157-65.

Hoglund, J., B. L. Ljungstrom, O. Nilsson and A. Uggla (1995). "Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *Anoplocephala perfoliata* in horse sera." Veterinary Parasitology **59**(2): 97-106.

Holste, J. E., D. D. Colwell, R. Kumar, J. E. Lloyd, N. P. Pinkall, M. A. Sierra, J. W. Waggoner, W. K. Langholff, R. A. Barrick and J. S. Eagleson (1998). "Efficacy of eprinomectin against *Hypoderma* spp in cattle." American Journal of Veterinary Research **59**(1): 56-8.

Hope, F. W. (1840). On insects and their larvae occasionnaly found in the human body. Transactions of the Royal Entomological Society of London 2**:** 256-271.

Horak, I. G., V. De Vos and B. D. De Klerk (1984). "Parasites of domestic and wild animals in South Africa. XVII. Arthropod parasites of Burchell's zebra, *Equus burchelli*, in the eastern Transvaal Lowveld." Onderstepoort Journal of Veterinary Research **51**(2): 145-54.

Humphrey, J. D., J. P. Spradbery and R. S. Tozer (1980). "*Chrysomya bezziana*: pathology of Old World screw-worm fly infestations in cattle." Experimental Parasitology **49**(3): 381-97.

Innocenti, L., M. Masetti, G. Macchioni and F. Giorgi (1995). "Larval salivary gland proteins of the sheep nasal bot fly, (*Oestrus ovis* L.), are major immunogens in infested sheep." Veterinary Parasitology **60**(3-4): 273-82.

Jordao, B. P., A. N. Capella, W. R. Terra, A. F. Ribeiro and C. Ferreira (1999). "Nature of the anchors of membrane-bound aminopeptidase, amylase, and trypsin and secretory mechanisms in *Spodoptera frugiperda* (Lepidoptera) midgut cells." Journal of Insect Physiology **45**(1): 29-37.

Jungblut, P. R., U. Zimny-Arndt, E. Zeindl-Eberhart, J. Stulik, K. Koupilova, K. P. Pleissner, A. Otto, E. C. Muller, W. Sokolowska-Kohler, G. Grabher and G. Stoffler (1999). "Proteomics in human disease: cancer, heart and infectious diseases." Electrophoresis **20**(10): 2100-10.

Kaboret, Y., P. Deconinck, J. Pangui, J. Akakpo and P. Dorchies (1997). "Lésion de la rhinoestrose spontanée à *Rhinoestrus usbekistanicus* (Gan, 1947) chez les ânes (*Equus asinus*) au Sénégal." Revue de Médecine Vétérinaire **148**: 123-126.

Karnowsky, M. J. (1965). "A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy." Journal of Cell Biology **27**(137A).

Keilin, D. (1944). "Respiratory system and respiratory adaptations in larvae and pupae of Diptera." Parasitology **36**: 1-66.

Keilin, D. and Y. L. Wang (1946). "Haemoglobin of *Gasterophilus* larvae. Purification and properties." Biochemical Journal **40**(5-6): 855-66.

Kettle, P. R. (1974). "The genus *Gasterophilus* in the horse in New Zealand." New Zealand Veterinary Journal **22**(4): 43-5.

Kirby, W. and W. Spence (1815). An Introduction to Entomology. London.

Lapointe, J. M., C. Celeste and A. Villeneuve (2003). "Septic peritonitis due to colonic perforation associated with aberrant migration of a *Gasterophilus intestinalis* larva in a horse." Veterinary Pathology **40**(3): 338-9.

Lecroisey, A., C. Boulard and B. Keil (1979). "Chemical and enzymatic characterization of the collagenase from the insect *Hypoderma lineatum*." European Journal of Biochemistry **101**(2): 385-93.

Lehane, M. J., H. M. Müller and A. Crisanti (1996). Mechanisms controlling the synthesis and secretion of digestive enzymes in insects. Biology of the Insect Midgut. M. J. Lehane and P. F. Billingsley. London, Chapman & Hall**:** 195-205.

Leite, A. C. and F. B. Scott (1999). "Scanning electron microscopy of the second-instar larva of *Gasterophilus nasalis*." Medical and Veterinary Entomology **13**(3): 288-94.

Leite, A. C., F. B. Scott and L. G. Evangelista (1999). "Scanning electron microscope observations on third-instar *Gasterophilus nasalis* (Diptera: Oestridae)." Journal of Medical Entomology **36**(6): 643-8.

