The use of serology in the control of *Ascaris suum* infections in pigs

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Proefschrift voorgelegd voor het behalen van de graad van Doctor in de Diergeneeskundige Wetenschappen

2018

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"*Sunshine and daylight are nearly as necessary for the complete development of pigs as for the flowering of plants. Piggeries should therefore be equipped with many windows...many disease-carrying parasites and parasitic plants will have the best flourishment conditions in places with subdued light. Because the resistance towards diseases is principally highest for pigs that are kept in light and airy pens, infirmity in pigs is mainly found in dim, humid and dirty pens... The pigs should therefore have the best possible access to sunlight and fresh air.*" Brief Instructions for Pig Breeding and Keeping by V. Cramer, veterinarian, 1856.

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List of abbreviations

ADG	Average daily growth
AI/AO	All-in-all-out
AsHb	*Ascaris suum* haemoglobin
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram
FCR	Feed conversion ratio
HPRO	Horse radish peroxidase
Ig	Immunoglobulin
L2	Second stage larva
L3	Third stage larva
L4	Fourth stage larva
ODr	Optical density ratio
PBS	Phosphate buffered saline
Th1	T-helper cell type 1
UV	Ultraviolet
LWS	Liver white spots

Chapter 1

Review on *Ascaris suum* and its importance in the pig industry

Pigs are known to be very intelligent and kind animals. With around 1 billion animals alive at the time, the domesticated pig is one of the most numerous mammals existing on this earth. As clever as these animals are, their host related parasites are equally as sophisticated. It is amazing how these parasites and their captivating life cycles are able to adapt to their host and environment, making it possible to survive for several years within their host. According to several studies, *Ascaris suum* is by far the most prevalent parasite infecting pigs in modern pig farms (Eijck and Borgsteede, 2005; Haugegaard, 2010; Joachim et al., 2001). Infections with *A. suum* have survived the transition from outdoor to conventional indoor conditions splendidly (Joachim et al., 2001; Roepstorff and Nansen, 1994; Roepstorff et al., 2011).

Although this parasite is still very prevalent and is known for its impact on the economic outcome of the pig industry, many farmers are unaware if their herd is exposed or not. And if so, they are mainly not treating consistently. Due to the subclinical nature of the disease combined with an -until recently- lack of appropriate diagnostic tools, ascariosis often remained undiagnosed. Subsequently this led to an underestimation of its actual prevalence. Nevertheless, it is important to understand the infection intensity in a herd, in order to apply an appropriate deworming program to eliminate adult worms in the pig, resulting in a decrease of egg excretion in the environment. In order to understand the problems associated with the presence of this parasite, a basic understanding of its life cycle and characteristics is necessary. Therefore, this chapter provides a general overview of *A. suum* and concentrates on its economic impact on pig farms.

1.1 The life cycle of *Ascaris suum*

The life cycle of *A. suum* is shown in Figure 1.1. Pigs get infected with *A. suum* when they swallow embryonated eggs that are present in the environment (Fig. 1.2 B). After uptake, an infective larva developed in the egg hatches out in the caecum of the pig. It is a third-stage larva covered by a second-stage cuticle (Fig. 1.2 C) (Fagerholm et al., 2000; Geenen et al., 1999). The larvae inside the egg are stimulated to secrete chitinases and proteinases, which presumably help them degrading the different layers of the eggshell from inside the egg (Geng et al., 2002; Hinck and Ivey, 1976). When the larvae get out of the egg, they penetrate the wall of the upper part of the colon and caecum (Murrell et al., 1997) and start their hepatotracheal migration. Subsequently, the L3 larvae are transported through the mesenterial blood veins to the liver. Here they obstruct the capillaries and subsequently destroy liver tissue, ultimately to reach the efferent blood vessels (Douvres et al., 1969; Murrell et al., 1997). Next, the larvae are carried via the blood stream to the lung, where they embolize in the pulmonary capillaries and penetrate the alveoli. Now, they move up the respiratory tree, get coughed up by the pig and eventually get swallowed again. From 8 days onwards, the L3s enter the small intestine for the second time. Here the first ecdysis takes place inside the host and the larvae mature into the L4 stage by day 14 post infection. After approximately 6 weeks, the worms have reached sexual maturity and adult females release up to hundreds of thousands of eggs per day into the host faeces (Fig. 1.2 A). Freshly shed eggs require a developmental period in the environment outside the host, which contains two moltings to an infective L3 larva (Fagerholm et al., 2000; Kirchgassner et al., 2008). This development takes in average 1 to 3 months. Numerous factors have an influence on the duration of the development, such as the temperature, humidity and seasonal climate (Connan, 1977; Geenen et al., 1999; Kim et al., 2012; Roepstorff, 2003). The eggs can persist in the external environment for 6 to 9 years (Roepstorff et al., 2011). The adult worms can populate the small intestine up to 1-2 years, unless expelled by the host immune response or through application of anthelmintics (Crompton, 2001).
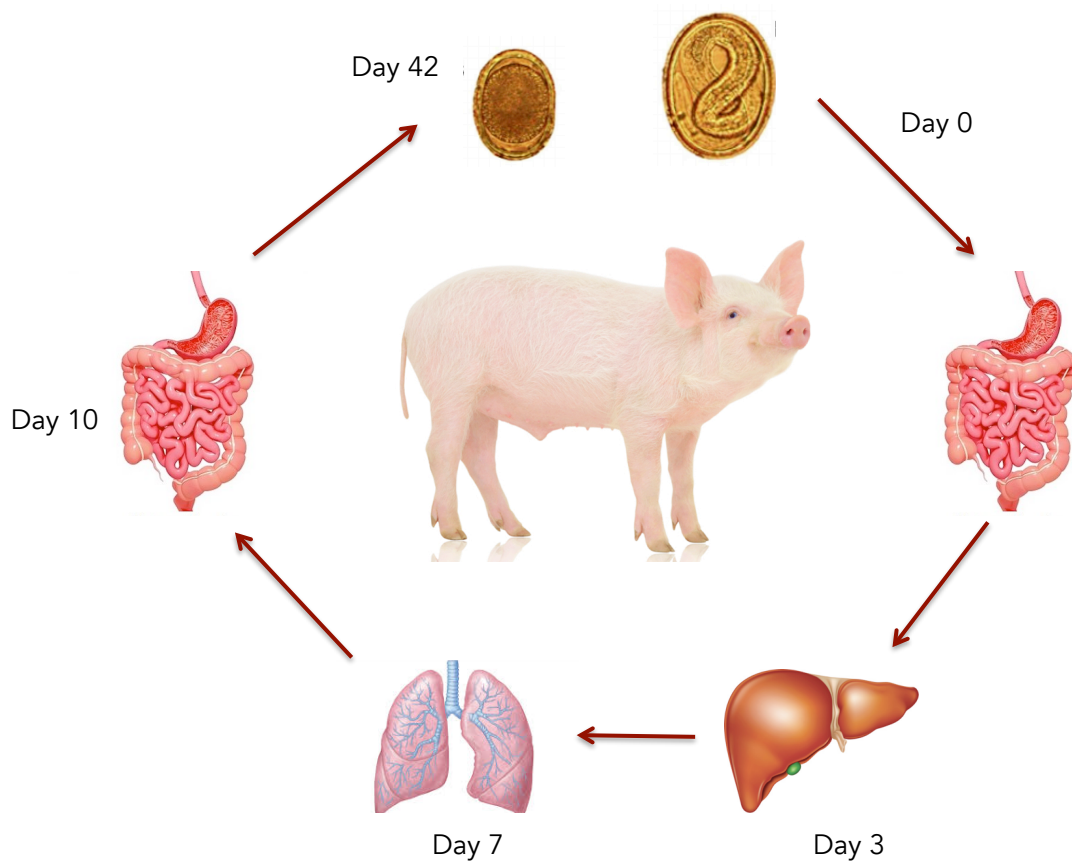
Figure 1.1: The life cycle of *A. suum* in pigs. Fertilized eggs are excreted with the faeces into the environment. When infective eggs are ingested by the host a third stage larva (L3) penetrates the wall of the caecum or colon (day 0) and starts its migration through the liver (day 3) and subsequently the lungs (day 7). Eventually the larva is coughed up, swallowed and reaches the small intestine where it can develop into adulthood. The adult worms shed eggs via the faeces into the environment (day 42).
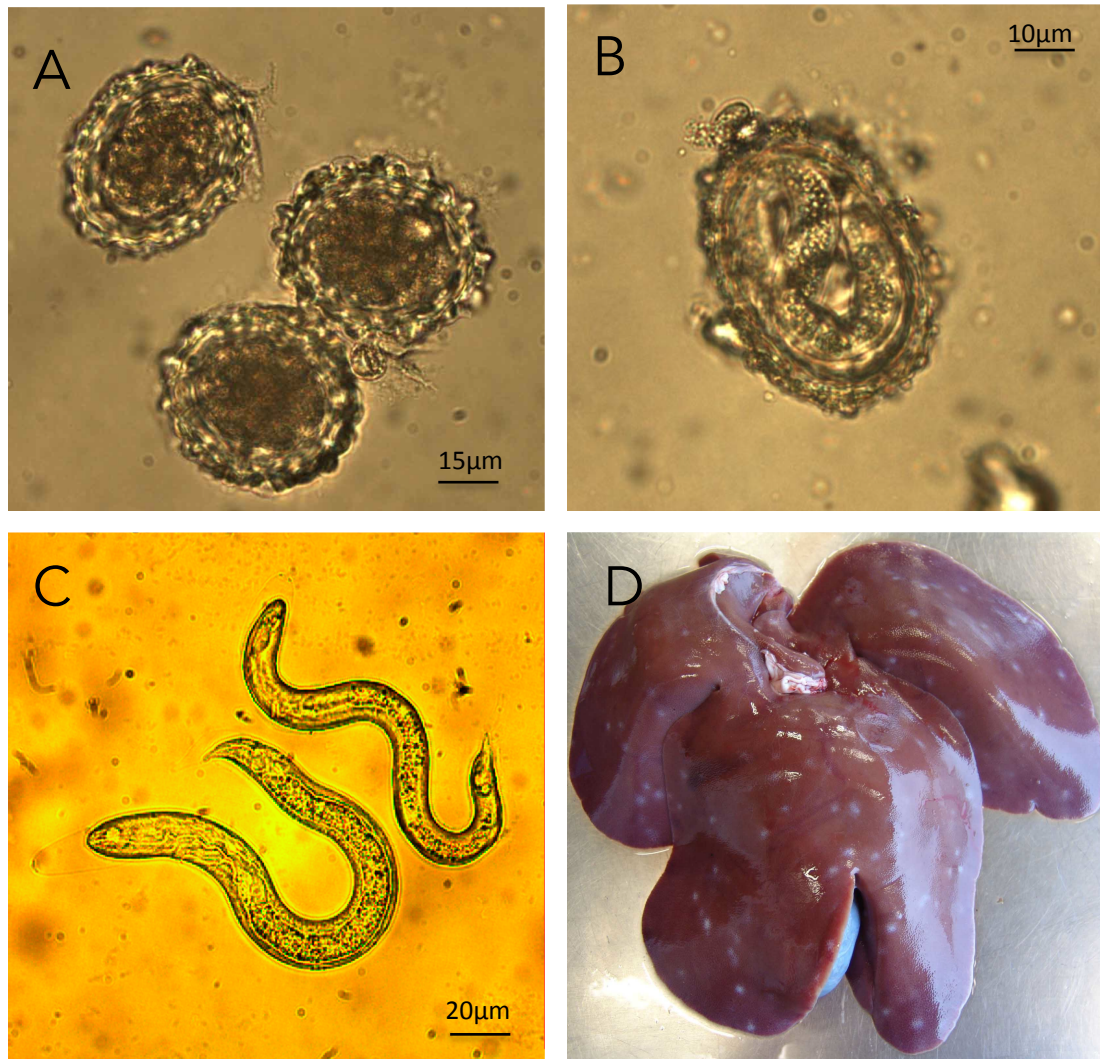
Figure 1.2: A: unembryonated eggs; B: fully embryonated, infective egg; C: L3 larvae, just hatched from the egg, covered by L2 cuticle; D: typical white spot lesions on the liver of a pig infected with *A. suum* (Pictures taken by J. Vlaminck).

1.2 Epidemiology of ascariosis

Prevalence

A. suum is still very prevalent today. It is the only helminth that is common in highly intensive production systems throughout the world (Roepstorff and Nansen, 1994). Few countries have up-to-date information on its prevalence, and different studies applied different methods to diagnose infections with *A. suum* in pigs, such as the percentage of rejected livers, the presence of worms in the intestines at the slaughterhouse or the detection of eggs

through coprological examination of faecal samples. Table 1.1 shows the results of studies that were performed in different countries and different types of stables, spread over a range of years. The table demonstrates that *A. suum* is still very prevalent and that the numbers of prevalence remain roughly the same over the years. If we have a closer look to China for example, there is an improvement in prevalence in 2005 in comparison to 2000, but in 2011 there is again an increase of 10%.

The prevalence and intensity vary with geographical region, production system (indoor/outdoor), age, breed, infrastructure of the stable (i.e. type of floor) and management system (i.e. all in/all out system). The implemented changes in farm management in 2013 to improve animal welfare can presumably increase the prevalence in the pig industry, i.e. group housing of pregnant sows. Sows in group have more nose-to-nose contact, as well as more oral contact with feces and urine (Maes et al., 2016). Old fecal samples were collected from pens to investigate the presence of eggs (Lai et al., 2011; Roepstorff, 1997; Roepstorff and Nansen, 1994), and based on these findings it could be concluded that the pens of fatteners and sows are the heaviest infected habitats (Nilsson, 1982). Despite the substantial presence of eggs in the farrowing pens (Joachim et al., 2001; Lai et al., 2011; Roepstorff, 1997; Roepstorff and Nansen, 1994), Roepstorff (1997) demonstrated that, even though the sows excreted considerable numbers of eggs in the farrowing pens, their piglets did not get patent *A. suum* infections. It was presumed that good hygiene in welfare pig farms and a dry microclimate in the farrowing pens prevented the eggs from embryonating and infecting the piglets. Prevalence within farm or within a group of pigs is usually dependent on age of the animals. Older growing fatteners have a higher prevalence than piglets and breeding sows (Roepstorff 1998). The prevalence is higher in gilts and younger parity females than in older sows. Over time, pigs that are exposed to *A. suum* develop an immunity (Urban et al., 1988).

There are some assumptions why *Ascaris* is still so prevalent in high intensity pig farms. The female adult worm can populate its host intestine several years, exceeding the average lifetime of a fattening pig easily. In addition, they are highly fecund, producing hundreds of thousands of eggs per day, which are shed via the faeces and contaminating the environment of the stable. These eggs are highly resistant and can survive in the stable climate

for several years. Even when anthelmintic drugs are applied and parasitic worms are expelled from the intestine, the pig keeps on reinfecting itself because it is continuously exposed to numerous eggs present in the environment. In addition, the anthelminthic drugs that are currently used do not have a remanent effect. Thus, even after treatment reinfections are possible through uptake of new eggs present in the stable. Finally, *A. suum* has a direct life cycle with an extra-intestinal migration phase, and therefore does not depend on intermediate hosts. The pre-patent period of 42 days is relatively short (Roepstorff, 2003), exceeding one fattening round amply.

Tabel 1.1: Results of prevalence studies throughout the world.

Year	Country	Sample size	% Infected farms*	Egg+	Worm+	Liver+	Reference
				% Infected pigs		
2000	China	100 outdoor pigs		37%			(Boes et al., 2000)
2001	Germany	13 farms		33%			(Joachim et al., 2001)
2004	Tanzania	70 pigs			44%		(Ngowi et al., 2004)
2005	The Netherlands	9 conventional farms	11%				
2005	The Netherlands	16 free range farms	50%				(Eijck and Borgsteede, 2005)
2005	The Netherlands	11 organic farms	73%				
2005	China	3,636 pigs		5%			(Weng et al., 2005)
2005	India	501 pigs			16%		(Gaurat and Gatne, 2005)
2008	Kenia	230 pigs			29%		(Nganga et al., 2008)
2010	USA	91 finishing farms	39%				(Pittman et al., 2010b)
2010	USA	40 farms (sow)	25%				(Pittman et al., 2010a)
2010	Switzerland	90 conventional farms	13%				(Eichhorn et al., 2010)
2010	Switzerland	20 free range farms	35%				
2010	Sweden	2.4 million pigs				5%	(Lundenheim and Holmgren, 2010)
2010	Denmark	79 farms, 1790 sows	76%	30%			(Haugegaard, 2010)
2011	China	916 pigs		15%			(Lai et al., 2011)
2011	Uganda	106 pigs from 56 households		40%	73%		(Nissen et al., 2011)
2011	Tanzania	13,310 pigs				4%	(Mellau et al., 2011)
2011	Tanzania	731 pigs			8%		(Mkupasi et al., 2011)
2012	England	34,168 pigs				4%	(Sanchez-Vazquez et al., 2012)
2012	Italy	667,028 finishing pigs				14%	(Luppi, 2014)
2012	Ireland	12,597 finishing pigs				12%	(Hidalgo, 2014)
2014	USA	61 farms	23%				(Duff, 2014)
2015	Portugal	6094 finishing pigs				60.3%	(Mateus et al., 2015)
2016	Mumbai	135 finishing pigs		33%			(Dadas et al., 2016)

* Based on EPG

1.3 Immunity development against infections with *A. suum*

To understand more of the characteristics of this parasite, insights on the immunity development are necessary.

Self-cure

The number of pigs showing liver lesions is not in accordance to the number of pigs with adult worms recovered from the intestine (Vlaminck et al., 2014). It seems that the number of adult worms is just a fraction in comparison to the amount of white spots. This is the consequence of a natural immune response of the pig. In pigs that are infected for the first time, usually up to 50% of the infective larvae administered reach the L4 stage 14 days after primar infection (Roepstorff et al., 1997). However, between days 17 and 21 p.i. most of these larvae are eliminated from the intestine. There is a self-cure reaction (Miquel et al., 2005; Roepstorff et al., 1997), causing the expulsion of more than 90% of the *A. suum* immature stages from the intestine before they even have the chance to develop into adult worms (Helwigh and Nansen, 1999; Masure et al., 2013b; Roepstorff et al., 1997). A consequence is that many pigs will never harbour adult worms, even though they are continuously reinfected. The effector mechanism behind the expulsion is currently unknown. It was assumed by Masure et al. (2013b) that faster gut movement is the origin, which prevents the larvae to remain in the small intestine.

Pre-hepatic barrier

Chronic natural and multiple inoculations with eggs have been associated with the development of pre-hepatic intestinal immunity, which stops freshly attained infective larvae from passing through the intestinal barrier and to reach the liver, preventing the formation of white spots (Eriksen et al., 1992; Masure et al., 2013a; Urban et al., 1988). Since the immune barrier is situated in the intestine, this type of resistance was referred to as the pre-hepatic barrier. In solidly immune pigs there is presumably a functional pre-hepatic barrier, which may result in no (or very limited) liver migration, even if animals are heavily exposed. Urban et al. (1988) demonstrated that this protective immunity develops at the level of the intestine. When *in vitro* hatched larvae were injected in the

mesenteric veins, they induced white spots. This in opposite to orally administered eggs, which failed to cause liver damage (Urban et al., 1988). What effector mechanism prevents larval penetration remains to be determined. Possibly there is a close interaction between histophysiological aspects and the immune response of the host, like tunica muscularis hypertrophy or an alteration of the surface mucosa (Stephenson et al., 1980). According to Masure et al. (2013a) it is the Th2 response of the host that prevents larvae from penetrating the gut, driven by mast cells and eosinophils, creating an inhospitable environment.

1.4 Pathogenesis and pathology of ascariosis

A large majority of the pigs having patent infections appear to be clinically healthy, even if the worm burden is high (Boes et al., 2010). However, pigs having ascariosis are more sensitive to opportunistic bacterial and viral infections (Curtis et al., 1987). In the lungs, the passage of migrating larvae can clear the way for pathogens, such as Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Swine Influenza Virus (SIV) and *Actinobacillus pleuropneumonia* (APP). However, Vlaminck et al. (2015) could not find any significant correlation between *Ascaris* and lung pathogens on the farms investigated.

Additionally, larval and adult worm burden enhance susceptibility to *Escherichia coli*, *Pasteurella multocida* and *Salmonella* spp. (Adedeji et al., 1989; Smith et al., 2011; Tjornehoj et al., 1992). Helminth infections may down-regulate the Th1 responses against bacteria and viruses, thereby reducing the efficacy of vaccinations (Urban et al., 2007), as recently described for *Mycoplasma hyopneumoniae* vaccination in pigs infected with *A. suum* (Steenhard et al., 2009).

Liver White Spots

Liver migration by L3 larvae is accompanied by severe pathology, thoroughly described by Roepstorff (2003). The consequences of *Ascaris* infection in the liver are well documented as liver white spots (LWS) (Fig 1.2 D). As the migrating larvae induce tissue damage to the liver, an inflammatory reaction is induced.

Subsequently, leucocytes infiltrate and cause pathological lesions, which are macroscopically visible as white stains on the liver (Frontera et al., 2003). The lesions are self-healing scar tissue and disappear with time, in most cases within 5-6 weeks post infection. Milk spots thus represent a recent *A. suum* infection (Roepstorff, 2003). Despite the appearance of these vanishing milk spots, the general texture of the liver is clearly hardened as a response to migration, which makes the livers less suitable for human consumption (Wismer-Pedersen et al., 1990).

Lung

6-8 days p.i. during the migration of *A. suum* larval stages through the lungs, pigs are subjected to respiratory stress, resulting in pneumonitis (Roepstorff et al., 1997). This is reflected as an increased breathing rate, dyspnea and a dry cough (Eriksen, 1981; Curtis et al., 1987; Matsuyama et al., 1998). On a histological approach, this has been correlated with the occurrence of fibroplastic lung lesions, inducing hemorrhagia and an inflammatory cell response (Figure 1.3) (Enobe et al., 2006). This appears to be one of the etiological organisms of the porcine respiratory disease complex (Liljegren et al., 2003).
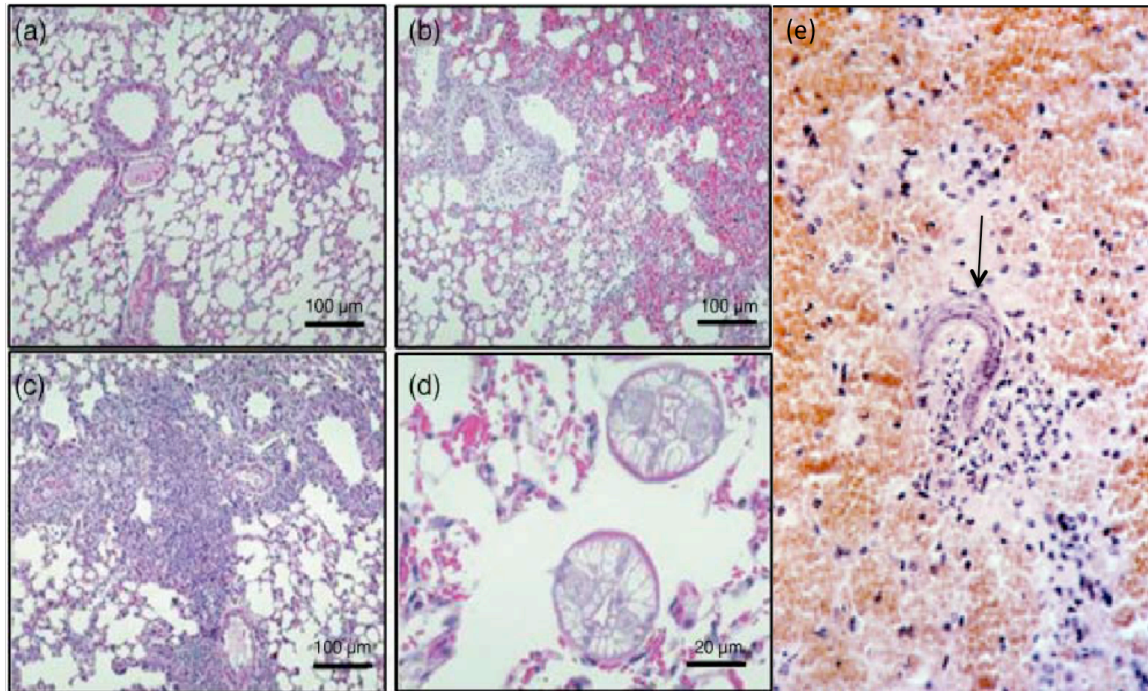
Figure 1.3: Histological alterations of lung tissue after *A. suum* infection. Representative lung section from normal (a) or infected mice after 8 (b) or 14 days (c), showing severe intra-alveolar hemorrhage (b) and intense inflammatory cell infiltration (c). Larvae are present in the alveolar space on day 8 of infection (d). H/E staining. *A. suum* larva in the lung (arrow) (e) (Enobe et al., 2006) (Pictures taken by Maria Fernanda Macedo-Soares).

Small intestine

At the onset of an infection with *A. suum*, as newly hatched third-stage larvae penetrate the cecum and proximal colon, marginal petechial bleedings of the mucosa are formed. Further on, the third-stage larvae molt to L4 when the nematodes return to the small intestine around day 10 p.i. (Roepstorff, 2003). Subsequently, a further molt takes place 2 weeks later to reach L5, ultimately developing into a sexually mature adult worm by day 42 p.i. The intestinal phase is associated with pathological changes in the mucosa (Stephenson et al., 1980), such as hypertrophy of the tunica muscularis and decreased villus height, resulting in a less efficient feed uptake.

1.5 Diagnosis of ascariosis

Infections with *A. suum* rarely cause clinical symptoms. These infections typically remain unrecognized by farmers and their veterinarians. There are different ways to diagnose the presence of *A. suum* infections on pig farms, such as the percentage of rejected livers, the presence of worms in the intestines at the slaughterhouse or the detection of eggs through coprological examination of faecal samples. However, each diagnostic method has its shortcomings. Some of the coprological techniques can create an overestimation of the prevalence (Permin et al., 1999). Low egg counts may be due to coprophagia and thus constitute to false positive results (Boes et al., 1997). In contrast, infections with only one worm or all worms of the same sex will result in an underestimation of the prevalence. In addition, the majority of the L4 larvae are rapidly expelled after arriving in the small intestine and only a small number of worms will mature into egg-producing adults (Roepstorff, 2003). Finally, white spots on the livers are lesions that are self-healing and disappear with time. Unfortunately, all of the tools fail in diagnosing an accurate prevalence.

Presence of worms

It is possible to observe expelled adult worms in the stable after anthelmintic treatment (Theodoropoulos et al., 2001). Alternatively, at slaughter the presence of adult worms in the small intestine can be registered. However, the larval stages in the intestine are small and hard to detect macroscopically. The detection of adult parasites is only a qualitative measure, and not a quantitative one, as the number of adult worms is not representative of the amount of migrating larvae the pig has been exposed to. This is the result of the protective immunity that causes an expulsion of the majority of the larvae.

Faecal examination

Since female adult worms produce eggs, the detection of eggs reflects the presence of adult worms in the intestine. For this, coprological techniques of sedimentation/flotation or McMaster can be applied (Thienpont et al., 1979). The sedimentation/flotation technique is a qualitative technique and is applied

to investigate whether parasites are present in the host or not. Alternatively, the McMaster test is used to investigate the quantity of eggs that are excreted, making it possible to determine an EPG (Eggs Per Gram of faeces), which provides more information on the infection pressure. When using coprological examinations for the detection of *A. suum* infections in pigs, there is a significant possibility of false positive and false negative egg counts. False positives could be the result of coprophagia (Boes et al., 1997). In general, EPG levels lower than 200 should be considered as false-positive. For diagnosis at farm level, misinterpreting a diagnosis because of a false negative detection is more important. Boes et al. (1997) showed that false negative results are possible when only worms of a single sex are present or only immature worms are present. Additionally, the presence of eggs in the faeces is not representative for the level of parasite exposure, as most larvae that completed their hepatotracheal migration will be expelled from the small intestine between day 17 and 21 post infection (Nejsum et al., 2009; Roepstorff et al., 1997). Furthermore, there seems to exist an inverse relationship between the number of adults found in the intestine and the amount of eggs given during a single experimental infection dose (Andersen et al., 1973; Roepstorff et al., 1997). With increasing infection dose, the elimination of adult worms from the gut is more effective (Roepstorff et al., 1997).

Liver White Spots

Hepatic white spots are typical and characteristic lesions caused by migrating *A. suum* larvae in pigs. After the migrating of larvae through the liver, the destroyed liver cells are replaced by interlobular depositions of fibrous tissue and cellular infiltrates, creating the typical white spots (Nakagawa et al., 1983; Perez et al., 2001). If white spots are present, it is very likely that an infection with *A. suum* took place (Vlaminck et al., 2014). However, it is possible that, while there are white spots present, no adult worms were to be found in the intestine or eggs in the faeces (Bernardo et al., 1990b). When exposure takes place during the last few weeks of the fattening round, for example when the animals were transferred to another stable, liver white spots can be determined at slaughter while adult worms were not able to develop yet (Vlaminck et al.,

2015). Another explanation could be that the worms were eliminated due to the natural immune responses of the host.

An absence of white spots on the other hand does not necessarily mean that pigs were not infected before. Milk spots have the property to heal, and 35 days after a primary inoculation with *Ascaris* eggs livers can appear to look normal again (Copeman and Gaafar, 1972). Hence, it only reflects recent larval migration (Eriksen et al., 1980). Livers might therefore look normal or only mildly affected at slaughter even though pigs could have been exposed to high numbers of infective eggs during the course of their life. Even more, when pigs are continuously exposed to migrating larvae, the number of white spots will gradually decline (Eriksen et al., 1992). Higher burdens with *A. suum* cause the pigs to infect themselves continuously, resulting in a natural immunity at the level of the intestine. This pre-hepatic barrier prevents the larvae from penetrating the gut wall to start their migration, the formation of white spots and subsequently, the development of adult worms (Eriksen et al., 1992; Masure et al., 2013a; Urban et al., 1988). Bearing this in mind, it is possible that chronically infected pigs appear to be utterly free from *Ascaris* at autopsy, which makes the amount of liver lesions a poor indicator of long-term *A. suum* exposure. This can result in a significant underestimation of *A. suum* infection levels in pigs. Although presence of liver white spots indicates the recent passing of migrating *A. suum* larvae, there exists no relationship between the number of lesions and the number of adult worms colonizing the gut (Nilsson, 1982).

Serology

Because of the natural immune responses active in most pigs which expel the majority of larvae from the gut after their hepatotracheal migration (Helwigh and Nansen, 1999; Roepstorff et al., 1997), there usually is no correlation between the adult worm load and antibody levels against parasite antigens in naturally or trickle-infected pigs (Roepstorff and Murrell, 1997; Nejsum et al., 2009). ELISA values actually reflect both the degree of larval exposure and the number of adult parasites that inhabit the pig's intestine (Vlaminck et al., 2014). Recently, Vlaminck et al. (2011) tested an ELISA using *A. suum* haemoglobin (AsHb), a

protein purified from the pseudocoelomic fluid, as an antigen. AsHb is being expressed from the lung stage onwards (Vlaminck et al., 2011). Consequently, larval migration in itself is sufficient to develop a measurable antibody response to the antigen. Thus, the actual presence of adults in the intestine is not required. This diagnostic antigen showed a high diagnostic sensitivity and specificity (99.5 and 100% respectively) in experimentally infected pigs (Vlaminck et al., 2012). The ELISA test could detect total IgG antibodies produced against AsHb from 6-8 weeks post-infection onwards. The test showed superior sensitivity for the detection of *A. suum* infections in comparison to stool examination and percentages of condemned livers. The *A. suum* infected pigs only showed increased IgG levels after a minimum of 6 weeks post infection, permitting the detection of infections in fattening pigs nearby the end of a fattening round.

1.6 Control of ascariosis

Chemotherapy and treatment regime

The drug administered for the control of *Ascaris* infections needs to comply with several criteria. Namely, the margin of safety of the used compound should be wide. The efficacy of the compound should include the different stages of *A. suum*. The spectrum of activity should be broad. The mode of administration is preferably easy to apply. Finally, the cost of the drug should be acceptable (Holland C., 2013). A review of the most commonly used anthelmintics and their effectiveness is shown in table 1.2. All compounds presented have proven to be highly effective against adult roundworms in pigs. Additionally, it demonstrates that levamisole, pyrantel, flubendazole and fenbendazole have shown activity against the migrating larvae of *A. suum* as well (Oakley, 1974; Stewart et al., 1984). The benzimidazole drugs (e.g. fenbendazole, flubendazole) have an excellent broad-spectrum activity and safety. The drugs bind nematode tubulin, resulting in cell lysis (Lacey, 1988). Anthelmintics of the macrocyclic lactone family (e.g. ivermectin, doramectin) potentiate the effect of the neurotransmitter gamma amino butyric acid (GABA) in parasitic nematodes (Brownlee et al., 1997; Feng et al., 2002), generating nematodicidal activity (Martin and Pennington, 1988). They have an excellent broad-spectrum activity against

nematodes and also against ectoparasites, such as hog lice (*Haematopinus suis*) and mange mites (*Sarcoptes scabiei var suis*) (Lee et al., 1980; Lichtensteiger et al., 1999; Logan et al., 1996; Stewart et al., 1981b; Yazwinski et al., 1997). In comparison with the other anthelmintics, macrocyclic lactones have a significantly longer residual effect. Levamisole has a rather narrow therapeutic index, and some care is required when medicating a group of animals with a large range of bodyweight (McKellar and Jackson, 2004).

Anthelmintics are available in a variety of formulations. Administration of the drug in either feed or water is the most economical way and easy to apply, but when there is a lot of variety in body weight in the animals belonging to the same treatment group it is hard to dose correctly. Nevertheless, most farmers prefer this way of application. Application by injection on the other hand, is more labour and time consuming and therefore more expensive, but the dosage is more accurate. One can take the animal welfare into consideration, since injection with a needle can be painful and stressful for the animals.

Routine anthelmintic treatments are commonly used these days in intensive pig rearing systems. Unfortunately, many treatments schemes vary according to farm and category of animal. Sows are usually treated 2 times per year in Belgian farms (based on www.Varkensloket.be). Treatment should preferably take place 14 days prior to movement into clean farrowing facilities (Pittman et al., 2015), to make sure that adult worms are removed from the small intestine and no egg-infestation occurs towards their piglets.

In Belgian farms, it seems that piglets are typically treated upon entering the fattening units and occasionally once more halfway through the fattening period (based on www.Varkensloket.be). Seeing that one fattening round takes approximately 16 weeks and the prepatent period for *Ascaris* is 6 weeks, treatment schemes like aforementioned will not be able to substantially decrease the infection intensity on a farm. Therefore, the frequency of treatments should be based on the prepatent period of the worm species. It is important to treat the pigs before the worms become adult and start shedding millions of eggs and contaminate the environment again. When a strict deworming scheme is applied, the infectivity of the environment will drop, as

newly arrived pigs will take up eggs from the environment. Additionally, eggs will lose their viability over time or swept away by cleaning actions. This ultimately results in a group of animals with a reduced or absent parasitic burden. Moreover, it is equally as important to persist the repeated application of anthelmintics by administering a strict schedule. If the environmental contamination in a stable is high, it will presumably take several fattening rounds to reduce the infection pressure in a significant way (Bakker, 1984).