Lello, E. (2006). Part B: Cuterebrinae Host-Parasite Interactions. The Oestrid Flies. Biology, Host-parasite relationships, impact and management. D.D. Colwell, Hall M.J.R. and Scholl P.J. Oxfordshire, United Kingdom, CABI Publishing**:** 179-189.

Lyon, S., H. C. Stebbings and G. C. Coles (2000). "Prevalence of tapeworms, bots and nematodes in abattoir horses in south-west England." The Veterinary Record **147**(16): 456-7.

Lyons, E. T., S. C. Tolliver and S. S. Collins (2006). "Prevalence of large endoparasites at necropsy in horses infected with Population B small strongyles in a herd established in Kentucky in 1966." Parasitology Research **99**(2): 114-8.

Lyons, E. T., S. C. Tolliver, J. H. Drudge, D. E. Granstrom, S. S. Collins and S. Stamper (1992). "Critical and controlled tests of activity of moxidectin (CL 301,423) against natural infections of internal parasites of equids." Veterinary Parasitology **41**(3-4): 255-84.

Magat, A. and C. Boulard (1970). "[Attempts at vaccination against bovine hypodermyiasis with a vaccine containing crude collagenase extracted from *Hypoderma lineatum* 1st stage larva]." C R Acad Sci Hebd Seances Acad Sci D **207**(5): 728-30.

Marchenko, V. P., V. A. Marchenko and E. S. Belousov (1991). "[The kinetics of specific serum antibodies in sheep infested with sheep bot larvae]." Parazitologiia **25**(4): 297-304.

Martoja, M. and C. Ballan-Dufrançais (1984). The Ultrastructure of the digestive and excretory organs. Insect Ultrastructure. R. C. King, Akai, H. New York, Plenum Press. **2:** 199-268.

Mateos, M., S. J. Castrezana, B. J. Nankivell, A. M. Estes, T. A. Markow and N. A. Moran (2006). "Heritable endosymbionts of Drosophila." Genetics **174**(1): 363-76.

Mazzon, L., A. Piscedda, M. Simonato, I. Martinez-Sanudo, A. Squartini and V. Girolami (2008). "Presence of specific symbiotic bacteria in flies of the subfamily *Tephritinae* (Diptera Tephritidae) and their phylogenetic relationships: proposal of '*Candidatus Stammerula tephritidis*'." International Journal of Systematics and Evolutionnary Microbiology **58**(Pt 6): 1277-87.

McAlpine, J. F. (1989). Phylogeny and classification of the Muscomorpha. In: Manual of Neoarctic Diptera Research Branch, Agriculture Canada.

Medjitna, T. D. E. (2003). Dynamique de l'infestation myiasigène par *Hypoderma* spp. (Diptera : Hypodermatidae) chez les bovins, sous conditions thérapeutiques. Neuchâtel, Université de Neuchâtel**:** 273.

Medjitna, T. D. E., B. Betschart and K. Pfister (2001). Progress towards eradication of hypodermosis in Switzerland. In: Mange and myiasis of livestock. COST Action 833. M. Good, M. J. R. Hall, B. Lossonet al. Ceske Budejovie, Czech Republic, European Commission, Brussel**:** 122-129.

Merck (2005). Flies. The Merck Veterinary Manual. C. M. Kahn. Whitehouse Station, N.J., U.S.A, Merck & Co.,INC.

Mock, D. E. (1973). "Eggs of the horse bot fly, *Gasterophilus intestinalis* (Diptera: gasterophilidae), On pastured cattle." Journal of Medical Entomology **10**(1): 34-7.

Moire, N., Y. Bigot, G. Periquet and C. Boulard (1994). "Sequencing and gene expression of hypodermins A, B, C in larval stages of *Hypoderma lineatum*." Molecular and Biochemical Parasitology **66**(2): 233-40.

Montenegro, H., A. S. Petherwick, G. D. Hurst and L. B. Klaczko (2006). "Fitness effects of *Wolbachia* and *Spiroplasma* in *Drosophila melanogaster*." Genetica **127**(1-3): 207-15.

Mukbel, R., P. R. Torgerson and M. Abo-Shehada (2001). "Seasonal variations in the abundance of *Gasterophilus* spp. larvae in donkeys in northern Jordan." Tropical Animal Health and Production **33**(6): 501-9.

Nelson, W. A. and J. Weintraub (1972). "*Hypoderma lineatum* (De Vill.) (Diptera: Oestridae): invasion of the bovine skin by newly hatched larvae." Journal of Parasitology **58**(3): 614-24.

Nicolas-Gaulard, I., N. Moire and C. Boulard (1995). "Effect of the parasite enzyme, hypodermin A, on bovine lymphocyte proliferation and interleukin-2 production via the prostaglandin pathway." Immunology **85**(1): 160-5.