Risk factors

Farm management can have a major impact on the epidemiology of *A. suum* in several ways. An overview of factors diminishing the infection levels on a farm is shown in Table 1.3. Undoubtedly, the type of production system that is applied on a farm will have a great influence (Nansen and Roepstorff, 1999). The majority of fattening farms in Belgium are conventional indoor farms, where fattening pigs are hold in pens, where a maximum density of 0,65 m² per animal is legally mandatory and an all in/all out system (AI/AO) is applied. Lower prevalence of *A. suum* is established when an AI/AO system is implemented (Roepstorff and Jorsal, 1990; Joachim et al, 2001; Roepstorff et al., 1999; Tielen et al., 1978). AI/AO systems make it possible to insert a period during which the stable is empty, making cleaning possible. This can reduce the horizontal spread of pathogens. Weaning age is a significant factor in having ascariasis (Roepstorff et al., 1999). Early weaning at 3 weeks of age or less makes the transmission of *A. suum* directly from dam to offspring unlikely, seeing that the time for embryo development to an infectious L3 is not long enough (Pittman et al., 2015). Indirect transmission should also be kept into consideration, such as exposure to infectious eggs remaining in the farrowing stable from previous groups (Roepstorff, 1997) or mechanical transmission from other farm areas or via boots, clothing and other materials. The type of floor present on a farm has an influence on the prevalence of *A. suum*. The majority of the Belgian fattening farms has semi- or fully slatted floors. It is advised to reduce the contact of pigs with their own faeces or that from previous inhabitants. Consequentially, housing of pigs on fully slatted floors is likely to reduce the chance of parasite infections in comparison to partially slatted floors (Roepstorff and Jorsal, 1990; Sanchez-Vazquez et al., 2010; Tielen et al., 1978). On the other hand,

contamination of the pens with urine diminishes egg development (Katakam, 2016). The presence of bedding increases the risk of fast development of the eggs and impedes the effectiveness of cleaning actions. However, according to Jankowska-Makosa and Knecht (2015) the presence of a deep manure layer makes it difficult or impossible for the parasitic eggs to develop, due to the unsuitable pH, lack of oxygen or destruction by rotting products and fermentation. Eggs are less likely to develop when the humidity is low. Considering that the lying area in a pig pen tends to be drier, it is advisable not to position the water supply there, which creates a favourable environment for the eggs to develop. Drinking nipples and troughs are preferably placed in the dung area, where the eggs are adversely affected by the presence of inhibiting pig urine. This will ultimately result in a decreased prevalence of *A. suum* (Roepstorff et al., 1999). Presumably, the rising temperatures in spring and summer allow simultaneous development of infectious stage larvae in the eggs that have spent the winter in the pig pens. This would result in an increase of infective eggs present in the environment and subsequently, liver condemnation rates are higher during summer and early autumn (Nilsson, 1982; Lai et al., 2011; Menzies et al., 1994; Sanchez-Vazquez et al., 2012). Joachim et al. (2001) indicated that in older pens, the prevalence of *Ascaris* infections was significantly higher (63.0%) than in the new pens (27.9%). Older pens with rough and uneven surfaces are more difficult to clean in comparison to new stables, which are more likely to have smooth walls and floor surfaces. Steam cleaning (Haas et al., 1998) and drying of the pens is highly recommended for killing nematode eggs. However, these methods are hard to achieve in practice. On breeding farms, washing the skin of sows to remove organic material before farrowing is often implemented in order to control infestations (Roepstorff and Nansen, 1998).

Since 2013, group housing of sows during gestation became mandatory in the European Union (EU 2008/120/EC). As a result, sows in group have more nose-to-nose contact, as well as more oral contact with feces and urine (Maes et al., 2016).

Table 1.3: List of factors associated with a decreased prevalence of *A. suum.*

Factor	Reference
Indoor system	Lai, 2011
	Roepstorff and Nansen, 1994
All in/all out	Joachim et al, 2001
	Roepstorff and Jorsal, 1990
	Roepstorff et al., 1999
	Tielen et al., 1978
Early weaning	Pittman et al., 2015
	Joachim et al, 2001
	Roepstorff and Jorsal, 1990
	Roepstorff et al., 1999
	Tielen et al., 1978
Slatted floor	Roepstorff and Jorsal, 1990
	Tielen et al., 1978
	Sanchez-Vazquez et al., 2010
Deep manure layer	Jankowska-Makosa and Knecht, 2015
New pens	Joachim et al., 2001
Water supply in dung area	Roepstorff et al., 1999
Season: winter	Nilsson, 1982
	Lai et al., 2011
	Menzies et al., 1994
	Sanchez-Vazquez et al., 2012
Steam cleaning	Haas et al., 1998
Washing of sows prior to farrowing	Roepstorff and Nansen, 1998
Individual housing	Maes et al., 2016

More organic farms have been established as a consumers' reaction against the industrialised husbandry (Roepstorff et al., 2011). However, in Belgium this type of husbandry is rather uncommon (less than 1%). Organic pig production differs in many ways from conventional production of pigs. Consumers prefer meat that is reared under biological conditions, using fewer chemicals and guaranteeing better animal welfare. There are restrictions concerning the use of antibiotics, and access to outdoor areas and spacious establishments indoor with plenty of bedding material are required. In addition, it is forbidden to restrain the animals and they should be fed organically produced feed. Under these conditions,

parasites are more prevalent. Studies showed that conventional pig farms had lower infection levels than free-range and organic farms (Eijck and Borgsteede, 2005). On farms where pigs have access to outdoor areas, there is a higher infection intensity, as well as a higher diversity of parasite species compared to intensive indoor systems (Lai, 2011; Roepstorff and Nansen, 1994). Since the use of traditional synthetic drugs is not allowed, farmers prefer a biological approach for the control of parasitic infections on their farms (van Krimpen et al., 2010). However, whether this is realistic is another matter. Although there are many plants that most likely have any nematocidal effects (Chitwood, 2002), studies by van Krimpen et al. (2010) involving infected pigs treated with *Papaya* fruits, *Bolbo* leaf, complete *Artemesia* plants or a herb mixture containing *Echinacea purpurea, Thymus vulgaris* and *Melissa officinalis* showed no significant reduction on the worm burden in comparison with untreated controls. Furthermore, Thapa et al. (2015) showed that the nematophagous fungi *Pochonia chlamydosporia* and *Purpureocillium lilacinum* only had a minor ovicidal effect on eggs of *A. suum in vitro*.

Table 1.2: Different anthelmintic drugs and their efficacy against different stadia of _A. suum._

Drug	Formulation	Dose rate	Effectiveness to adult _A. Suum_	Effectiveness to larval stages	Reference
Dichlorvos	Orally	43 mg/kg, 1d	100%		(Marchiondo and Szanto, 1987)
	In feed	17 mg/kg, 1d	100%		(Marti et al., 1978)
Piperazine	In water	200 mg/kg, 1d	100%		(Steffan et al., 1988)
			100%		(Marchiondo and Szanto, 1987)
			100%		(Stewart et al., 1981a)
Fenbendazole	In feed	3 mg/kg, 3d		99% to liver and lung stage L3	(Stewart et al., 1984)
			100%		(Corwin et al., 1984)
			92,40%	Reduced liver lesions	(Marti et al., 1978)
		2.5 mg/kg, 3d		100% to L4 stage	(Stewart and Rowell, 1986)
		30 ppm, 10d	100%		(Vanparijs et al., 1988)
Flubendazole	In feed	5 mg/kg, 1d	100%		
		1.5 mg/kg, 5d	100%	85% to L4 stage	(Bradley et al., 1983)
Oxibendazole	Orally	15 mg/kg, 1d	100%	92% to L4 stage	(Pecheur, 1983)
	In feed	40 ppm, 10d	100%	100% to L4 stage	
Levamisole	Injection	7.5 mg/kg, 1d	100%	92.3% to L4 stage and 63.5% to lung stage L3	(Oakley, 1974)
	In feed	8 mg/kg, 1d	97.7%		(Marti et al., 1978)
Pyrantel	In feed	96 g/ton feed, 24d	100%	97-100% to lung stage L3	(Kennedy et al., 1987)
		106 mg/kg feed, cont.	100%	Reduced liver lesions	(Kennedy et al., 1980)
		2 ppm, 7d	100%		(Primm et al., 1992)
Ivermectine	In feed	100 µg/kg, 7d	97.7%	100% to L4 stage	(Alva-Valdes et al., 1989)
		200 µg/kg, 7d	100%	100% to L4 stage	
		300 µg/kg, 7d	98.7%		(Marchiondo and Szanto, 1987)
	Injection		100%		(Schillhorn and Gibson, 1983)
			97.5%		(Lichtensteiger et al., 1999)
	In water	300 µg/kg, 1d	100%	100% to L4 stage	(Schillhorn and Gibson, 1983)
			100%	100% to L4 stage	(Stewart et al., 1996)
Doramectin	Injection	300 µg/kg, 1d	100%		(Mehlhorn et al., 1993)
			100%		(Yazwinski et al., 1997)
			100%		(Lichtensteiger et al., 1999)
Oxfendazole	Orally	30 mg/kg, 1d	100%		(Alvarez et al., 2013)

Control of larval development and *Ascaris* eggs in the environment

Once a stable is contaminated with *Ascaris* eggs, it is hard to obtain a parasite-free environment. *Ascarid* eggs have been called "one of the most resistant biological structures" (Wharton, 1980). The eggs are highly resistant to chemicals, such as strong acids and bases, and environmental influences. This is due to their complex shell, which is composed of 4 layers, namely an inner lipoprotein layer, a thicker chitine layer, a vitelline layer and a thick outer albuminous uterine layer. Because of their resistance, *A. suum* eggs are commonly used to test potential disinfection chemicals. In one study, the eggs of *A. suum* were resistant to 11 commonly used disinfectants in swine facilities, including quaternary ammonium, phenol, glutaraldehyde based products and sodium hydroxide (van den Burg and Borgsteede, 1987). Resistance to povidone-iodine has also been reported (Labare 2013).

Several attempts to inactivate *A. suum* larvae using fatty acids (Butkus et al., 2011), high hydrostatic pressure (Rosypal et al., 2007), low-pressure UV radiation (Brownell and Nelson, 2006), and ammonia (Pecson and Nelson, 2005) were not practical or available for everyday use. In a study by Oh et al. (2016) the efficacy of some disinfectants was evaluated on the inactivation of *A. suum* egg development *in vitro*. The embryonic development of decorticated (i.e. removed from the albumin layer which causes the eggs to adhere to each other) *A. suum* eggs was investigated for 3 and 6 weeks of incubation at 25°C after exposure to different disinfectants. At 6 weeks of incubation, decorticated eggs exposed to all disinfectants tested showed embryonation regardless of exposure time, except for 10% povidone iodine, which completely inhibited the embryonation of decorticated *A. suum* eggs at 6 weeks of incubation. However, results of the embryogenesis of intact undecorticated *A. suum* eggs showed a significant reduction from over 90% to below 5% from 5 min exposure to 10% povidone iodine solutions, but failed to completely inactivate the eggs. This study demonstrated that many commercially available disinfectants failed to inactivate larval development *in vitro*.

1.7 Impact of *A. suum* on technical performance

Over the years several studies tried to measure the negative economic impact caused by infections with *A. suum*. It is difficult to determine economic losses due to parasites that mostly induce subclinical parasitism or subtle symptoms. There are numerous interactions between the worm and its host on the one hand, and the interference of secondary pathogens, organ damage, the production system and alterations to immunity on the other hand. Additionally, between farms there can be variations that have an influence on the prevalence of *A. suum* (Roepstorff and Jorsal, 1990), such as type of floors (Joachim et al., 2001), climate in the stables, farm management (f.e. all-in/all-out system), genetic differences and gender of the animals (Jankowska-Makosa and Knecht, 2015; Bernardo et al., 1990a; Boes et al., 2010). This makes it challenging to define one unambiguous outcome. Losses due to ascariasis in pigs can be summarized as farm economic losses due to clinical effects (although limited), reduced growth and feed conversion efficiency, extension of the fattening round and costs of control (e.g. use of anthelmintics); abattoir operator losses due to condemnation of livers and lower meat quality; potential interference with vaccinations and higher risk of co-infections. Other factors can influence the economic outcome of a production round other than the presence of *A. suum*. In a study conducted by Zimmerman et al. (1973) protein deficiency in the feed, absent of treatment with pyrantel and a challenge with *Mycoplasma* each reduced the average daily growth and increased feed conversion. It is clear that the economic impact of ascariosis is not always in a direct way. Paving the way for other pathogens and lower meat quality also results in (sometimes marginal) losses. Due to secondary diseases, the time before slaughter weight is reached can increase.

The presence of *A. suum* on a farm reduces its productivity in several ways. Various studies investigating the influence of ascariosis on the average daily growth (ADG), feed conversion ratio (FCR) and meat quality are shown in Table 1.4. Importantly, some of these studies are performed under natural conditions, while others are based on experimental infections.

In numerous studies artificially infected animals were compared to uninfected control animals. It has been assumed that the larval migration was the phase of infection that affected the growth of pigs most severely (Spindler, 1947). However, Stephenson et al. (1980) infected pigs with post migratory larvae taken from rabbit intestines and showed that adult worms depressed growth rate and feed intake of pigs that had not been subjected to larval migration. Experimental studies in pigs given 20.000 *A. suum* eggs have shown that digestion coefficients were affected adversely only during the maturation phase and not during the migratory phase or early intestinal phase of infection (Hale et al., 1985). Similarly, pigs that received 3 doses of 200 eggs had lower growth rates than the uninfected controls, but only after mature worms were present in the gut, based on the observation of eggs in the faeces. The growth depression was related to the amount of adult worms found in the gut at necropsy (Forsum et al., 1981). However, no significant differences were found in ADG and FCR in experimentally infected pigs with 1.000 infective *A. suum* eggs every other day over a 52-day period (Urban et al., 1989). Bernardo et al. (1990a) and Jankowska and Knecht (2015) described a decrease in ADG when burdens with *A. suum* were higher, based on coprological methods. However, Bernardo et al. (1990a) considered the decrease of ADG in natural *A. suum* infected fatteners to be a result of pneumonia and not the parasite itself. Bearing in mind that *A. suum* paves the way for secondary lung pathogens, the effect on the ADG by *A. suum* infections was presumably indirect. To investigate the influence of different infection doses, increasing doses of infective eggs in experimentally infected pigs with 0, 23, 226 or 2.256 eggs/kg bodyweight were applied and demonstrated a significant linear effect on feed efficiency (Hale et al., 1985), but did not influence the depression in ADG in a significant way. Similarly, Stewart and Hale (1988) demonstrated that experimental infections with 23, 226 or 2.256 eggs/kg bodyweight reduced daily gain and increased feed conversion in a linear, though non-statistical way. According to the results obtained by Kipper et al. (2011), parasitized pigs needed 5 days extra to reach slaughter weight in opposite to non-challenged animals. Presumably, the reduction of feed intake is the key response characteristic of the host to the manifestation of parasites (Kyriazakis et al., 1998).

To demonstrate whether anthelmintic drugs improve the economic outcome of infected animals, several studies compared treated animals with untreated controls or animals that received a placebo. Pigs were infected experimentally three times with 2000 *A. suum* eggs 11 days apart followed by fenbendazole treatment (Stewart et al., 1985). This study found no differences in ADG or FCR. The study did report a 21% lower weight gain in untreated naturally exposed pigs in the finishing phase (days 53-110) in a contaminated outside yard (days 0-52) as compared to pigs reared under similar conditions but treated twice with either fenbendazole or ivermectin during the exposure phase. To support this, a group trickle infected with *A. suum* for the same period but kept in a clean, concrete pen did not exhibit reduced performance in the finishing phase. The conclusion was that a strict control of environmental contamination and strategic application of anthelmintics is needed to avoid long-term effects of infection. However, despite treatment, pigs are immediately exposed to new infections and new larvae migrate in their bodies. Lassen et al. (2017) investigated two groups of pigs that were diagnosed to be naturally exposed to *A. suum* based on serology and faecal samples. One group was treated with 2.5 mg fenbendazole/kg bodyweight in water for 2 consecutive days 1 and 6 weeks after arriving in the fattening stables, whereas the other group received a placebo. A reduced % of liver rejection was detected, namely 69.8% fewer in treated animals than in the placebo pens. Fenbendazole did not improve the ADG as hypothesized. However, untreated animals with eggs present in their faeces at week 6 had a decreased ADG of 61.8 g/day. Patent infections thus had an influence on the overall ADG. Kanora (2009) demonstrated that administering a deworming program of 30 ppm flubendazole during 5 days every 5 weeks during 4 rounds resulted in a reduction of affected livers and in a positive effect on the ADG, compared to the untreated controls. However, this was not in a statistically way except in one farm out of four. Boes et al. (2010) compared natural exposed fattening pigs that were treated with flubendazole (Flubenol) to animals treated with a placebo. The prevalence of LWS did not differ significantly between the treated and the non-treated pigs, nor the ADG, FCR and lean meat percentage.

In addition, Knecht et al. (2011) investigated the effect on meat quality. A population of 120 pigs was coprologically diagnosed if they were infected with *A. suum*. Subsequently, the EUROP classification was conducted in the slaughterhouse. The meatiness was negatively correlated to the average number of helminth eggs in a statistical way.

Despite this brief summary in Table 1.4, a lot of the performed studies have very different outcomes. This could be explained by the fact that there is a variety of infection levels between the conducted studies. The diagnostic tools used to detect whether an *A. suum* infection is present or not varies between different studies, and unfortunately their sensitivity differ substantially (cfr. 1.5 of this chapter). In addition, some of the tools are only able to detect patent infections, while others detect the level of exposure as well. Vlaminck et al. (2015) concluded that serological screening could be used to indicate the presence of roundworm infection in fattening pigs. This study showed, besides an increase of affected livers, a negative correlation between both serology and % affected livers to the ADG in natural exposed fattening pigs.

Table 1.4: Overview of different studies performed on the influence of ascariosis on technical performance parameters.

Infection type	Infection dose	Diagnosis	ADG decrease	FC increase	Meat quality decrease	Reference
Experimental	200 2 week old larvae		not significant	not significant		(Stephenson et al., 1980)
Experimental	600 eggs/pig		-			(Forsum et al., 1981)
Experimental	60,000 eggs/pig		10% (not significant)	13%		(Hale et al., 1985)
Experimental	2,256 eggs/kg bodyweight		80g (not significant)	13% (not significant)		(Stewart and Hale, 1988)
Experimental	1,000 eggs every other day over 52d (N=26)		not significant	no influence		(Urban et al., 1989)
Natural		worm count and ELISA	-	no influence		(Urban et al., 1989)
Natural		EPG, worm count and WS	<1%			(Bernardo et al., 1990a)
Natural		EPG	15.1-34.7g			(Kanora, 2009)
Natural		EPG	no influence	no influence	no influence	(Boes et al., 2010)
Natural		Meta-analysis	31%*	17%*		(Kipper et al., 2011)
Natural		EPG			1,56%*	(Knecht et al., 2011)
Natural		EPG	60g		3.2%	(Jankowska-Makosa and Knecht, 2015)
Natural		ELISA	-			(Vlaminck et al., 2015)
Natural		EPG and ELISA	61,8g		no influence	(Lassen et al., 2017)

* Infection with a variaty of endoparasites

1.8 Conclusion

The prevalence of infections with *A. suum* remains very high, even though a range of effective anthelmintics is available. Considering the subclinical nature and an -until recently- lack of appropriate diagnostic tools, there are still a lot of uncertainties regarding this nematode that need to be unravelled. Although numerous papers emphasize the economic losses caused by infections with *A. suum*, it has been difficult to demonstrate changes in parameters generally associated with subclinical gastrointestinal parasitism in a statistically meaningful way.

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Objectives

Today, *Ascaris suum* is still the most prevalent parasite infecting swine in the modern pig farm industry. This is mainly due to the subclinical nature of this parasite and, second, a lack of adequate diagnostic tools to measure infection levels of the animals. Recently, research showed that serology seems to be a useful tool to measure exposure of fattening pigs to *Ascaris*. Therefore, the overall goal of this thesis was to investigate how serology could be further implemented in practise.

The first objective was to evaluate whether serology could also be used to measure exposure of weaned piglets to *A. suum* (**Chapter 2**). Piglets are often routinely treated with anthelmintics to avoid the import of *A. suum* at the onset of the fattening phase. As this is currently done without any form of diagnosis, serology could offer an interesting tool in this process. It can provide more information about the infection levels and the timing of a first anthelmintic treatment can be based on the results.

The second objective was to investigate whether serology could be used to measure the effect of *Ascaris* infections on technical performance parameters of fattening pigs (**Chapter 3**). Although several studies stated that exposure to *Ascaris* can result in significant economic losses, it remains difficult to measure or calculate these potential losses in practise. Research on other parasitic diseases has shown that a quantitative diagnostic tool such as serology can be used to determine an 'economic threshold' and therefore we wished to investigate whether this is also applicable to *Ascaris*.

Chapter 2

Evaluation of serology to measure exposure of piglets to *Ascaris suum* during the nursery phase

2.1 Introduction

Ascaris suum is a widespread parasitic nematode that infects pigs (Nansen and Roepstorff, 1999; Roepstorff et al., 1998). Despite the availability of effective anthelmintics, prevalences of *A. suum* on farm level remain high throughout the world (Thamsborg et al., 2010). *Ascaris* infections can occur in all age groups of pigs, but housing, production system and management factors often determine which age group shows the highest intensity of infection (Boes et al., 2010). In most cases *Ascaris* infections are subclinical of nature without clear disease symptoms (Vlaminck et al., 2014). In the case of highly exposed animals the main clinical symptoms are respiratory problems (Urquhart et al., 1996) caused by the larvae migrating through the lungs (Boes et al., 2010).

Several studies have shown that exposure to *Ascaris* can result in significant losses for the pig producers. These losses can be directly caused by *Ascaris*, such as reduced growth and feed conversion (Hale et al., 1985), costs of anthelmintics, condemnation of affected livers (Perez et al., 2001) and lower carcass quality (Kanora, 2009). In addition, exposure to *Ascaris* can also indirectly affect the health status of the animals due to the immunomodulatory effect of this parasite, making the animals more susceptible to co-infections and/or interfere with vaccine efficacy (Steenhard et al., 2009).

Ascaris infections can be diagnosed post-mortem by the presence of worms in the small intestine or the presence of white spots on the livers (Vlaminck et al., 2012) or *in vivo* by detecting eggs in faecal samples (Roepstorff, 1998). Unfortunately, all these approaches have severe shortcomings. First, most pigs will expel the L4 larvae when they arrive back in the small intestine after the hepato-tracheal migration. Therefore, only a small number of pigs exposed to *Ascaris* will actually harbour egg-producing adults (Masure et al., 2013; Roepstorff et al., 1997). As a result of this strong over-dispersion, diagnosis based on the presence of worms or eggs in the faeces could severely underestimate true exposure and infection levels. Second, liver white spots are scar tissue, which heals and disappears over approximately 35 days (Copeman and Gaafar, 1972). Therefore, the number of liver white spots is only a reflection of recent larval migration and does not always reflect the level of long-term *A. suum* exposure (Nejsum et al., 2009b).

Furthermore, the required visual assessment of the livers at the slaughterhouse also makes this parameter somewhat subjective.

As an alternative, the use of serology for diagnosis has recently been investigated. Vlaminck et al. (2012) reported on a serodiagnostic test that is based on the antibody recognition of a haemoglobin protein (Hb), which is being expressed from the lung stage onwards and mainly produced by adult *A. suum*. Evaluation of the test on commercial farms showed that it had a better sensitivity for the detection of *A. suum* infections in comparison to faecal egg counts (Vlaminck et al., 2012) and that antibody levels measured at the end of the fattening period reflected infection intensity (Vlaminck et al., 2015). Based on the serological result, the deworming strategy applied in the fatteners can subsequently be adjusted if necessary.

Most commonly, in Belgian farms piglets remain suckling in the farrowing stable until 3 weeks of age, after which they are weaned and transferred to clean and warm (30°C) nursery facilities for a duration of 7 weeks. Subsequently, they are transferred to the fattening units at approximately 10 weeks of age, weighing 22 kg in average. At arrival, piglets are often routinely treated with anthelmintics to avoid the import of *A. suum* with these animals. As this is currently done without any form of diagnosis, serology could offer an interesting tool in this process. It can provide more information about the level of exposure at the onset of the fattening phase and the timing of a first anthelmintic treatment can be based on the results. The aim of the current study therefore was to investigate whether serology could also be used to measure exposure of piglets to *A. suum* during the nursery phase. For this, a 7 week nursery phase was mimicked during which piglets were trickle infected with different doses of *A. suum* eggs and their serum subsequently analysed on 2 ELISA tests based on the antibody recognition of either a haemoglobin protein purified from the pseudocoelomic fluid of adult *A. suum* worms or a water-soluble protein homogenate of the 3rd stage larvae that migrate through the lungs (L3-lung). In a second phase, the most promising serodiagnostic test was used in a seroprevalence study in Belgium to get more insights into the level of exposure to *A. suum* of piglets during the nursery phase.

2.2. Material and methods

2.2.1 Ethics statement

All animal experiments were conducted in accordance with the E.U. Animal Welfare Directives and VICH Guidelines for Good Clinical Practice, and ethical approval to conduct the studies were obtained from the Ethical Committee of the Faculty of Veterinary Medicine at Ghent University (Identification number EC2015/55 and EC2013/143) who approved the document.

2.2.2 Parasite material

Adult female *A. suum* worms were collected from the intestines of naturally infected pigs at the local abattoir. The eggs were obtained by dissection of the worm uteri and suspended in a 2% potassium dichromate solution ($K_2Cr_2O_7$) as an oxidizing agent, to a volume of 50ml and placed in a culture flask. The eggs were incubated restricted from light at 27°C approximately 30 days until fully embryonated and then used to infect the piglets.

Infective L3 lung larvae were obtained by removing the lungs from 10 week old pigs experimentally infected with 250 000 eggs 7 days post infection. The collected lung tissue was cut using scissors into pieces of approximately 0,5-1 cm3 and poured upon a Baermann filter filled with phosphate buffered saline (PBS) at 37°C and kept overnight, where the larvae were allowed to migrate out of the tissue (Urban et al., 1981). After collection at the bottom of the funnel, the parasites were washed 3 times with PBS, grounded in liquid nitrogen and the pellet resuspended in PBS with a protein inhibitor mix (Vlaminck et al., 2016). After centrifugation, the supernatant was collected and the protein concentration determined by BCA.

2.2.3 Animal experiments

In a first study, 3 groups (n=10) of 4-week old pigs were individually orally infected on a daily basis using a 5 ml syringe during 7 consecutive weeks, in order to mimic the nursery phase. The piglets received an infection dose of approximately 10, 100 or 500 eggs/day. One group of 10 piglets served as a negative control group. Blood was collected every week, using 5 ml serum

tubes. The first collection took place before any infection occurred, at the age of 4 weeks. At the end of the study, at an age of approximately 11 weeks, all animals were euthanized by electrocution, followed by exsanguination. Intestinal content of each piglet was collected and sieved over a 220-μm sieve in order to determine the number of *Ascaris* worms present. A second identical study was subsequently performed with 4 groups (n=10) of 4-week old pigs that were individually orally infected using a probe, with approximately 20, 40, 60 or 80 eggs/day for 7 consecutive weeks. In addition to the number of worms present in the intestines, the number of liver white spots were also quantified in the second study, based on macroscopically assessment.

All piglets from both studies came from the same conventional farrow-to-finish farm and were randomly selected from different litters. All piglets were weaned at 4 weeks of age and simultaneously transferred to our facilities at the onset of the trial, weighing 7.8 kg in average.

Finally, an additional 100 individual serum samples were collected from *A. suum* naïve piglets at an age of 10 weeks. To avoid accidental infection with *A. suum*, 3 weeks old piglets born from worm-free sows based on ELISA were housed in a new stable that had not been used before. In addition, the animals received three treatments with Flubendazole in the drinking water during the 7 weeks of housing to further avoid accidental infection. All piglets had access to feed and water ad libitum throughout the experimental period.

2.2.4 Analysis of the serum samples

Serum samples were individually analysed on two different ELISA tests which were based on the haemoglobin purified from the pseudocoelomic fluid from adult *A. suum* worms and the water-soluble protein homogenate from L3 larvae migrating through the lungs (L3-Lung ELISA). The haemoglobin ELISA was performed essentially as described in Vlaminck et al. (2012). For the ELISA test based on the L3-lung larvae, plates were coated overnight with 5 μg/ml L3-lung protein homogenate at 4°C. The wells were washed three times with wash buffer (PBS with 0.05% Tween 20) followed by the addition of blocking solution (150μl/well of 5% milk powder in PBS) for 2h at 4°C. Sera were added at a dilution of 1/200 in wash buffer. After 1 hour at room

temperature, the conjugate was added (HPRO-conjugated goat anti-pig IgG) at a dilution of 1/12,500 in wash buffer + 5% milk powder. The plates were incubated for 1h at room temperature. O-phenylenediamine 0.1% in citrate buffer (pH 5.0) served as a substrate and optical density (OD) was measured at 492 nm. In order to compensate for variation between different plates, a negative and positive control sample was included on each plate. The negative control (NC) was a pooled serum sample taken from 10 naïve piglets without previous exposure to *A. suum*. The positive control (PC) was a pooled serum sample from experimentally infected piglets with 100 eggs/day. Both NC and PC were obtained from the animals from the first infection trial after 7 consecutive weeks of infection. Reactivity to the antigen is shown in ODR (Optical Density ratio) (ODR sample=(OD sample – OD NC)/ (OD PC – OD NC)).

2.2.5 Maternal transfer of anti-*Ascaris* antibodies

To determine whether anti-*Ascaris* antibodies detected in weaned piglets were maternally derived, serum was collected from 6 sows and 5 of their 1 week old piglets in the farrowing stable and subsequently analysed on the L3-Lung ELISA. In addition, serum was collected from 20 piglets 3 days after birth and used to measure both total immunoglobulin levels using the Ig immunocrit method (Vallet et al., 2013), as a measure for colostrum uptake, as well as anti-*Ascaris* antibody levels with the L3-Lung ELISA.

2.2.6 Seroprevalence study

A seroprevalence study with the L3-Lung ELISA was performed on 68 nursery farms in Flanders, Belgium. Serum was collected on each farm from 10 weaned piglets at the end of the nursery phase (approximately at 10 weeks of age). All 10 sampled piglets were housed in the same nursery unit of the respective farms. Serum was analysed on the L3-Lung ELISA as described above.

2.2.7 Statistical analysis

To see whether anti-*Ascaris* antibodies were maternally derived, a parametric Pearson's r correlation test was performed. Probability (P) values <0.05 were considered to indicate significant correlations. In order to calculate the diagnostic threshold of the L3-Lung ELISA, a ROC analysis was performed. All analysis was performed using Prism Version 5.0b.

2.3. Results

2.3.1 Seroconversion in experimentally infected piglets

The serum samples collected during study 1 were analysed on both the haemoglobin as well as the L3-Lung ELISA. As shown in Figure 2.1 A, almost no seroconversion was measurable on the haemoglobin test for any of the infection doses. In contrast, a clear and dose-dependent seroconversion was measurable with the L3-Lung ELISA in comparison to uninfected control piglets (Figure 2.1 B). At necropsy, the intestinal content of all piglets was collected, sieved and the worms counted microscopically. The results of the worm counts are shown in Table 2.1. Importantly, none of the worms collected had developed past the 4th larval stage (based on the size which is approximately 2mm).

To further refine the minimum level of exposure measurable with the L3-Lung ELISA, a second experimental infection study was performed in which the piglets were infected with 20, 40, 60 or 80 eggs/day. Identical as in study 1, seroconversion was measurable from approximately 4 weeks post infection onwards (results not shown). The average number of worms recovered at necropsy for the different groups are summarized in Table 2.1. In addition, the average number of white spots visible on the livers was also counted and the results are summarized in Table 2.1.

Table 2.1: Worm counts and the liver white spot data collected at necropsy of 11 weeks old piglets after 7 consecutive weeks of daily infection (eggs/day).

Groups	Arithmetic mean (range)
Worm counts Study 1	
10 eggs/day (N=9)	0.6 (0-2)
100 eggs/day (N=10)	4.7 (0-11)
500 eggs/day (N=7)	2.6 (1-7)
Worm counts Study 2	
20 eggs/day (N=10)	6.5 (0-21)
40 eggs/day (N=10)	7.3 (0-30)
60 eggs/day (N=10)	9.9 (0-60)
80 eggs/day (N=10)	6.9 (0-23)
Liver white spots Study 2	
20 eggs/day (N=10)	96.9 (65-141)
40 eggs/day (N=10)	137.8 (73-175)
60 eggs/day (N=10)	131.5 (73-205)
80 eggs/day (N=10)	164 (58-243)

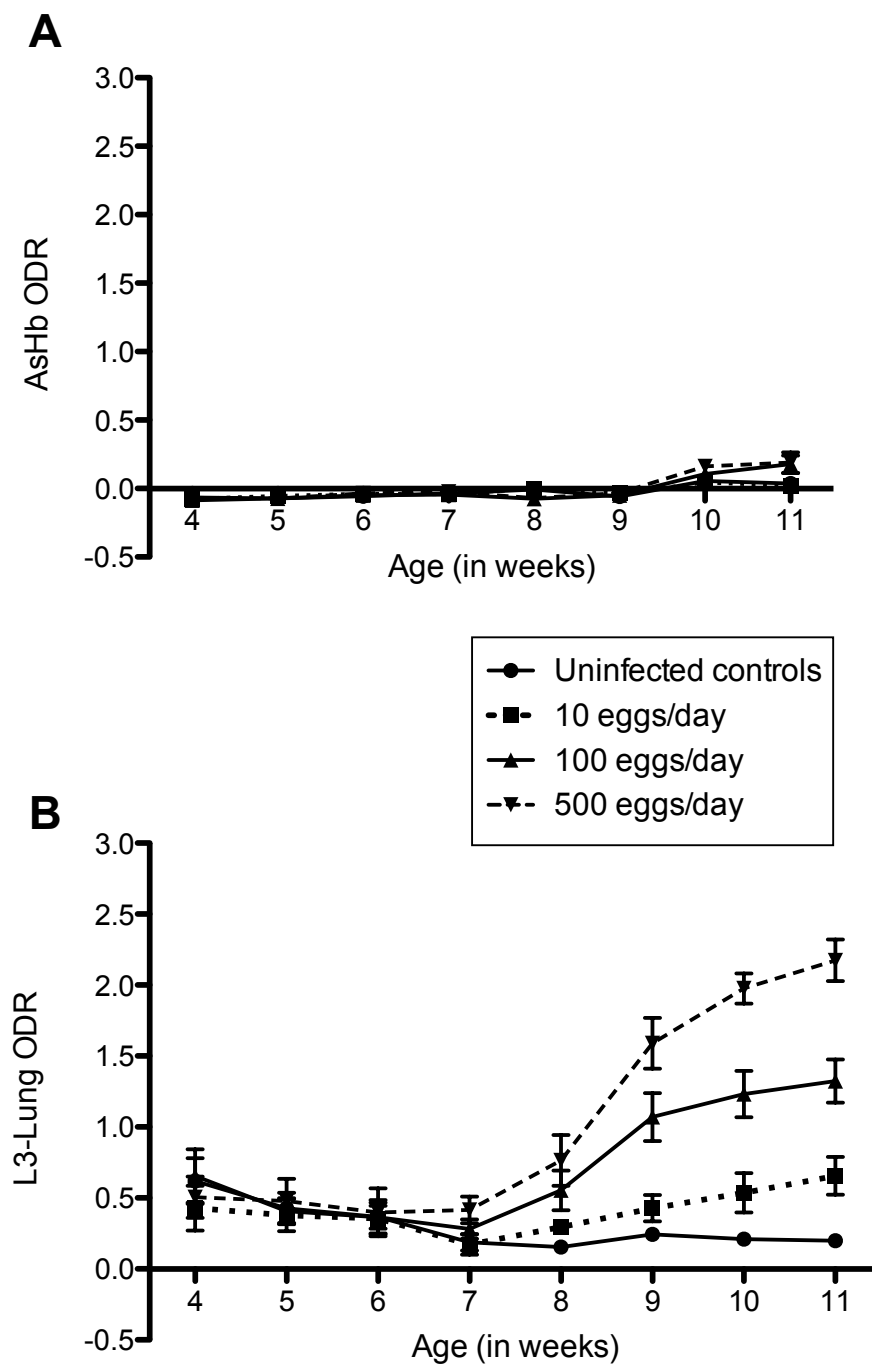**Fig. 2.1: Anti-*Ascaris* antibody levels detected in the serum of piglets experimentally infected with 10, 100 or 500 *A. suum* eggs/day for 7 consecutive weeks. Parasite specific antibody levels were measured with the haemoglobin- (panel A) and the L3-Lung ELISA test (panel B). Results are shown as optical density ratio (ODR) compared to negative and positive control samples (Mean with SEM).**