Nirmala, X., V. Hypsa and M. Zurovec (2001). "Molecular phylogeny of Calyptratae (Diptera: Brachycera): the evolution of 18S and 16S ribosomal rDNAs in higher dipterans and their use in phylogenetic inference." Insect Molecular Biology **10**(5): 475-85.

O'Donnell, I. J., P. E. Green, J. A. Connell and P. S. Hopkins (1980). "Immunoglobulin G antibodies to the antigens of *Lucilia cuprina* in the sera of fly-struck sheep." Australian Journal of Biological Sciences **33**(1): 27-34.

Otranto, D. (2001). "The immunology of myiasis: parasite survival and host defense strategies." Trends in Parasitology **17**(4): 176-82.

Otranto, D., D. D. Colwell, P. Milillo, V. Di Marco, P. Paradies, C. Napoli and S. Giannetto (2004). "Report in Europe of nasal myiasis by *Rhinoestrus* spp. in horses and donkeys: seasonal patterns and taxonomical considerations." Veterinary Parasitology **122**(1): 79-88.

Otranto, D., P. Milillo, G. Capelli and D. D. Colwell (2005). "Species composition of *Gasterophilus* spp. (Diptera, Oestridae) causing equine gastric myiasis in southern Italy: parasite biodiversity and risks for extinction." Veterinary Parasitology **133**(1): 111-8.

Otranto, D. and J. R. Stevens (2006). Molecular phylogeny and identification. The Oestrid Flies: Biology, Host-Parasite Relationship, Impact and Managment. M. J. R. H. D.D. Colwell, P.J. Scholl, CAB International**:** 51-66.

Otranto, D., J. R. Stevens, E. Brianti and P. Dorchies (2006). "Human and livestock migrations: a history of bot fly biodiversity in the Mediterranean region." Trends in Parasitology **22**(5): 209-13.

Panadero-Fontan, R., C. Lopez-Sandez, F. Parra-Fernandez, P. Morrondo-Pelayo, P. Diez-Banos and D. D. Colwell (2002). "Detection of circulating hypodermin C: an antigen capture ELISA for diagnosis of cattle grub (Diptera: Oestridae) infestations." Veterinary Parasitology **108**(1): 85-94.

Panadero, R., C. Lopez, D. Carballo, R. Casais, A. Paz, P. Morrondo and P. Diez-Banos (2000). "Assessment of a recombinant antigen versus natural hypodermin C for the serodiagnosis of hypodermosis in cattle." Parasitology Research **86**(1): 67-8.

Panadero, R., C. Lopez, M. Mezo, P. Morrondo and P. Diez-Banos (1997). "Effect of early treatment with ivermectin and doramectin on the dynamics of antibody response in cattle naturally infested by *Hypoderma lineatum* and *H. bovis*." Veterinary Parasitology **73**(3-4): 325-34.

Pandey, V. S. and J. Cabaret (1993). "Stomach parasites of donkeys in Morocco: habitat and interspecific interactions." Veterinary Parasitology **49**(2-4): 331-7.

Pandey, V. S., H. Ouhelli and A. Verhulst (1992). "Epidemiological observations on *Gasterophilus intestinalis* and *G. nasalis* in donkeys from Morocco." Veterinary Parasitology **41**(3-4): 285-92.

Pape, T. (2001). "Phylogeny of Oestridae (Insecta: Diptera)." Systematic entomology **26**: 133-171.

Pape, T., G. Dahlem, C. A. de Mello Patiu and M. Giroux. (2006). "The World of Flesh Flies (Diptera: Sarcophagidae)." Http://www.zmuc.dk/entoweb/sarcoweb/sarcweb/sarc.

Pappin, D. J. (1997). "Peptide mass fingerprinting using MALDI-TOF mass spectrometry." Methods in Molecular Biology **64**: 165-73.

Patton, W. S. (1922). "Notes on the myiasis-producing Diptera of man and animals." Bulletin of Entomological Research **12**: 239-261.

Perkins, D. N., D. J. Pappin, D. M. Creasy and J. S. Cottrell (1999). "Probability-based protein identification by searching sequence databases using mass spectrometry data." Electrophoresis **20**(18): 3551-67.

Pesce, A., M. Nardini, S. Dewilde, D. Hoogewijs, P. Ascenzi, L. Moens and M. Bolognesi (2005). "Modulation of oxygen binding to insect hemoglobins: the structure of hemoglobin from the botfly *Gasterophilus intestinalis*." Protein Science **14**(12): 3057-63.