2.3.2 Determination of the sensitivity and specificity of the L3-Lung ELISA

In order to calculate the diagnostic threshold of the L3-Lung ELISA, serum samples were collected from 100 *Ascaris*-naïve piglets and analysed on the L3-Lung ELISA together with the serum samples from all experimentally infected animals collected at the end of the study. The results, shown in Figure 2.2, were subsequently used for a ROC analysis. The diagnostic threshold for an optimal discrimination between *Ascaris*-positive and *Ascaris*-negative samples was set at an ODR of 0.250. With this threshold, the test showed a specificity of 99 % and a sensitivity of at least 90 % for infection doses of minimum 20 eggs/day. For an infection dose of 10 eggs/day, the sensitivity dropped to 40 %.
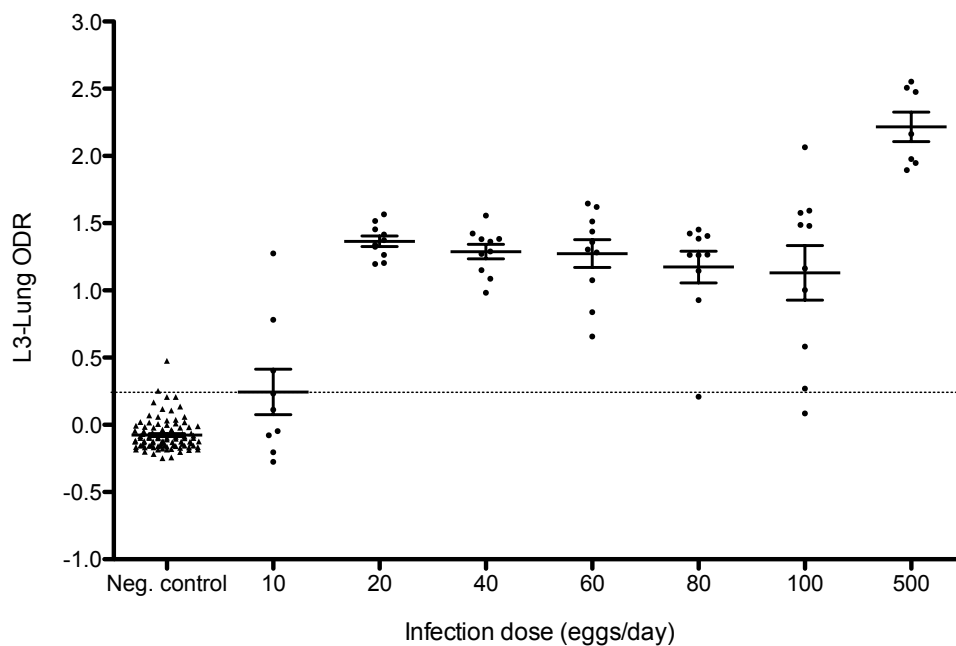
Fig. 2.2: Comparison of anti-*Ascaris* antibody levels in the serum of 100 *Ascaris*-negative piglets and piglets experimentally infected with 10, 20, 40, 60, 80, 100 or 500 eggs/day for 7 consecutive weeks. Parasite specific antibody levels after 7 weeks of exposure were measured on the L3-Lung ELISA and shown as optical density ratio (ODR) compared to negative and positive control samples. The horizontal dotted line at ODR 0.250 indicates the optimal diagnostic threshold as calculated by a ROC analysis (Mean with SEM).

2.3.3 Seroprevalence study in Belgian nurseries

Blood was collected on 68 Belgian nursery farms. On each farm, 10 piglets belonging to the same nursery stable were sampled at the end of the nursery phase and their serum individually analysed on the L3-Lung ELISA. The results, as summarized in Figure 2.3, show that on 38 % of the stables (N=26) all 10 piglets tested seronegative. For the remaining 62 % of the stables (N=42), the percentage seropositive piglets ranged from 10 to 100 %.
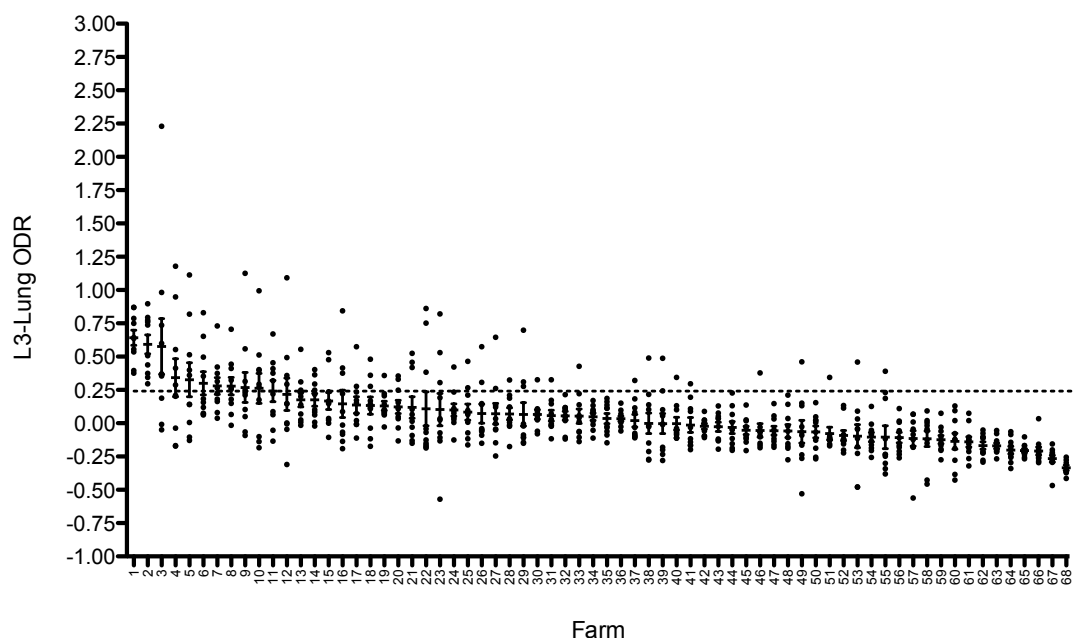

Fig. 2.3: Anti-*Ascaris* antibody levels detected in 10 piglets derived from 68 different farms in Belgium. Serum was collected at the end of the nursery phase at an age of approximately 10 weeks and analysed on the L3-Lung ELISA. The horizontal dotted line indicates the diagnostic threshold of ODR 0.250 (Mean with SEM).

2.3.4 Detection of maternal derived anti-*Ascaris* antibodies in piglets

Analysis of the serum samples collected at the start of the experimental infection studies showed that some piglets already tested positive for anti-*Ascaris* antibodies (Figure 2.1 B). To investigate whether these anti-*Ascaris* antibodies were maternally derived, the dynamics of these antibodies were analysed in the 10 negative control piglets that were part of the experimental infection study. Analysis on the L3-Lung ELISA showed that at the age of weaning, 5 piglets tested seropositive (Figure 2.4 A). In the following weeks, the anti-*Ascaris* antibody levels in these animals declined until all animals tested seronegative at an age of 7 weeks. Subsequently we investigated whether a correlation existed between anti-*Ascaris* antibodies in sows and their piglets. For this, serum samples were collected from 6 sows and 5 of their respective piglets and analysed on the L3-Lung ELISA. The results, shown in Figure 2.4 Panel B, indicated that piglets born from sows with low anti-*Ascaris* antibody levels tested lower on the L3-Lung ELISA compared to piglets born from sows with high anti-*Ascaris* antibody levels. Finally, anti-*Ascaris* antibody levels in 3-days old piglets born from seropositive sows correlated significantly (P<0.0001) with the level of maternally derived Ig's (Figure 2.4 C), further suggesting the maternal origin of the anti-*Ascaris* antibodies detected at weaning.
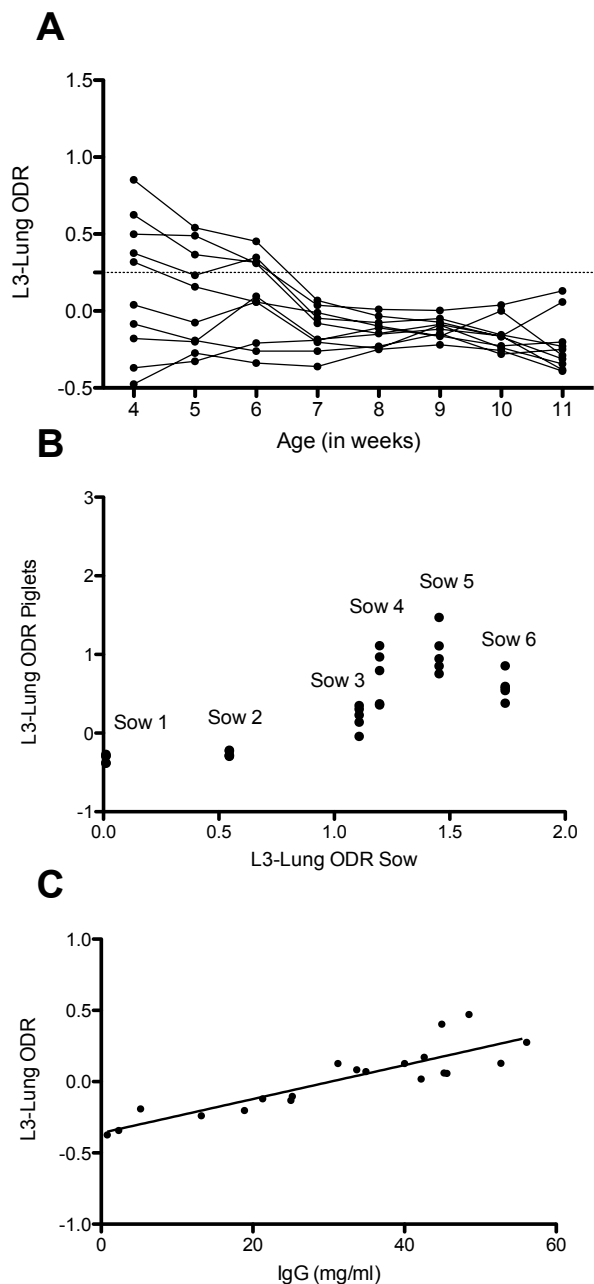
Fig. 2.4: A: Anti-*Ascaris* antibody levels, analysed on the L3-Lung ELISA, detected in 10 individual *Ascaris*-free piglets from 4 weeks of age until 11 weeks of age. The horizontal dotted line indicates the diagnostic threshold of ODR 0.250. B: Correlation between anti-*Ascaris* antibody levels in 6 sows and their 1-week-old piglets as measured on the L3-Lung ELISA. C: Correlation between total Ig levels in serum 3 days after birth, as a measure for colostrum intake at birth, and anti-*Ascaris* antibody levels measured by the L3-Lung ELISA (*r*=0.880; P<0.0001).

2.4 Discussion

The data presented in this study shows that an ELISA based on the water-soluable protein homogenate of the L3–lung stage of *A. suum* can be used to detect the exposure of piglets to *A. suum* during the nursery phase. The L3-Lung ELISA showed a 90 % sensitivity and 99 % specificity at a minimum infection dose of 20 *A. suum* eggs/day and higher. However, when piglets were infected with 10 eggs/day, the sensitivity of the test dropped substantially.

Interestingly, seroconversion was not measurable with the ELISA test based on the haemoglobin antigen. In a similar type of study performed in 10-weeks old fattening pigs infected with a daily dose of 100 eggs, a clear seroconversion was shown on the haemoglobin test within the first 6 weeks of exposure (Vlaminck et al., 2012). The haemoglobin protein used in the ELISA is expressed from the L3-lung stage onwards, but the highest expression was detected in the L4 and adult worms (Vlaminck et al., 2012). Remarkably, in the current study, none of the worms recovered from the intestines of the artificially infected piglets at necropsy had developed further than the L4 stage. The absence of adult *A. suum* worms as well as the few amount of L4 larvae in these animals could explain the lack of seroconversion on the haemoglobin test. However, the animals were continuously infected for 7 weeks and although many animals will expel the developing larvae from their gut as a result of the expulsion reaction (Masure et al., 2013), it is surprising that none of the piglets carried adult worms. Also, the number of larvae recovered from the intestines was lower compared to what would typically be recovered from fatteners. In a study from Nejsum et al. (2009a) pigs were infected with 25 eggs/kg/day, divided over 2 infections per week, starting at an age of 10 weeks. Seven weeks post infection there were eggs present in the faeces determined by McMaster technique in two-third of the pigs. In the current study all piglets remained negative based on egg counts despite daily infection. The dose-dependent seroconversion on the L3-Lung ELISA and the presence of a high number of liver white spots in study 2 indicates that the low number of larvae recovered was not caused by a low infectivity of the eggs used in the two studies. Whether there is an age dependent immunological or physiological reason for this lower establishment of *A. suum* in piglets is currently unclear. Roepstorff (1997) demonstrated that piglets did not get patent *A. suum* infections even though

the sows excreted considerable numbers of eggs. It was suggested that good hygiene and a dry microclimate in the farrowing pens completely prevented egg embryonation and thus transmission to the piglets. Kelley and Nayak (1965) reported partial protection of piglets against larval migration after intake of colostrum from egg-immunized sows and by passive transfer of hyper immune sera. Although some piglets in the current study clearly tested positive for maternally derived anti-*Ascaris* antibody levels (Figure 4A), others tested negative and still these animals did not seem to carry more L4-larvae in their intestine at time of necropsy. On the other hand, in a study performed by Boes et al. (1999) piglets infected on day 4 and day 7 after birth with 50 eggs/day harboured adult worms in their intestines at necropsy at 10 weeks of age. Andersen et al. (1973) reported that lower doses of 50 *A. suum* eggs distributed to young piglets induced more patent infections than doses of 1.000, 5.000 or 10.000 *A. suum* eggs, so potentially the trickle infection approach used in the current study may have triggered a stronger immune response leading to the expulsion of the developing worms in the intestine.

A serological survey performed in Belgium indicated that on a consequential number of farms the piglets were exposed to *A. suum* during the nursery phase. Although the impact of such infections on the health status and performance of these young developing animals is currently unclear, the data does show that many nursery facilities are apparently contaminated with *A. suum* eggs. The potential source of this contamination remains unclear. This could either be the sows, which can excrete eggs in the farrowing units and infect their piglets and/or the piglets themselves through contamination of the nursery units via remaining faeces when transferred from the farrowing stable. Of course, mechanical transmission from other farm areas via boots, clothing and other materials is also a possibility.

2.5 Conclusion

Current study shows that serology can be used to measure exposure of piglets to *A. suum*. Based on the results, additional control measures could be implemented to, first, lower the exposure of the piglets to *A. suum* during the nursery phase and, second, avoid further transmission to the fattening units. Importantly however, the timing of serum collection for serological analysis should be carefully chosen as the results have shown that maternally derived anti-*Ascaris* antibodies can persist in piglets until an age of approximately 6 to 7 weeks. Finally, considering that the sensitivity is only 40% for an infection dose of 10 eggs/day, one should be aware that marginally low infection levels could not be detected with this tool.

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Chapter 3

Effect of strategic deworming on *Ascaris suum* exposure, measured through serology, and technical performance parameters in fattening pigs

3.1. Introduction

Ascaris suum is a widespread parasitic nematode that causes infection in pigs worldwide (Dold and Holland, 2011; Nansen and Roepstorff, 1999; Roepstorff et al., 1998). A single *A. suum* female worm may produce close to two million eggs per day and these eggs can remain viable in the environment for several years (Roepstorff et al, 2011). After oral uptake, the L3-stage larvae will hatch from the egg in the gastrointestinal tract, penetrate the caecum wall and migrate via the blood stream to the liver and subsequently the lungs. Here, the larvae are coughed up, swallowed and subsequently arrive back in the small intestine. During this migration, damage is caused in the respective organs (Roepstorff et al., 1997). In the liver, the characteristic lesions caused by the inflammatory response to the larvae are called hepatic white spots and are a major reason for liver rejection at slaughter. The acute phase of a severe infection is characterized by frequent coughing (Boes et al., 2010) due to pneumonia. The damage caused in the lungs can pave the way for opportunistic bacterial and viral infections (Adedeji et al., 1989; Curtis et al., 1987; Tjornehoj et al., 1992). Finally, it has also been shown that *A. suum* impairs the effects of a *Mycoplasma hyopneumoniae* vaccine resulting in increased pulmonary lesions (Steenhard et al., 2009).

Several studies state that infections with *A. suum* result in significant economic losses such as decreased average daily growth (ADG) (Bernardo et al., 1990; Lassen et al., 2017; Pedersen et al., 2002; Stewart et al., 1972; Urban et al., 1989; Vlaminck et al., 2015) in combination with an increased feed conversion ratio (FCR) (Hale et al., 1985; Kipper et al., 2011; Stewart and Hale, 1988; van Krimpen et al., 2010; Zimmerman et al., 1973) and lower meat quality (Jankowska-Makosa and Knecht, 2015; Knecht et al., 2012; Knecht et al., 2011). However, pigs harbouring patent infections, even with large numbers of adult worms, often appear to be clinically healthy (Boes et al., 2010). The consequence of this is that pig farmers and veterinarians underestimate both the infection levels as well as the potential impact on production.

In recent years, several studies have investigated the use of serology to more accurately measure the level of exposure of pigs to *A. suum*. First, Vlaminck et al. (2012) reported on a serological test that is based on the antibody recognition of a haemoglobin protein (AsHb) mainly produced by the late

larval and adult stages of *A. suum*. More recently, Vandekerckhove et al. (2017) described an ELISA test that is based on the recognition of antigens present in a water-soluble protein homogenate of L3 larvae that migrate through the lungs. Interestingly, applying these tests on commercial fattening farms in both Belgium and Spain showed significant correlations between *Ascaris* antibody levels and technical performance parameters, such as daily weight gain and feed conversion ratio (Vlaminck et al., 2015; Martínez-Pérez et al., 2017), suggesting that serology could potentially also be used to estimate economic impact of *Ascaris* infection.

Based on these observations, the aim of the current study was to monitor the effect of strategic anthelmintic treatment in fattening pigs on both *A. suum* antibody levels and technical performance parameters over time. For this, two groups of 9 fattening stables with different levels of contamination with *A. suum* eggs were monitored for 7 consecutive fattening rounds. In each stable a deworming program was applied using fenbendazole every 6 weeks in order to interrupt the development of adult worms in the fattening pigs. For every stable and every fattening round, technical performance parameters such as average daily growth, feed conversion ratio, days in fattening and percentage of condemned livers as well as anti-*Ascaris* antibody levels were monitored in the fattening pigs.

3.2. Material and methods

3.2.1 Selection of the fattening farms

A total of 41 commercial fattening stables that were part of the integration of the cooperative Covavee cvba (Belgium) were visited and screened for potential inclusion in the study. Of these 41 stables, eventually 2 groups of 9 stables were selected for inclusion based on the availability of production data for each fattening round and the possibility to treat animals via drinking water. Group 1 consisted of 9 stables in which the fattening pigs tested seropositive for *Ascaris* on the AsHb ELISA (Average ODR > 0,5), indicative for the presence of *Ascaris* eggs in the stable, whereas group 2 consisted of 9 stables in which the fattening pigs tested seronegative for *Ascaris* (Average ODR < 0,5), indicating a low or absent environmental contamination level (Vlaminck et al., 2012). Some characteristics of the selected stables, such as the type of floor and stable capacity, are summarized in supplementary table

3.1, together with the AsHb ELISA results obtained in round 0. All pigs were typically fattened from approximately 10 weeks old with an average weight of approximately 22 kg until slaughter weight was reached (approximately 110 kg). An all-in/all-out management system was employed in all stables.

3.2.2 Deworming program

The stables were monitored for a period of 7 consecutive fattening rounds. The first of these 7 fattening rounds (i.e. round 0), during which no intervention took place in the deworming strategy applied on the farms, served as a historical control. The deworming schedules applied on the farms before the onset of the trial are elucidated in Table 3.1.

From the second fattening round onwards (i.e. round 1) a deworming program using 200 mg/ml fenbendazole oral suspension for use in drinking water (Panacur® AquaSol) for 2 days was implemented every 6 weeks for 6 consecutive rounds. Deworming was applied at week 0 (onset), 6 and 12 of the fattening period.

3.2.3 Collection of blood

Blood was collected approximately 14 weeks after onset of the fattening round from 10 animals randomly selected from different pens in the investigated stable. Samples were collected in 5 ml serum tubes that were subsequently centrifuged at 4,000 g during 10 minutes at 4°C. Serum was collected and stored at -20°C until used.

Table 3.1 Characteristics of the stables belonging to group 1 and 2.

	Nr. Stable	ODR	Capacity stable	AI/AO	Type of floor		Deworming history		Way of administration	
					Fully slatted	Semi slatted	Number of treatments	Treatments schedule	In feed	Drinking water
Group 1	1	0,599	390	x	x		2	onset (week 0) and week 12	x	
	2	0,62	450	x	x		2	onset and week 6	x	
	3	0,545	288	x	x		2	week 2 and 7	x	
	4	0,533	570	x	x		2	onset and week 6	x	
	5	0,622	200	x	x		2	week 7 and week 14	x	
	6	0,583	230	x		x	2	onset and week 8	x	
	7	0,565	400	x	x		2	onset and week 6		x
	8	0,759	160	x	x		0	/	/	/
	9	0,601	400	x	x		1	week 10		x
Group 2	10	0,351	400	x	x		3	week 3, week 8 and week 13	x	
	11	0,354	300	x		x	3	week 4, week 10 and week 14	x	
	12	0,479	550	x	x		3	onset, week 6 and week 12	x	
	13	0,207	430	x	x		2	week 4 and week 12	x	
	14	0,326	310	x	x		2	onset and week 7	x	
	15	0,385	530	x		x	2	no schedule		x
	16	0,292	350	x	x		2	no schedule		x
	17	0,461	400	x	x		2	week 3 and week 10	x	
	18	0,484	550	x	x		2	week 2 and week 6		x

3.2.4 Slaughter line data and technical performance indicators

After approximately 16 weeks, when the animals weighed around 110 kg, animals were transported to a commercial slaughterhouse. For each fattening round and for each stable, technical performance parameters such as percentage of mortality, average daily growth (Formula: Finish weight- start weight/ (number of days in fattening - number of animals), expressed as gram/day), feed conversion ratio (Feed intake/(ADG), expressed in kg) and days in fattening were obtained from the producer Aveve. The FCR is a measure of an animal's efficiency in converting feed into body mass and reflects the mass of feed needed to produce 1 kg of bodyweight. It was corrected to 20-110 kg of bodyweight, which is determined as the reference weight by Aveve, in order to compensate for growth variation between the animals of one fattening round.

Official meat inspection personnel assessed percentage of affected lungs, percentage of meatiness (percentage lean meat according to the EUROP scale) and liver lesions during routine post-mortem meat inspection at the slaughterhouse. The percentage of condemned livers was calculated as the percentage of livers with one liver white spot or more.

3.2.5 Analysis of the serum samples

The AsHb and L3-Lung ELISA tests were performed as described by Vlaminck et al. (2012) and Vandekerckhove et al. (2017) respectively. In order to compensate for variation between different plates, a negative and positive control sample was included on each plate. The negative control (NC) was a pooled serum sample from 10 piglets without previous exposure to *A. suum*. The positive control (PC) for the AsHb test was a pooled serum sample from pigs after 18 weeks of daily infection with 100 *A. suum* eggs. The positive control (PC) for the L3-lung test was a pooled serum sample from piglets after 7 weeks of daily infection with 100 *A. suum* eggs. Reactivity to the antigen is shown in ODR (Optical Density ratio) (ODR sample=(OD sample – OD NC)/ (OD PC – OD NC)). Serological results are expressed as the arithmetic mean of the ODR values of all 10 samples per stable.

3.2.6 Statistical analysis

Spaghetti plots were prepared for each parameter to visualize the trends of the variables over time and paired, non-parametric statistical analysis was performed using the Friedman test combined with a posthoc Dunn's multiple comparison test to evaluate significant changes over time. Correlations between the different diagnostic variables (AsHb ELISA, L3-Lung ELISA and % of liver white spots (LWS)) were investigated using the non-parametric Spearman's rank correlation test. Probability (P) values <0.05 were considered to indicate significant changes or correlations. These calculations were performed in Prism Version 5.0b.

Subsequently we aimed to estimate the effect of *Ascaris suum* infection intensity on farm production parameters by producing models. Hierarchical linear mixed models were applied to the data because the serial measurements are nested within the farm levels over time. Different models were prepared for each production parameter or dependent variable (ADG, FCR or DIF). For each dependent variable, three different models were created, each time including a single different diagnostic test or explanatory variable (AsHb and L3-Lung ELISA or LWS) since diagnostic methods are highly correlated. The data on the percentage of livers showing white spots per farm was highly zero-inflated which required transformation of the parameter into an ordinal parameter with value 0 for when LWS were absent and value 1 if LWS were present (> 0%).

Differences in trajectories of economic parameters between farms are expected. Adding a random intercept is thus a conceptual necessity for repeated measures analysis. This allows the baseline score of each production parameter of each farm to be taken into account. The factor Farm was thus incorporated as a random effect. The basic model we started from was: $Y \sim X + (\sim 1|Farm)$, where Y is any of the response or dependent variables (ADG, FCR, DIF) and where X is any of the explanatory or independent variables (AsHb and L3-Lung ELISA or LWS). The change in economic parameters and explanatory variables is associated with the sampling round and this relationship is not always completely linear. It can be expected that changes in these parameters are more pronounced during the first treatment rounds and that they reach equilibrium over time as environmental contamination with parasite eggs is reducing. Therefore, it was also tested whether the inclusion of a time and/or time2 (quadratic

effect) fixed parameter increased the model fit. All models were fit in R (R Core Team, 2016) using the hglm package for hierarchical general linear models (Ronnegard et al., 2010). The package fits generalized linear models with random effects, where the random effect may come from a conjugate exponential-family distribution (normal, gamma, beta or inverse-gamma). Model fit was evaluated using the conditional Akaike Information Criterion (cAIC) as the calibrating parameter. The best model is selected as the model with lowest cAIC value (Vaida and Blanchard, 2005).

3.3. Results

3.3.1 Effect of repeated deworming on *Ascaris* infection intensity

The *Ascaris* infection levels in all stables were monitored over 7 consecutive rounds using the AsHb- and the L3-Lung ELISA and the percentage of livers showing white spots (LWS). The results of the ELISA analyses are represented as the average ODR of 10 animals analysed individually per herd per round. The LWS are reported as percentages for the whole herd. The results obtained for the *Ascaris*-positive stables (group 1) are shown in Figure 3.1 panels A, C and E respectively. A significant decrease in anti-*Ascaris* antibody levels over time was observed with both serological tests. Similarly, the percentage of affected livers decreased substantially after only one round of deworming. In contrast, no significant decrease in anti-*Ascaris* antibody levels and percentage of affected livers could be detected for the *Ascaris*-negative/low stables (group 2) (Figure 3.1 panels B, D and F). Finally, a significant correlation was found between the results for the 2 serological tests and LWS for the positive stables (Table 3.2). For the stables of group 2, a significant correlation was only found between the results of the 2 serological tests (Table 3.2).

Group 1 (*Ascaris*-positive herds)

Group 2 (*Ascaris*-negative/low herds)
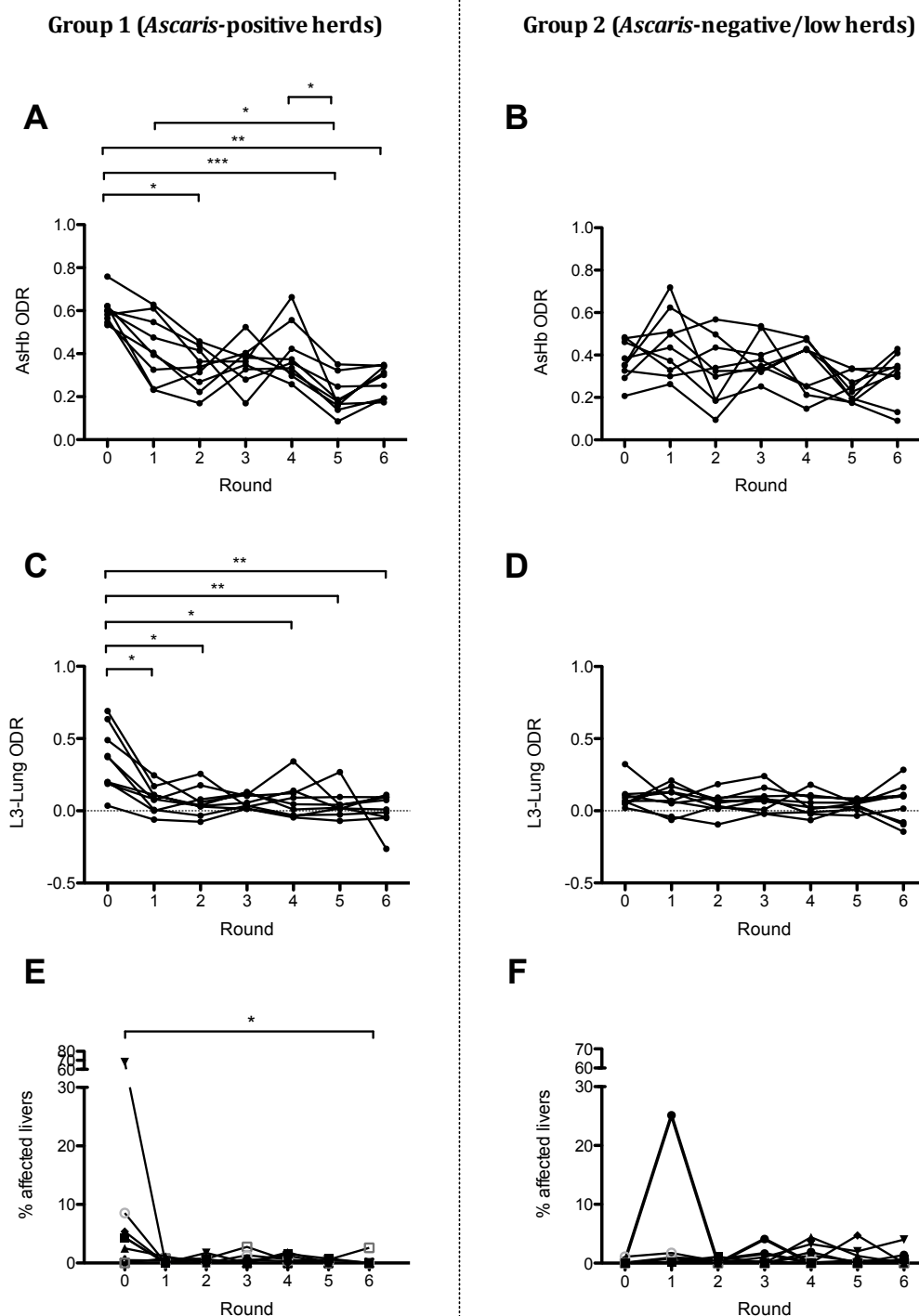
Fig. 3.1: Evolution of the Anti-*Ascaris* antibody levels as measured by the AsHb- (panels A and B), the L3-Lung ELISA (panels C and D) and percentage of livers with white spots (panels E and F) for *Ascaris* positive stables (panels A, C, E) and stables negative/low for *Ascaris* (panels B, D, F). Serological results are shown as the average serological result of 10 animals. (Statistical analysis: * P<0.05, ** P<0.01, *** P<0.001).

Table 3.2: Correlation coefficients between the results for the serology (AsHb and L3-Lung) and Liver white spots (LWS) for the stables of both groups, as calculated by Spearman correlation analysis (*P<0.05).

		L3-Lung	LWS
Group 1	**AsHb**	0,694*	0,386*
	L3-Lung		0,560*
Group 2	**AsHb**	0,574*	0,156
	L3-Lung		-0,093

3.3.2 Effect of *Ascaris* infections on technical performance parameters

No significant changes could be observed for the percentages of affected lungs, meatiness or mortality. The average daily growth (ADG), feed conversion ratio (FCR) and number of days in fattening (DIF) for all the stables of both groups are shown in Figure 3.2. In both the *Ascaris* positive and negative group, production data was missing for 1 time point for 3 stables as a result of random absence of information rather than attrition from the study. Missing values were omitted from the datasets before statistical evaluation. No significant changes were observed over time for any of the parameters monitored.

To investigate the relative effect of *Ascaris* infections on performance parameters, hierarchical linear mixed models were prepared for each of the three performance parameters (ADG, FCR and DIF) in combination with each of the three explanatory variables (AsHb ELISA, L3-Lung ELISA and LWS). The outcomes of the fixed effects estimates of the best modelled interactions for the positive stables are presented in Table 3.3. The results indicated that the level of L3-Lung antibody reactivity was a significant predictor of decreased ADG, increased FCR and prolonged DIF for the *Ascaris*-positive stables. This was not observed for the AsHb antibody reactivity or the percentage affected livers. An identical analysis performed on the data of the *Ascaris* negative stables also did not reveal any significant results (Table 3.4).

Group 1 (*Ascaris*-positive herds)

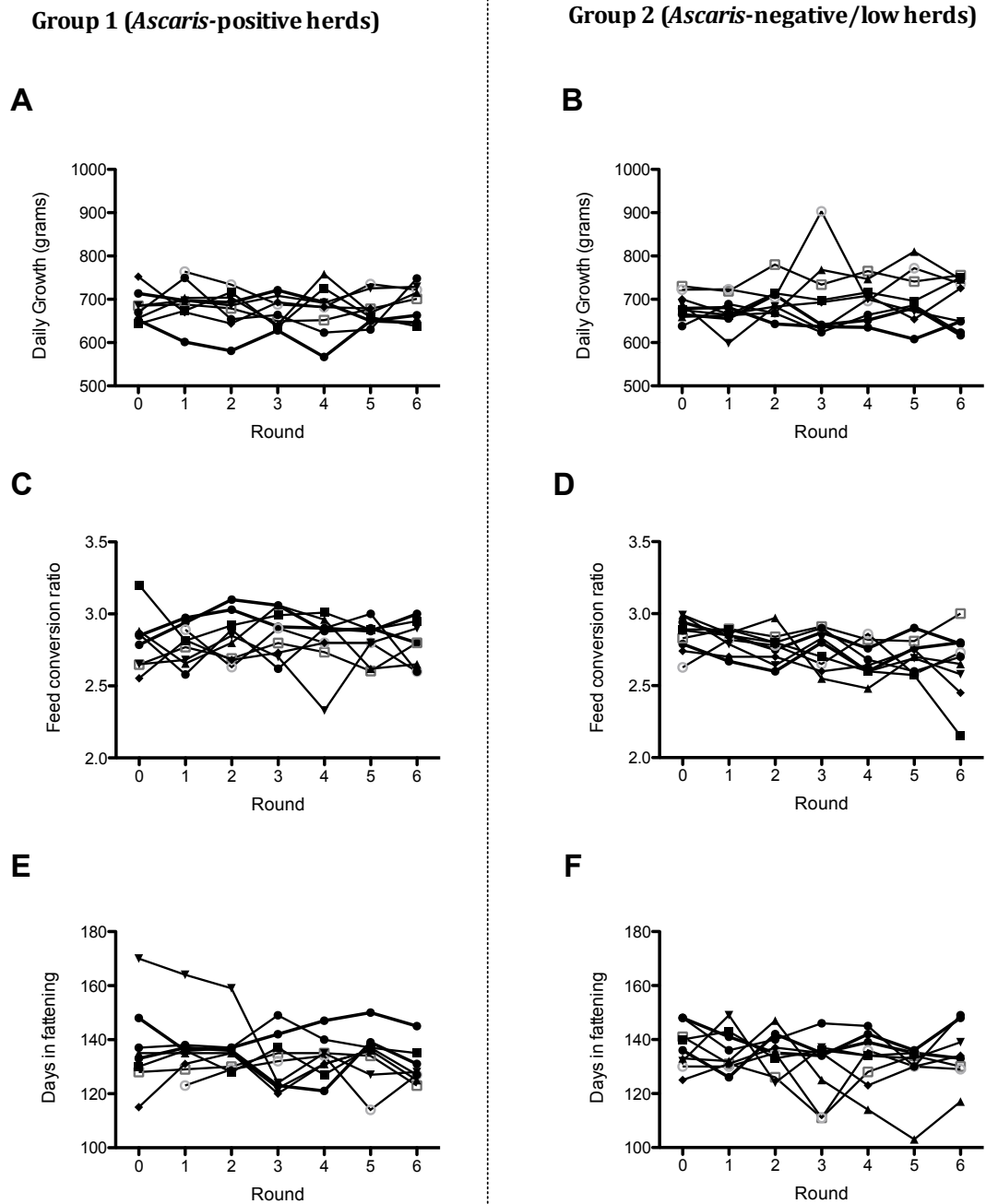**Group 2 (*Ascaris*-negative/low herds)**

Fig. 3.2: The results obtained for daily growth (panels A and B), feed conversion ratio (panels C and D) and the number of days in fattening (panels E and F) for *Ascaris* positive stables (panels A, C, E) and stables negative/low for *Ascaris* (panels B, D, F).