Peyresblanques, J. (1964). "[Ocular Myiasis.]." Annales d'Oculistique (Paris) **197**: 271-95.

Principato, M. (1988). "Classification of the main macroscopic lesions produced by larvae of *Gasterophilus* spp. (Diptera:Gasterophilidae) in free-ranging horses in Umbria." The Cornell Veterinarian **78**(1): 43-52.

Principato, M. (1989). "Observations on the occurrence of five species of *Gasterophilus* larvae in free-ranging horses in Umbria, central Italy." Veterinary Parasitology **31**(2): 173-7.

Principato, M. and M. Tosti (1988). "Scanning electron microscope observations on the anterior thoracic and post-abdominal spiracles of *Gasterophilus* larvae (Diptera: Gasterophilidae)." International Journal for Parasitology **18**(2): 191-6.

Proudman, C. J. and A. J. Trees (1996). "Use of excretory/secretory antigens for the serodiagnosis of *Anoplocephala perfoliata* cestodosis." Veterinary Parasitology **61**(3-4): 239-47.

Pruett, J. H. and C. C. Barrett (1985). "Kinetic development of humoral anti-*Hypoderma lineatum* antibody activity in the serum of vaccinated and infested cattle." Southwestern Entomologist **10**: 39-48.

Pruett, J. H., K. B. Temeyer and B. K. Burkett (1988). "Antigenicity and immunogenicity of *Hypoderma lineatum* soluble proteins in the bovine host." Veterinary Parasitology **29**(1): 53-63.

Reinemeyer, C. R., P. J. Scholl, F. M. Andrews and D. W. Rock (2000). "Efficacy of moxidectin equine oral gel against endoscopically-confirmed *Gasterophilus nasalis* and *Gasterophilus intestinalis* (Diptera: Oestridae) infections in horses." Veterinary Parasitology **88**(3-4): 287-91.

Report (1998). Report on the animal health situation in Greenland, Danish Veterinary Service and Greenland Home Rule Department for Environment and Nature Veterinary Service.

Ribbeck, R., G. Ilchmann and T. Hiepe (1984). "[Immunodiagnosis of gasterophilosis]." Angewandte Parasitologie **25**(2): 78-83.

Robertson, R. H. (1980). "Antibody production in cattle infected with *Hypoderma* spp." Canadian Journal of Zoology **58**(2): 245-51.

Rose, C. H. (1990). Gastrointestinal nematodes of sheep in Greenland. . Copenhagen, Denmark, Royal Veterinary and Agricultural University. **PhD:** 79.

Rothman, J. E. and L. Orci (1992). "Molecular dissection of the secretory pathway." Nature **355**(6359): 409-15.

Roy, D. N. (1937). "The physiology of digestion in larvae of Gasterophilus equi " Parasitology **29**: 150-162.

Sanchez-Andrade, R., J. L. Romero, J. L. Suarez, J. Pedreira, P. Diaz, M. Arias, A. Paz-Silva, R. Panadero, P. Diez-Banos, P. Morrondo and A. Scala (2005). "Comparison of *Oestrus ovis* metabolic and somatic antigens for the immunodiagnosis of the zoonotic myasis oestrosis by immunoenzymatic probes." Immunological Investigations **34**(1): 91-9.

Sandeman, R. M. (1996). Immune responses to flies and mosquitoes. The Immunology of Host-Ectoparasitic Arthropod Relationship. S. K. Wikel, CAB International**:** 175-203.

Schaëfer, C. W. (1979). "Feeding habits and hosts of Calyptrate flies (Diptera: Brachycera: Cyclorrhapha)." Entomologia Generalis **5**: 193-200.

Scholl, P. J. (1993). "Biology and control of cattle grubs." Annual Review of Entomology **38**: 53-70.

Scholl, P. J. (2006). Management and Control of Oestrid Flies. The Oestrid Flies. Biology, Host-parasite relationships, impact and management. D.D. Colwell, Hall M.J.R. and Scholl P.J. Oxfordshire, United Kingdom, CABI Publishing**:** 210-220.

Scholl, P. J., M. R. Chapman, D. D. French and T. R. Klei (1998). "Efficacy of moxidectin 2% oral gel against second- and third-instar *Gasterophilus intestinalis* De Geer." Journal of Parasitology **84**(3): 656-7.

Sequeira, J. L., R. A. Tostes and T. C. Oliveira-Sequeira (2001). "Prevalence and macro- and microscopic lesions produced by *Gasterophilus nasalis* (Diptera: Oestridae) in the Botucatu Region, SP, Brazil." Veterinary Parasitology **102**(3): 261-6.