Table 3.3: Summary of fixed effects estimates for each modelled interaction between response variables (ADG, DIF and FCR) and fixed explanatory variable or diagnostic test (L3 Lung ELISA, AsHb ELISA, LWS) for the *Ascaris*-positive stables (group 1). Significant results are shown in bold.

Explanatory Variables	Response Variables	Group 1			
		Estimate	SE	t-value	P-value
		ADG			
L3 Lung	Intercept	761.3	23.8	32.0	<0.001
	L3lung	**-128.4**	34.3	-3.7	<0.001
	Time	-35.8	10.6	-3.4	<0.01
	Time^2	3.8	1.3	3.1	<0.01
AsHb	Intercept	746.3	35.7	20.9	<0.001
	AsHb	-53.0	41.8	-1.3	NS
	Time	-24.7	12.1	-2.1	< 0.05
	Time^2	2.7	1.4	2.0	0.057
LWS	Intercept	715.6	22.1	32.4	<0.001
	LWS	-0.5	0.57	-0.9	NS
	Time	-20.2	11.3	-1.8	0.079
	Time^2	2.4	1.4	1.8	0.086
		DIF			
L3 Lung	Intercept	132.1	2.1	62.6	<0.001
	L3lung	**20.9**	7.6	2.8	<0.01
	Time	/	/	/	/
	Time^2	/	/	/	/
AsHb	Intercept	128.6	3.6	35.6	<0.001
	AsHb	14.7	7.8	1.9	0.064
	Time	/	/	/	/
	Time^2	/	/	/	/
LWS	Intercept	131.8	2.5	52.6	<0.001
	LWS	**5.2**	2.3	2.2	<0.05
	Time	/	/	/	/
	Time^2	/	/	/	/
		FCR			
L3 Lung	Intercept	2.61	0.10	27.38	<0.001
	L3lung	**0.38**	0.14	2.68	< 0.05
	Time	0.09	0.04	2.12	< 0.05
	Time^2	-0.01	0.01	-1.90	NS
AsHb	Intercept	2.82	0.06	50.08	<0.001
	AsHb	0.01	0.12	0.07	NS
	Time	/	/	/	/
	Time^2	/	/	/	/
LWS	Intercept	2.80	0.04	74.03	<0.001
	LWS	0.04	0.04	1.15	NS
	Time	/	/	/	/
	Time^2	/	/	/	/

Table 3.4: Summary of fixed effects estimates for each modelled interaction between response variables (ADG, DIF and FCR) and fixed explanatory variable or diagnostic test (L3 Lung ELISA, AsHb ELISA, LWS) for the *Ascaris*-negative/low stables (group 2).

Explanatory Variables	Response Variables	Group 2			
		Estimate	SE	t-value	P-value
		ADG			
L3 Lung	Intercept	682.8	10.9	62.48	<0.001
	L3lung	27.08	74.91	0.361	NS
	Time	/	/	/	/
	Time^2	/	/	/	/
AsHb	Intercept	678.44	19.4	34.9	<0.001
	AsHb	16.79	43.4	0.39	NS
	Time	/	/	/	/
	Time^2	/	/	/	/
LWS	Intercept	684.6	9.1	75.7	<0.001
	LWS	0.45	1.5	0.3	NS
	Time	/	/	/	/
	Time^2	/	/	/	/
		DIF			
L3 Lung	Intercept	134.9	2.0	67.2	<0.001
	L3lung	-0.4	14.8	-0.0	NS
	Time	/	/	/	/
	Time^2	/	/	/	/
AsHb	Intercept	134.4	3.7	36.4	<0.001
	AsHb	1.0	8.7	0.1	NS
	Time	/	/	/	/
	Time^2	/	/	/	/
LWS	Intercept	135.0	1.9	70.5	<0.001
	LWS	-0.4	2.2	-0.2	NS
	Time	/	/	/	/
	Time^2	/	/	/	/
		FCR			
L3 Lung	Intercept	2.78	0.03	99.3	<0.001
	L3lung	0.32	0.20	1.58	NS
	Time	/	/	/	/
	Time^2	/	/	/	/
AsHb	Intercept	2.75	0.05	53.3	<0.001
	AsHb	0.15	0.12	1.29	NS
	Time	/	/	/	/
	Time^2	/	/	/	/
LWS	Intercept	2.82	0.03	101.9	<0.001
	LWS	-0.0	0.03	-0.03	NS
	Time	/	/	/	/
	Time^2	/	/	/	/

3.4. Discussion

The results obtained in this study clearly indicated an improvement in terms of exposure of the animals to *A. suum* after the implementation of a strategic deworming program, as measured by 2 different serological tests and the percentage of affected livers. The decline in anti-*Ascaris* antibody levels was especially detectable in the stables that originally tested positive for *Ascaris*. The aim of the 6-weekly treatment program was to avoid the development of adult worms and, as a consequence, the secretion of new *Ascaris* eggs in the stables. The decrease in antibodies against the haemoglobin antigen, which is mainly produced by the intestinal stages of *Ascaris*, could be explained by a reduced exposure to the adult worms. On the other hand, the decline in antibody reactivity towards the L3-lung extract suggests that the animals were less exposed to the migratory larvae. This effect was already visible after one round of strategic deworming, indicating that environmental contamination levels were immediately reduced after the beginning of the treatment program.

Despite the improvement in *Ascaris* infection levels over time in the seropositive herds of group 1, there were no significant changes in the technical performance parameters. These results are somewhat in contrast to previous studies that have shown an effect of *Ascaris* on daily weight gain and feed conversion ratio (Bernardo et al., 1990; Hale et al., 1985; Kipper et al., 2011; Lassen et al., 2017; Pedersen et al., 2002; Stewart and Hale, 1988; Stewart et al., 1972; Urban et al., 1989; van Krimpen et al., 2010; Zimmerman et al., 1973). Importantly, however, the *Ascaris*-positive stables in the current study were selected based on serology, which has shown to be more sensitive to detect exposure to *Ascaris* in comparison to faecal egg counts (Vlaminck et al., 2012). Other studies that looked at the effect of *Ascaris* on production mostly used faecal egg counts as a parameter to select *Ascaris*-positive herds. As a consequence, perhaps the infection intensities in the stables included in the current study were too low to observe an improvement in the technical performance parameters. It is important to bear in mind that every farm is subjected to dynamic parameters that change continuously, such as type of feed and housing (Hale et al., 1985; Zimmerman et al., 1973) and bacterial and viral infections (Bernardo et al., 1990) may potentially also have masked the effects of the *Ascaris* infection in

the current study. Unfortunately, in this study only one fattening round per investigated stable was included as a historical control round.

Vlaminck et al. (2015) and Martinez-Pérez et al. (2017) previously observed significant correlations between anti-*Ascaris* antibody levels and technical performance parameters in fatteners. In the current study, hierarchical linear mixed models indicated that the level of L3-Lung antibody reactivity was a significant predictor of decreased ADG, increased FCR and prolonged DIF for the animals housed in the *Ascaris*-positive stables. The results basically indicated that an increase of 1 ODR on the L3-Lung test was indicative for a drop in ADG with approximately 128 g, an increase of the numbers of days in fattening with 21 days and an increase of FCR with 0,38. A similar trend was also observed for the AsHb ELISA test, although this did not reach significancy.

3.5 Conclusion

The outcome of this study showed that a strategic treatment program with fenbendazole oral suspension was effective to reduce the exposure of fatteners to *A. suum* and that this effect was measurable by serology.

There was no improvement detectable of the technical performance parameters in the investigated stables included in our study. Nonetheless, the results indicated that anti-*Ascaris* antibody levels were a significant predictor of decreased technical performance of the animals and thus that serology can be used to estimate economic losses due to *Ascaris*.

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Chapter 4

General Discussion

The main objective of this thesis was to investigate the further implementation of serology for *Ascaris* in practise. More specifically to gain more insights concerning the economic impact of infections with *A. suum* on the pig industry and whether these losses can be measured through serology. Furthermore, we also wished to investigate whether serology could be useful to detect *A. suum* infections in weaned piglets. In this final chapter, some of the findings of the performed studies are discussed.

4.1 Serology to diagnose exposure to *A. suum* in piglets

In **Chapter 2** the main goal was to investigate whether serology could be used to measure exposure of weaners to *A. suum*. A total of 70 4-week old piglets were infected on a daily base during 7 consecutive weeks. Serum was collected on a weekly base. Initially, all collected sera were exclusively analysed on the AsHb ELISA. Surprisingly, the test was unable to detect any anti-*Ascaris* antibodies, even after several weeks of infection. In contrast, Vlaminck et al. (2012) showed the ability of the AsHb ELISA test to detect total IgG antibodies in 10-week-old fattening pigs 7 weeks p.i. The basic assumption in our study was that a minimum of 6 weeks of infection was sufficient time to develop antibodies, irrespective of the age of the animals that were infected. However, this was not the case. Interestingly, at the time of slaughter there were very few worms present in the gut of the infected piglets. Although this explains the lack of seroconversion on the AsHb ELISA, the concern was whether an infection actually took place. There were, however, white spots present on the livers, indicative of larval migration. This was further confirmed by a dose dependent seroconversion on the ELISA test based on the migrating L3-lung larvae. The mechanisms that prevented the development of adult worms in the piglets and whether it is an age dependent immunological and/or physiological reason is unclear.

Interestingly however, even before any infection took place, some piglets already had high antibody levels against *A. suum* at the time of weaning. After further investigation, it could be demonstrated that these antibodies were maternally derived. These findings are in correspondence to the results obtained by Kelley and Nayak (1965) who showed that piglets born from *Ascaris*-immunized sows had anti-*Ascaris* antibodies present in their blood that they received through the colostrum. To what extent such maternally derived antibodies provide the piglets protection against *A. suum* infections

remains unclear. In the study of Kelley and Nayak (1965) piglets born from sows that were immunized by oral administration of *Ascaris* eggs during gestation had less migrating larvae through the lungs following experimental infection than piglets from non-immunized sows. In addition, in a study performed by Boes et al. (1999) piglets from sows that have been exposed to *A. suum* were cross-suckled with piglets from helminth-naïve sows within 4 hours of birth, before any colostrum uptake took place. The exposed sows were inoculated during gestation with *A. suum* eggs. Subsequently, all piglets were inoculated 3 times with 50 *A. suum* eggs on day 4, 7 and 14 after birth. The piglets suckling control sows harboured more worms at slaughter than the piglets suckling exposed sows. These results clearly indicate that maternal immunity against *A. suum* infections can be transferred from sow to piglet via colostrum. However, whether the antibodies have a role in this is currently unclear. Some piglets in our study tested positive for the maternally derived antibodies at the time of weaning, while others tested negative. Still, these last-mentioned animals did not seem to carry more L4-larvae in their gut at time of necropsy than the antibody positive animals. This could suggest that other factors are likely also playing a role in the maternal immunity.

In addition to maternal immunity, it is also possible that the trickle infection approach used in our study may have triggered a strong intestinal immune reaction, preventing development into adult worms as a result of the expulsion of the L4 larvae in the intestine. Perhaps it would be advisable to repeat this study with a single lower infection ratio, rather than administering trickle infections. Andersen et al. (1973) revealed that the single administration of a low number of *A. suum* eggs rather induced patent infections. The lowest infection dose used in that experiment of 50 eggs was able to establish the highest amount of adult worms in the intestine. Although there were trickle infections administrated in our research, the worm counts showed in Table 2.1 of this thesis are similar to the findings of Andersen et al. (1973), with the highest counts detected in the piglets that were infected with 40 and 60 eggs/day. One could argue whether it is the infection dose rather than the infection intensity that is more likely to induce patent infections in piglets. With further increasing infection doses, the amount of counted worms diminished. However, 6 piglets out of 10 that received only 10 eggs/day did not develop any worms. This can explain why

the L3-Lung test was less able to detect antibodies in piglets infected with the respective dose.

Seroprevalence in nurseries

In the seroprevalence study performed in 68 Belgian nurseries, blood was collected from 10 piglets housed in the same nursery stable. This sampling strategy was based on research performed by Vlaminck (2013), which showed that randomly sampling of 10 animals from the same stable is sufficient to reflect the general infection pressure in that stable. A higher number of samples would not provide any added information, as the mean ODR values stay roughly the same when sampling more than 10 animals, and it would unnecessary increase the cost of analyses. If a lower number of samples is collected, the chances of gaining a false negative result increases significantly considering there is a heterogeneity within the pig population in their response to infections with *A. suum.*

Around 60% of the investigated stables in the seroprevalence study contained seropositive piglets, indicating that these piglets have come in contact with *Ascaris* eggs in one or the other way. The origin of these eggs and whether they are originally secreted by the sows or by the infected piglets themselves is currently unclear. Roepstorff (1997) demonstrated that the age of weaning of growing piglets significantly increased the risk of ascariasis. Apparently, weaning after the age of 6 weeks favours finishers to be positive for *A. suum,* due to the additional time for the eggs secreted by the sows to embryonate in the farrowing stable. Vice versa, Pittman et al. (2015) suggested that early weaning at 3 weeks of age or less diminishes the risk of *A. suum* transmission from the sow to her newborn piglets, based on the insufficient time for the embryo to develop into an infective L3 larva, which takes approximately 1 to 3 months. On the majority of the farms in Belgium, sows are weaned between approximately 3 to 4 weeks of age. Mostly piglets are then transferred to the nursery units post weaning, or they spent an additional time in the farrowing units, in absence of the sow. Whether or not the potential source of the contamination is mainly the sow remains unravelled. In addition to the time of weaning, also the age of the sows can be an important factor. Transmission to offspring is most likely from gilts than sows (Pittman et al., 2015). Multiparous sows are more likely to have a solid immunity, developing a pre-hepatic barrier. The chance of

infecting their offspring diminishes with increasing age. Based on this, it would be interesting to compare seroprevalence levels in piglets from gilts with those from sows of different parities, to examine if age of the sow has indeed an influence. If so, the more susceptible piglets from gilts could be placed in separate stables from the piglets born out of multiparous sows, to diminish the infection pressure.

Implementation of the L3-Lung test in practise

Considering that an ELISA test based on L3-lung larvae has the ability to detect exposure both in weaners as well as in fatteners, this test could be implemented as a useful diagnostic tool to detect *A. suum* infections on a farm. However, to obtain the antigen material from the migrating larvae, infected pigs must be euthanized. Alternatively, a recombinant antigen could be produced in the future. The remaining concern would be if the recombinantly produced antigen would be recognized by the anti-*Ascaris* antibodies raised against the native antigens. Still, this tool could be implemented standardly to screen piglets in the nursery phase, before they are transported to the fattening stables. This would allow the farmer and veterinarian to apply an adjusted deworming scheme, rather than treating the animals at the onset of the fattening round without further knowledge of their infection levels. Importantly however, the sensitivity of the test dropped substantially to 40% for infection doses of 10 eggs/day or lower. Thus, the test could fail to detect these lower infection levels, but one could argue whether such low levels are negligible or not, in terms of animal health and economic impact. Nevertheless, even if a stable appears to be *Ascaris* negative based on the results of the L3-lung test, one should be cautious to reduce the number of anthelmintic treatments after all.

Currently, there is no anthelmintic resistance described with regard to *A. suum*. To maintain this great advantage, it would be strongly recommended to standardly screen the pigs before deworming, preferably in different age groups present on a farm. This information could gain more insights in the actual prevalence and epidemiology of this parasite. In addition, based on serology the option to postpone a first treatment of the fatteners is possible, whereas two treatments may be sufficient to control infections during one

fattening round. Needless to say, this would be beneficial both economically as well as within the context of the prevention of anthelmintic resistance.

Of course, diagnosing exposure to *Ascaris* through serology also comes with a cost. This includes the cost for both the test itself as well as the collection of the blood samples by a veterinarian. However, one could say this is a small price to pay in order to control this parasite. Alternatively, taking saliva samples by placing ropes for example in the stables could be used to find antibodies in a cheap and animal-friendly way. This tool is currently used to detect Porcine Circovirus (PCV2) and Porcine Respirator and Reproductive Syndrome virus (PRRSV) in swine. Unfortunately, preliminary research has shown that this approach is not applicable to detect anti-*Ascaris* antibodies.

4.2 Serology as a tool to estimate economic impact of *A. suum*

Chapter 3 describes the evolution of the technical results in 18 stables on 18 fattening farms after the implementation of a deworming program during 6 consecutive rounds. Blood was collected at the end of each fattening round. Sera were tested on both the AsHb ELISA as well as the L3-Lung test. For each fattening round and for each stable, technical performance parameters were obtained from the producers.

Serology as a tool to estimate technical performance parameters

There was no improvement detectable of the technical performance parameters in the investigated stables of this study. Apparently, an enhancement in production parameters is hard to demonstrate, despite the improvement in *Ascaris* infection levels over time in the seropositive herds. Perhaps the infection intensities were too low to observe an improvement. All farms that tested seropositive on the AsHb ELISA at the onset of the trial had average ODR levels between 0,500 and 0,800. The ODR levels of the farms of group 2 were situated between 0,200 and 0,500. It would be interesting to investigate whether the effects on economic parameters do become visible when this study is repeated in herds with *Ascaris* antibody levels higher than ODR 0,800. However, such herds are rather exceptional in Belgium. Based on serum samples analysed from 392 Belgian fattening farms between 2013 and 2016, 63% of the farms had an average ODR lower than 0,500 (P. Geldhof, personal communication).