Shefstad, D. K. (1978). "Scanning electron microscopy of *Gasterophilus intestinalis* lesions of the equine stomach." Journal of the American Veterinary Medical Association **172**(3): 310-3.

Shewell, G. E. (1987a). Sarcophagidae. Manual of Nearctic Diptera. J. F. McAlpine. Ottawa, Research Branch Agriculture Canada. **2:** 1159-1186.

Shewell, G. E. (1987b). Calliphoridae. Manual of Nearctic Diptera. J. F. McAlpine. Ottawa, Research Branch Agriculture Canada. **2:** 1133-1145.

Shin, Y. S., G. W. Shin, Y. R. Kim, E. Y. Lee, H. H. Yang, K. J. Palaksha, H. J. Youn, J. H. Kim, D. Y. Kim, A. E. Marsh, J. Lakritz and T. S. Jung (2005). "Comparison of proteome and antigenic proteome between two *Neospora caninum* isolates." Veterinary Parasitology **134**(1-2): 41-52.

Simpson, R. J. (2003). Proteins and Proteomics. A Laboratory Manual. . New-York, Cold Spring Harbor Laboratory Press.

Sinclair, I. J. and D. A. Wassall (1983). "Enzyme-linked immunosorbent assay for the detection of antibodies to *Hypoderma bovis* in cattle." Research in Veterinary Science **34**(2): 251-2.

Slansky, F. (2007). "Insect/mammal associations: effects of Cuterebrid bot fly parasites on their hosts." Annual Review of Entomology **52**: 17-36.

Soulsby, E. J. L. (1982). Helminths, arthropods and protozoa of domestical animals. Eastbourne, United Kingdom, Baillière, Tindall & Cassell.

Stevens, J. R., J. F. Wallman, D. Otranto, R. Wall and T. Pape (2006). "The evolution of myiasis in humans and other animals in the Old and New Worlds (part II): biological and life-history studies." Trends in Parasitology **22**(4): 181-8.

Suarez, J. L., A. Scala, J. A. Romero, A. Paz-Silva, J. Pedreira, M. Arias, P. Diaz, P. Morrondo, P. Diez-Banos and R. Sanchez-Andrade (2005). "Analysis of the humoral immune response to *Oestrus ovis* in ovine." Veterinary Parasitology **134**(1-2): 153-8.

Tabouret, G., L. Bret-Bennis, P. Dorchies and P. Jacquiet (2003a). "Serine protease activity in excretory-secretory products of *Oestrus ovis* (Diptera: Oestridae) larvae." Veterinary Parasitology **114**(4): 305-14.

Tabouret, G., C. Lacroux, O. Andreoletti, J. P. Bergeaud, Y. Hailu-Tolosa, H. Hoste, F. Prevot, C. Grisez, P. Dorchies and P. Jacquiet (2003b). "Cellular and humoral local immune responses in sheep experimentally infected with *Oestrus ovis* (Diptera: Oestridae)." Veterinary Research **34**(2): 231-41.

Tabouret, G., F. Prevot, J. P. Bergeaud, P. Dorchies and P. Jacquiet (2001). "*Oestrus ovis* (Diptera: Oestridae): sheep humoral immune response to purified excreted/secreted salivary gland 28 kDa antigen complex from second and third instar larvae." Veterinary Parasitology **101**(1): 53-66.

Tarry, D. W. (1986). "Progress in warble fly eradication." Parasitology Today **2**(4): 111-6.

Tarry, D. W., I. J. Sinclair and D. A. Wassall (1992). "Progress in the British hypodermosis eradication programme: the role of serological surveillance." The Veterinary Record **131**(14): 310-2.

Tatchell, R. J. (1958). "The physiology of digestion in the larvae of the horse bot-fly, *Gasterophilus intestinalis* (De Geer)." Parasitology **48**(3-4): 448-58.

Telfer, W. H. and J. G. Kunkel (1991). "The function and evolution of insect storage hexamers." Annual Review of Entomology **36**: 205-28.

Tellam, R. L., C. Eisemann, R. Casu and R. Pearson (2000). "The intrinsic peritrophic matrix protein peritrophin-95 from larvae of *Lucilia cuprina* is synthesised in the cardia and regurgitated or excreted as a highly immunogenic protein." Insect Biochemistry and Molecular Biology **30**(1): 9-17.

Tellam, R. L., G. Wijffels and P. Willadsen (1999). "Peritrophic matrix proteins." Insect Biochemistry and Molecular Biology **29**(2): 87-101.

Terra, W. R., F. P. Espinoza-Fuentes, A. F. Ribeiro and C. Ferreira (1988). "The larval midgut of the housefly (*Musca domestica*): ultrastructure, fluid fluxes and ion secretion in relation to the organization of digestion." Journal of Insect Physiology **34**: 463-472.

Terra, W. R. and C. Ferreira (2005). Biochemistry of digestion. Comprehensive Molecular Insect Science. L. I. Gilbert, Iatrou, K., Gill, S.S. Oxford, Elsevier **4:** 171-224.

Terra, W. R., C. Ferreira and J. E. Baker (1996). Compartmentalization of digestion. Biology of the Insect Midgut. M. J. Lehane and P. F. Billingsley. London, Chapman & Hall**:** 206-235.

Tolliver, S. C., E. T. Lyons and J. H. Drudge (1987). "Prevalence of internal parasites in horses in critical tests of activity of parasiticides over a 28-year period (1956-1983) in Kentucky." Veterinary Parasitology **23**(3-4): 273-84.

Tong, N. T., J. M. Imhoff, A. Lecroisey and B. Keil (1981). "Hypodermin A, a trypsin-like neutral proteinase from the insect *Hypoderma lineatum*." Biochimica et Biophysica Acta **658**(2): 209-19.

Traversa, D. and D. Otranto (2006). "A new approach for the diagnosis of myiasis of animals: the example of horse nasal myiasis." Veterinary Parasitology **141**(1-2): 186-90.

Ulrich, R. G., D. A. Buthala and M. J. Klug (1981). "Microbiota Associated with the Gastrointestinal Tract of the Common House Cricket, *Acheta domestica*." Applied and Environmental Microbiology **41**(1): 246-254.

Viviers, P. L., M. A. Elazhary, A. Lagace, R. S. Roy and A. Tremblay (1974). "[An experimental study on the variation of serum antibody levels relative to the life cycle of the horsefly]." Canadian Journal of Comparative Medecine and Veterinary Science **38**(3): 336-40.

Vogelsang, E., P. Gallo and T. H. Mayaudon (1955). "Elementos bacteriostaticos en las larvas de *Gastrophilus* (nota previa)." Rev. Med. Vet. Parasit. Caracas **14**: 41-44.

Wigglesworth, V. B. (1972). Digestion and Nutrition. The Principles of Insect Physiology. New-York, Chapman & Hall**:** 836.

Wood, D. M. (1987). Oestridae. Manual of Nearctic Diptera. J. F. McAlpine. Ottawa, Canada, Research Branch Agriculture Canada. **2:** 1147-1158.

Wood, D. M. (2006). Morphology of Adult Oestridae. The Oestrid Flies. Biology, Host-parasite relationships, impact and management. D.D. Colwell, Hall M.J.R. and Scholl P.J. Oxfordshire, United Kingdom, CABI Publishing**:** 78-92.

Xiao, L., R. P. Herd and G. A. Majewski (1994). "Comparative efficacy of moxidectin and ivermectin against hypobiotic and encysted cyathostomes and other equine parasites." Veterinary Parasitology **53**(1-2): 83-90.

Yasuda, K., T. Ishii, H. Suda, A. Akatsuka, P. S. Hartman, S. Goto, M. Miyazawa and N. Ishii (2006). "Age-related changes of mitochondrial structure and function in *Caenorhabditis elegans*." Mechanisms of Ageing and Development **127**(10): 763-70.

Yeates, D. K. and B. M. Wiegmann (1999). "Congruence and controversy: toward a higher-level phylogeny of Diptera." Annual Review of Entomology **44**: 397-428.

Zick, M., R. Rabl and A. S. Reichert (2009). "Cristae formation-linking ultrastructure and function of mitochondria." Biochimica and Biophysica Acta **1793**(1): 5-19.

Zumpt, F. (1962). "The genus Gyrostigma Brauer (Diptera: Gasterophilidae)." Zeitschrift für Parasitenkunde **22**: 245-260.

Zumpt, F. (1965). Myasis in man and animals in the old world, Butterworths, London, United Kingdom.