Since infection levels associated with ODR values of 0,800 or higher are rare in Belgium, one could make use of experimentally infected animals. Hale et al. (1985) for example observed a linear increase of the FCR with infection levels raising from 600 to 6000 and 60.000. Furthermore, pigs infected with the 60.000 eggs were 13% less efficient than negative controls. In theory, one could repeat such study and add serology as a parameter. However, in order to mimic the natural situation as close as possible, the animals would need to be trickle infected rather than receiving a single infection, which is technically not straightforward.

In our study, 9 out of the 18 farms investigated had only one fattening stable. The other 9 farms had two stables or more. Originally, the aim was to include such additional stables in the study as some kind of internal control stables,

in which we did not interfere with the anthelmintic treatment schedules normally applied. This way the outcome from stables where the deworming schedule of 3 treatments per fattening round was applied could be compared to that from the internal control stables. Unfortunately, during the course of our study, farmers started to implement the same schedule as that of the investigated stables, biasing these results and making it impossible to include the control stables in our research.

Vlaminck et al. (2015) and Martinez-Pérez et al. (2017) observed significant correlations between anti-*Ascaris* antibody levels and some of the technical performance parameters monitored. However, it would be bluntly to conclude that there is a complete causal relationship. Ample studies investigated the negative economic impact caused by infections with *A. suum*, as described in chapter 1. However, every farm is subjected to dynamic parameters that change continuously. Unfortunately, in this study only one fattening round per investigated stable was included as a historical control round. Ideally, this should have been more rounds, to rule out influences that can change the economic outcome of a stable over different rounds, for example the season, type of feed, outbreak of viral or bacterial diseases and genetics of the pigs (Zimmerman et al., 1973, Pittman et al., 2015). However, there was not enough time to include extra historical rounds. In addition, internal control stables could have given extra insights on the dynamics of a farm. As aforementioned, we could not rely on the results obtained in the control stables.

Interestingly, hierarchical linear mixed models indicated that the L3-Lung ELISA was a significant predictor of decreased ADG, increased FCR and prolonged DIF for the animals belonging to group 2. The results showed that an increase of 1 ODR on the L3-Lung test was indicative for a drop in ADG with approximately 128 g, an increase of the numbers of days in fattening with 21 days and an increase of FCR with 0,38. Thus, serology is a useful tool to estimate economic losses due to *Ascaris suum* infections.

Implementation of serology in practise

Nine farms were screened as being seropositive at the onset of the study (ODR>0,500). In these herds a clear decrease in antibody recognition was noticeable. At the end of the study all seropositive farms became seronegative. Then, the question could arise whether it is profitable to maintain the implementation of 3 treatments in these 'negative' herds. It would be advisable to screen seronegative farms before further application of deworming takes place. Preferably, this would take place at the end of the weaning phase and at the end of the fattening phase. In weaners, the L3-Lung test as described in chapter 2 could be applied. If the animals test negative on the L3-lung test, one could decide not to implement 3 but 2 anthelmintic treatments during that respective fattening round, postponing the first treatment after onset of the fattening round. However, as mentioned in 5.1, the test could fail to detect low infection levels of 10 eggs/day or less, so it would be advisable to serologically screen the fatteners again at the end of the fattening round, to rule out that a potential infection ultimately did take place. This procedure could be repeated as long as no new infection occurs, preventing an unnecessary amount of 3 treatments and diminishing the chance of anthelmintic resistance.

Farm management factors

In addition to an anthelmintic treatment, implementing a good farm management and taking infrastructural precautions can assist in the control of *A. suum*. Housing of pigs on fully slatted floors should reduce the chance of parasite exposure in comparison to partially slatted floors (Sanchez-Vazquez et al., 2010). Vandekerckhove et al. (2014) detected that antibody levels analysed on the AsHb ELISA were significantly higher in stables with partial slatted floors than fully slatted floors (P < 0.05). Solid floors contribute to be a risk factor, as it enlarges the change of contact with faeces, possibly containing infective eggs. Implementing a good biosecurity (i.e. aspects to prevent pathogens of entering and spreading within a group of pigs) on a farm is important. Herds with a better biosecurity seem to have better production values (Laanen et al., 2013). In addition, an AI/AO management system was applied on all investigated farms. This system makes it possible for the animals of the same fattening round to be slaughtered within the

same time period, which results in an empty stable before a new batch of animals arrives. During this period thorough cleaning and disinfection is possible (Edwards et al., 2014). According to Martinez-Pérez et al. (2017), an AI/AO system was associated with a significantly lower prevalence of *A. suum*.

Former obliged changes in the management on farrow-to-finish farms can bring new risk factors to the surface. Namely, in 2013 it became mandatory in Belgium for pregnant sows to be housed in group. Surely this is beneficial for the animal welfare, but it induces conditions that are favourable to the transmission of parasite infections. Sows and gilts that are in different stages of gestation are mixed, in case of dynamic grouping. As there is more contact possible between the animals, there is also more exposure to faeces (Haugegaard, 2010), increasing the prevalence levels.

All the aforementioned should be taken into account in order to further control parasite infections pig herds in the future.

4.3 Conclusion

The main goal in pig production systems is to produce as many healthy pigs as possible, and as fast as possible. Although it is striking how difficult it is to show a reduced productivity in a significant way due to *Ascaris,* still the control of *Ascaris* infections in pig production systems remains crucial. Without any control strategies in place, infection levels can rapidly build up ultimately leading to reduced production efficiency. The results obtained during this PhD research project have shown that serology can be a useful diagnostic tool in the control of this parasite, both in piglets as well as fattening pigs. Furthermore, anti-*Ascaris* antibody levels have shown to be a significant predictor of decreased technical performance of the animals, indicating that serology can be used to estimate economic losses due to *Ascaris*.

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SUMMARY

Ascaris suum is by far the most common parasite infecting pigs in modern pig farms. Although this parasite is still very prevalent and is known for its impact on the economic outcome of the pig industry, due to the subclinical nature of the disease combined with a lack of appropriate diagnostic tools, ascariosis often remains undiagnosed. Nevertheless, it is important to understand the infection intensity in a herd, in order to apply an appropriate deworming program.

In recent years several studies have investigated the use of serology to more accurately measure the level of exposure of pigs to *A. suum*. Baring this in mind, this thesis focused mainly on how serology could be further implemented in practise.

In **chapter 2** of this thesis we evaluated whether serology can be used to measure exposure of piglets to *A. suum* during the nursery phase. Experimental infection studies were performed in which 7 groups of 10 piglets of 4 weeks of age were orally infected with either 10, 20, 40, 60, 80, 100 and 500 *A. suum* eggs/day during 7 consecutive weeks. Serum was collected on a weekly basis to monitor seroconversion on serology. A dose-dependent seroconversion was measurable with the L3-lung ELISA starting from 4 weeks post-infection onwards, whereas this was not measurable with the AsHb ELISA. The results of a seroprevalence study sampling 10 piglets on 68 different nursery farms in Belgium showed that 38 % of the farms analysed all piglets tested seronegative, whereas for the remaining 62 % of the farms the percentage seropositive piglets ranged from 10 to 100 %. This indicates contamination of the nursery facilities with *A. suum* eggs. The outcome of this study shows that serology can be used to measure exposure of nursery piglets to *A. suum*, thereby providing an additional tool in the control of this widespread parasite.

Chapter 3 focused on the effect of a strategic deworming program on *Ascaris* infection levels and technical performance parameters in fattening pigs. Eighteen stables on 18 different fattening farms were monitored for a period of 7 consecutive fattening rounds. A deworming program using 200mg/ml fenbendazole oral suspension in drinking water for 2 days every 6 weeks was implemented for 6 consecutive fattening rounds. For each fattening round and for each stable, technical performance parameters

including average daily growth, feed conversion, days in fattening and the percentage of affected livers were obtained from the producers. Blood was collected in each stable from 10 animals at the end of each fattening round and evaluated using serology. The results clearly indicated an improvement in terms of exposure of the animals to *Ascaris suum* after the implementation of a strategic deworming program, as measured by 2 different serological tests. The decline in anti-*Ascaris* antibody levels was especially detectable in the stables that originally tested positive for *Ascaris*. There was no improvement detectable of the technical performance parameters in the investigated stables. Yet, hierarchical linear mixed models indicated that the L3-Lung ELISA was a significant predictor of decreased ADG, increased FCR and prolonged DIF for the animals that tested positive for *Ascaris*.

The control of parasite infections remains an important aspect. Serology showed to be a useful diagnostic tool to assess the worm status on a farm of both piglets and fattening pigs, making it possible to control this parasite in a more decisive way by applying an adequate treatment strategy. Additional control measures through farm management and stable infrastructure could be implemented to lower the exposure of the piglets to *A. suum* during the nursery phase and to avoid further transmission to the fattening units. Furthermore, anti-*Ascaris* antibody levels were a significant predictor of decreased technical performance of the animals. Thus, serology can also be used to estimate economic losses due to *Ascaris*.

SAMENVATTING

Ascaris suum is een intestinale rondworm die wereldwijd varkens infecteert en kan bijdragen tot omvangrijke economische verliezen in de varkensindustrie. Het merendeel van de infecties met *A. suum* verlopen subklinisch, waardoor ze moeilijk vast te stellen zijn en vaak gemist worden door de veehouder. Tot voor kort was er een gebrek aan adequate diagnostische middelen. Een goede diagnose is nochtans onmisbaar in het kader van een goed ontwormingsprogramma.

Recente studies hebben aangetoond dat serologie in staat is om de levels van blootstelling van de varkens aan *A. suum* te detecteren. De focus van deze thesis lag daarom voornamelijk op het implementeren van serologie in de praktijk.

In **hoofdstuk 2** werd nagegaan of serologie kan gebruikt worden om de blootstelling van gespeende biggen aan *A. suum* te bepalen op de batterijen. Zeven groepen van 10 biggen van 4 weken oud werden experimenteel geïnfecteerd met 10, 20, 40, 60, 80, 100 and 500 *A. suum* eieren/dag gedurende 7 opeenvolgende weken. Elke week werd er bloed genomen om na te gaan of er seroconversie kon worden vastgesteld op basis van serologische tests. Een dosis-afhankelijke seroconversie kon gedetecteerd worden vanaf 4 weken volgend op de eerste infectie, en dit aan de hand van de L3-Long ELISA. Dit kon echter niet gedetecteerd worden op basis van de AsHb ELISA. Voor een seroprevalentie onderzoek werden er 10 biggen gescreend op 68 verschillende batterijen in België. Op 38% van de geanalyseerde bedrijven waren alle biggen seronegatief, maar op de overige 62% van de bedrijven lag het aantal seropositieve biggen tussen 10 en 100%. Dit geeft aan dat de biggenbatterijen mogelijks gecontamineerd zijn met *A. suum* eieren. Deze studie toont aan dat serologie kan toegepast worden om blootstelling aan *A. suum* in biggen op de batterijen aan te tonen en zo te helpen bij de controle van deze veelvoorkomende parasiet.

In **hoofdstuk 3** werd de focus gelegd op wat het effect is van een strategisch ontwormingsprogramma bij afmestvarkens op de infectielevels van *Ascaris* en op de technische productiegegevens. Er werden op 18 verschillende afmestbedrijven 18 stallen gescreend gedurende 7 opeenvolgende afmestronden. In elke stal werd er een strikt ontwormingsschema toegepast met 200mg/ml fenbendazole in het

drinkwater opgelost gedurende 2 dagen, en dit om de 6 weken, gedurende 6 afmestronden. Voor elke ronde en elke stal werden de technische productieparameters zoals gemiddelde dagelijkse groei, voederconversie, aantal dagen in afmest en aantal afgekeurde levers verkregen via de producenten. Daarnaast werd er bloed genomen van 10 mestvarkens op het einde van elke afmestronde en geanayseerd op basis van serologische tests. Uit de resultaten bleek dat er een duidelijke verbetering was in de blootstelling aan *A. suum* na invoering van het strikt ontwormingsprogramma. De daling in de anti-*Ascaris* antistoflevels was vooral duidelijk in de bedrijven die aanvankelijk positief testten op de AsHb ELISA. Er konden geen significante veranderingen worden vastgesteld in de technische productiegegevens in de onderzochte stallen. Aan de hand van een hiërarchisch lineair mixed model kon aangetoond worden dat de L3-Long ELISA op significante wijze een daling in de gemiddelde dagelijkse groei, een stijging in de voederconversie ratio en een toename in aantal dagen dat de dieren in een afmestronde zaten kon voorspellen, en dit voor de dieren die aanvankelijk positief testten op de aanwezigheid van *Ascaris*.

Het blijft belangrijk om parasitaire infecties onder controle te houden, zowel voor economische redenen als in het kader van het dierwelzijn. Serologie maakt het mogelijk om een bedrijf zorgvuldig te screenen en zo een idee te krijgen van de wormstatus op een bedrijf, zowel van de biggen als van de afmestvarkens. Op deze manier kan er accuraat behandeld worden en kunnen infecties met *A. suum* onderdrukt worden. Daarnaast kan blootstelling gecontroleerd worden via een aangepast management en stalinrichting om zo een besmetting in de batterijen te voorkomen en bijgevolg de verspreiding naar de afmeststallen te vermijden. Anti-*Ascaris* antistoflevels kunnen een daling in de technische productiegegevens op significante wijze voorspellen. Serologie kan dus ook gebruikt worden om eventuele productieverliezen veroorzaakt door *A. suum* in te schatten.

DANKWOORD

En of 5 jaar voorbij kan vliegen… Mijn thesis is eindelijk klaar! Ik wil bij deze enkele mensen bedanken.

Peter, bedankt om zo'n geweldige promotor te zijn, steeds met tonnen geduld. Bedankt om er voor me te zijn, bedankt dat je deur steeds open stond en bedankt om de tijd te nemen om me verdunningen uit te leggen! Je bent de fijnste west-vlaamse linkshandige mens die ik ken!

Edwin, bedankt voor de fijne tijden en om me te vertrouwen om je lessen over te nemen en kliniek te geven. Je was een ontzettend fijne collega!

Thanks to the members of my examination commission, Prof. Dr. Stig Milan Thamsborg, Prof. Dr. Dominiek Maes, Dr. Tamara Vandersmissen en Dr. Hilde van Meirhaeghe for your expertise and your reviews of my thesis.

Bedankt aan iedereen die geholpen heeft tijdens mijn onderzoeken. Bedankt aan mijn 18 veehouders, Aveve, MSD, Merial, DGZ Veepeiler en KRKA.

Bedankt aan Renée en Katrijn, om me de liefde voor de varkenshouderij bij te brengen.

Johnny, toch even een woordje aan jou gericht aangezien je de biologische vader bent van "ons" Serasca-kindje. Bedankt om aan mij te denken toen je me belde 5 jaar geleden. Je hebt je taak als surrogaat-broer met verve volbracht.

Dries, Leentje en Fre: jullie waren mijn eerste bureaugenoten. Merci voor de fijne tijden, zowel tijdens als buiten de werkuren. We zien elkaar ongetwijfeld nog, om te gaan shoppen, of sushi te eten, of een pint te gaan pakken!

Iris, Stijn en Nathalie: de kern van de parasitologie. Zonder jullie staan we nergens! Iris, merci om me steeds te helpen met mijn verdunningen, ook al mocht dat niet van Peter ☺ Stijn, merci om me steeds te helpen met de varkentjes. Nathalie, merci om de Serasca analyses van me over te nemen en zoveel meer.

Ana, mi chica! You were one of the first people I spoke to on my first day here at the lab. I'm so happy we could do this together, I am very proud of you! Hasta luego, claro!

Oonagh: Stille waters, diepe gronden. Ik vind je stiekem één van de grappigste mensen die ik ken. Merci voor de leuke feestjes, er volgen er ongetwijfeld nog!

Brecht: onze pupil, wat ben ik blij dat we jou in het labo hebben binnen gekregen ☺ Je zal dat ongetwijfeld goed doen! Zorg goed voor Nathalie!

Charlotte, Sien, Ellen, Tianyu, Karen, Santosh, Rika: it was a pleasure to know you guys! We'll meet again!

Jimmy, Aurélie, Marieke, Piet, Zhen Zhen, Bruno, Pierre, Fiona, Wouter: thanks for being such great colleagues! Best of luck!

Dirk, Isabelle en Marijke: het secretariaat-team! Altijd daar voor advies, IT-problemen of gewoon voor een praatje.

Aan mijn nieuwe collega's bij Boehringer Ingelheim: bedankt om in mij te geloven en mij een job aan te bieden! Ik ben heel blij dat ik voortaan bij de BI familie mag horen!

Aan mijn vrienden, bedankt om in mijn leven te zijn, ik zou jullie niet kunnen missen! En aan m'n mokje, veel succes met je nieuwe woonst!

Sonja en Lisanne, zonder jullie zou ik niet de persoon zijn die ik vandaag ben. 22 jaar and still counting… Van onschuldige meisjes naar getrouwde vrouwen met kinderen en/of katten. Ik zie jullie zoooo graag!

Mijn schoonouders, bedankt om mij in jullie gezin op te nemen met zoveel warmte en liefde. Steph, you hot mama! Wat ben ik blij dat jij mijn schoonzus bent! Op naar nog vele reisjes samen met liters sangria. Noortje floortje, you rock!

Mijn broers, mien beste moaten. Ik ben erg trots om jullie grote zus te mogen zijn! Blijf vooral wie je bent, you're perfect.

Mijn ouders… Woorden schieten me tekort om te beschrijven wat jullie voor mij betekenen. Ik denk dat niemand zo trots is vandaag als jullie nu zijn. Bedankt om altijd in mij te geloven en mij onvoorwaardelijk te steunen in alles wat ik doe. Ik zie jullie graag!

Mijn twee mannen… I love you to the moon and back, and back, and back, … Zoetje, bedankt om in mijn leven te zijn en me graag te zien zoals ik ben. Ik ben zo blij om jouw vrouwtje te zijn! Ik had me geen betere papa voor ons zoontje kunnen wensen!

Ten slotte wil ik dit boekje graag opdragen aan mijn bompa, die ongetwijfeld de reden is dat ik dierenarts ben geworden.
"I don't believe in an interventionist God…
…I believe in some kind of path
That we can walk down, me and you"

Elise