10 Abbreviations

µg	microgram
µl	microliter
1-D	One Dimensional
2-D	Two Dimensional
ABTS	2.2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid
AcOH	Acetyl Hydroxide
alm	apical lamellar membrane
APS	Amoniumpersulfate
bl	basal lamina
BSA	Bovine Serum Albumin
C	Cuticle
CC	Columnar Cell
CG	Cephalic Ganglia
CIE	Counterimmunoelectrophoresis
COST	European Cooperation in the field of Scientific and Technical Research
DAB	3.3-Diaminobenzidine
Dd	Deferent duct
DD	Double Immunodiffusion
ddH$_2$O	Distilled and deionised water
DIG-ELISA	Diffusion-in-gel Enzyme-Linked Immunosorbent Assay
e.g.	*Exempli gratia* (example given)
EC	Epithelial Cell
ECL	Enhanced Chemiluminescence
Ed	Efferent duct
EDTA	Ethylenediamino-tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ENC	Endocrine Cell
ENPT	Endoperitrophic space
EPT	Ectoperitrophic space
FG	Foregut

FPLC	Fast Performance Liquid Chromatography
g	gram
G. intestinalis	*Gasterophilus intestinalis*
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
gly	glycogen
H	Haloed vesicle
H. bovis	*Hypoderma bovis*
H. lineatum	*Hypoderma lineatum*
Hb	Hemoglobin
HG	Hindgut
HPS-70	Heat Shock Protein 70
hyA	Hypodermin A
hyB	Hypodermin B
hyC	Hypodermin C
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IH	Indirect Hemagglutination
Inc	Intermediate cell
KDa	Kilo Dalton
L1	First larval stage
L2	Second larval stage
L3	Third larval stage
LB	Basal Labyrinth
LCE	Larval crude extract
LCE2	Larval Crude Extract of second larval stage
LCE3	Larval Crude Extract of third larval stage
li	lipidic droplet
lmv	long microvilli
LMW	Low Molecular Weight
LSP-2	Larval Serum Protein
LU	Lumen
M	Molar
mA	milliAmper

MALDI	Matrix-Assisted Laser Desorption/Ionization
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight
MeOH	Methanol
MG	Midgut
MGant	Anterior Midgut
mi	mitochondria
Mice-Hb	Mice immunised with *Gasterophilus intestinalis* hemoglobin recombinant molecule
Mice-L1	Mice immunised with L1 crude larval extracts
Mice-L2	Mice immunised with L2 crude larval extracts
Mice-L3	Mice immunised with L3 crude larval extracts
ml	millilitre
mM	millimolar
Morg	Microorganism
MS	Mass Spectrometry
MT	Malpighian Tubules
mt	microtubules
mu	muscle
mv	microvilli
mvb	multivesicular body
MW	Molecular Weight
N	Nucleus
N	Number
N_2	Nitrogen
O. Ovis	*Oestrus ovis*
OD	Optical Density
OE	Oesophagus
OEc	Oesophageal cell
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween
PEPCK	Phosphoenolpyruvate Carboxykinase
PM	Peritrophic Membrane
PMF	Peptide Mass Fingerprint

PMSF	Phenylmethansulfonyl Fluoride
pov	pinching off vesicle
PROV	Proventriculus
PVc	Proventricular cell
rb	residual body
RER	Rough Endoplasmic Reticulum
RGC	Regenerative Cell
sbl	stretched basal lamina
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
se	septate junction
SEM	Scanning Electron Microscopy
SEM	Standard Error of the Mean
SG	Salivary Gland
SGc	Salivary Gland cell
smi	swollen mitochondria
smv	short microvilli
spp.	Species
T	Tracheae
T°	Temperature
TBS	Tris Buffered Saline
TEM	Transmission Electron Microscopy
Th1	T Helper Cell 1
TIA	Thin layer immunoassay
Tris	Tris-(hydroxymethyl)-aminomethan
TTBS	Twin Tris Buffered Saline
v	vesicle
Va	Vacuole
vc	coated vesicle
x g	*gravities* (centrifugal force)

11 Figures

12 Tables

13 Acknowledgements

Je dédie ce travail à Reza, grâce à lui j'ai pu réaliser mes rêves. Son soutien dans tous les domaines est depuis longtemps mon moteur et sans lui je n'y serais jamais parvenue.

Durant les nombreuses années nécessaires pour réaliser ce travail, j'ai eu la chance de rencontrer et de collaborer avec de nombreuses personnes qui m'ont toutes permis, directement ou indirectement, d'atteindre ce but.

Je tiens tout d'abord à témoigner ma reconnaissance au Professeur Kurt Pfister pour cette incroyable collaboration. Ses encouragements, son soutien et sa gentillesse m'ont permis à aller au-delà de ce que je croyais être mes limites.

Je remercie le Prof. B. Betschart de m'avoir accueille dans son laboratoire, pour son écoute et ses conseils tout au long de mon projet.

Je également le Prof. Diehl pour son engagement à la fin du projet et de m'avoir ouvert les yeux sur le domaine fascinant de la microscopie.

Un grand merci à P. Kopacek pour son accueil en Tchéquie sa gentillesse et ses conseils scientifiques. Je remercie toute son équipe pour leur accueil.

Je remercie chaleureusement Pierre Brocard et Jean-François Graf d'avoir eu l'amabilité d'accepter de faire partie du jury de thèse. Et merci à tout le jury pour leurs commentaires très enthousiastes !

Je tiens à remercier tout particulièrement :

- Le Prof C. Deeg pour son accueil dans son laboratoire à Munich, son immense aide à la réalisation du projet de protéomique et tous les conseils prodigués pour l'article. Merci à toute son équipe qui a contribué à la réussite du projet.
- Michèle Vlimant. Sans elle le projet de microscopie n'aurait jamais vu le jour et le manuscrit ne serait pas ce qu'il est !!
- Le Professeur Tainturier de l'Ecole Nationale Vétérinaire de Nantes d'avoir prélevé et envoyé les sérums de juments, de poulains et les colostrums.
- I am grateful to the Dr Marner Nolsøe for the blood samples she collected in Greenland and for the time she spent answering all my questions.
- Le personnel du Centre Intégratif de Génomique à l'Université de Lausanne, spécialement au Professeur Alexandre Reymond qui m'a accueilli et donné accès à toutes les facilités de son laboratoire.
- Tous les laborantins qui ont participé à ce projet : Morgane Nadège, Ludivine, Maude, Elodie, Lucia, Fanny, Didier, Régis. Merci Pitou pour ton aide durant toutes ces années, surtout avec les souris.
- Mes collègues Christine, Muriel, Christian Reno, David, JC et tous les autres pour la complicité et les fou-rires
- Romain, ton aide a été des plus précieuses et tu m'as sauvé bien des fois de la cata !!
- Josiane pour son aide indispensable à la réalisation d'une superbe bibliographie, mais aussi pour son immense gentillesse
- Natacha et Brigitte, les plus sympathiques et serviables des secrétaires

Sans la participation des agriculteurs, des éleveurs de chevaux, des vétérinaires et des bouchers, ce projet n'aurait pas vu le jour et je leur suis infiniment reconnaissante de m'avoir toujours aussi bien accueilli. Je remercie spécialement :

- Christophe Ackermann pour son engagement dans tous les projets que j'ai pu entreprendre et son amitié. Merci pour mon Baïkal
- Peter Hofmann, Ueli, M. Friedli et M. Hofer pour leur chaleureux accueil chaque fois que je viens au Jugendheim de Prêles.
- M. Mahé, M. Bilat et M. Sauser ainsi que tout leur personnel pour leur formidable accueil dans leur abattoir et de m'avoir autant facilité le travail.
- Benjamin Muller pour tout ce qu'il m'a apporté, supporté, sa participation à ce projet et son affection.
- Daniella pour ta présence, ton amitié et tes réponses aux problèmes «techniques», tard le soir au King.

Je suis très reconnaissante envers le Dr E. Boillat de m'avoir engagée dans son laboratoire, de m'avoir permis d'acquérir de nouvelles connaissances et de la confiance qu'il m'a témoigné. Merci chers «anciens» collègues Mathew, Léti, Karin, Raffaella, Marie-Thérèse, Anne, Laurence, Nathalie, Christine, Mme Falthis et M Dufour de m'avoir appris autant de choses et d'avoir fait preuve d'une telle patience.

Je remercie du fond de cœur toutes les personnes hors cadre professionnel qui m'ont soutenu et apporté beaucoup d'énergie pour réaliser ce travail. Je pense aux familles Arhab et Moumni pour leur amour, soutien et joie qu'ils m'apportent depuis de très nombreuses années. Egalement un grand merci à la famille Ait-el-Djoudi, spécialement à Karim pour son infaillible soutien dans toutes les causes.

Je remercie mon amie Sonia d'avoir réalisé les cycles pour mon manuscrit et pour son soutien. Merci Adrien pour la lecture et la correction du manuscrit.

Merci à tous mes amis pour leur soutien et présence de tous les instants, je pense spécialement à Vanessa, Richard, Stéphanie, Sandra. Dinah merci de m'avoir autant soutenue durant la période de rédaction, pour ton amitié et tes petits plats.

Mathieu merci pour ton soutien scientifique, sans toi il y a beaucoup de choses que je n'aurais pas pu réaliser, merci pour ta présence et nos grandes discussions.

Paulette, tu crois en moi parfois plus que moi. Je te raconte tout depuis toujours et cette thèse tu l'as vécue avec moi. Merci pour tout.

Le mot merci n'est pas assez grand pour dire à ma famille à quel point je leur suis reconnaissante de tout ce qu'ils m'apportent depuis toujours et à quel point je pense à eux dans tout ce que j'entreprends.