FACULTY OF MEDICAL AND HEALTH SCIENCES
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Ecology and viability of eggs of the pig round worm (*Ascaris suum*) – on-farm and laboratory studies

KIRAN KUMAR KATAKAM

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Author: **Kiran Kumar Katakam**

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Name of the department: Section for Parasitology and Aquatic Diseases,
Department of Veterinary Disease Biology,
Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

Supervisors: **Professor Stig Milan Thamsborg** (main supervisor)
Section for Parasitology and Aquatic Diseases,
Department of Veterinary Disease Biology,
Faculty of Health and Medical Sciences,
University of Copenhagen, Denmark.

Professor Anders Dalsgaard
Section for Food Safety and Zoonoses,
Department of Veterinary Disease Biology,
Faculty of Health and Medical Sciences,
University of Copenhagen, Denmark.

Associate professor Helena Mejer
Section for Parasitology and Aquatic Diseases,
Department of Veterinary Disease Biology,
Faculty of Health and Medical Sciences,
University of Copenhagen, Denmark.

Assessment committee:

Dr. Annette Olsen (Chairperson)

Section for Parasitology and Aquatic Diseases,

Department of Veterinary Disease Biology, University of Copenhagen, DK

Dr Jaap Boes

Knowledge Centre for Agriculture, Denmark.

Professor Johan Höglund

Swedish University of Agricultural Sciences, Sweden.

Preface

The current PhD thesis is based on the research work performed between 2009 to 2013 at Section for Parasitology and Aquatic Diseases in close collaboration with Section for Food Safety and Zoonoses, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences. The Ph.D. stipend was financed by the Faculty of Health and Medical Sciences, University of Copenhagen and its Ph.D. Research School, RECETO and the research was performed as part of the projects PATHOS (funded by the Strategic Research Council of Denmark) and PAROL (funded by the Danish AgriFish Agency, Ministry of Food, Agriculture and Fisheries). Professor Stig Milan Thamsborg was my main supervisor, Professor Anders Dalsgaard and Associate Professor Helena Mejer were my co-supervisors. Dr. Allan Roepstorff acted as my main supervisor for first one year.

My Ph.D. work comprised two minor studies on viability testing of *Ascaris suum* eggs using staining technique referred to as staining study and hatching technique referred to as hatching study (reported as part of thesis), a study on antagonistic effect of microfungi on the viability of thick-shelled Ascarid eggs, performed jointly with S. Thapa and others (reported elsewhere) and 4 papers/manuscripts included in the thesis, referred to as follows:

Paper 1. **Katakam, K. K.,**Roepstorff, A., Popovic, O., Kyvsgaard, N. C., Thamsborg, S. M., and Dalsgaard, A. (2013). Viability of *Ascaris suum* eggs in stored raw and separated liquid slurry. Parasitology, 140(03), 378-384.

Paper 2. **Katakam K.K.,** Mejer H., Dalsgaard A., Kyvsgaard, N. C. and Thamsborg S. M. Survival of *Ascaris suum* and *Ascaridiagalli* eggs in liquid manure at different ammonia concentrations and temperatures. Submitted to Veterinary Parasitology.

Paper 3. **Katakam K.K.,** Thamsborg S. M., Kyvsgaard, N. C., Dalsgaard A. and Mejer H. Development and survival of *Ascaris suum* eggs in deep litter of pigs. Submitted to Parasitology.

Paper 4. **Katakam K.K.,** Thamsborg S. M., Dalsgaard A. and Mejer H. Environmental contamination and transmission of *Ascaris suum* in Danish organic pig farms (manuscript)

Kiran Kumar Katakam,
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Abbreviations

DAPI	4', 6 diamidino-2-phenylindole
DM	Dry matter
DNA	Deoxyribonucleic acid
EPG	Eggs per gram
g	Gram
h	Hour
kg	Kilogram
l	Litre
m	Metre
mg	milligram
min	minute
mM	milli mole
L_3	Third stage larva
L_4	Fourth stage larva
$NH_{3\,(aq)}$	Un-ionised ammonia in liquid phase
$NH_{3\,(g)}$	Un-ionised ammonia in gaseous phase
$NH_4^{+}{}_{(aq)}$	Ionised ammonia in liquid phase
P	Probability
pi	Post infection
PI	Propidium iodide
RNA	Ribonucleic acid
RH	Relative humidity
rpm	Rotations per minute
spp.	Species
TAN	Total ammonia nitrogen
T_{50}	Time for 50% inactivation
T_{99}	Time for 99% inactivation
µm	micrometre

Summary

Ascaris suum is a highly prevalent parasitic helminth in pig production systems worldwide. In most industrialized countries production systems are highly intensive and fully in-doors (low prevalence of parasites) but there is a growing market for pigs produced out-door or under organic pig farming conditions (high prevalence of parasites). In organic pig production there is a number of factors related to housing and management practices that predispose for *A. suum* infections. These practices include restricted use of anthelmintics, late weaning of piglets (after min seven weeks of age) and access to outdoor runs. Provision of bedding material, which might be conducive for development and survival of free-living stages of helminths, is mandatory in organic production systems but the amount and type of bedding material vary between farm and housing systems.

The differences in *A. suum* egg development in different housing systems have been poorly described. Bio-degradation of organic matter in deep litter housing systems produces physico-chemical conditions which may affect the development and survival of *A. suum* eggs, but little is known about such effects. Understanding the ecology and transmission dynamics of *A. suum* may help prevent infections in organic farming. Pig slurry is used to fertilize crops in organic as well as conventional farming and may be treated to inactivate a variety of pathogens present in it before application. To achieve adequate pathogen inactivation, slurry may need to be stored for around one year which may not be ideal logistically and economically. Chemical treatment of slurry may reduce storage time needed to adequately inactivate pathogens. The eggs of *A. suum* are commonly used as a conservative indicator in estimating pathogen inactivation during slurry treatment as they are more resistant to environmental stress than bacterial and viral pathogens. The eggs of the poultry ascarid, *Ascaridia galli* are ubiquitous, resistant and available even in places where the eggs of *A. suum* cannot be found, e.g., areas without pig keeping, but little is known about the suitability of *A. galli* eggs as a conservative hygiene indicator. Currently, viability of *A. suum* eggs is estimated by their ability to embryonate which takes up to six weeks depending on temperature. There is a need to develop more rapid viability tests and in addition, embryonation cannot be used as a viability criterion for already embryonated eggs. A quantitative egg hatch test may thus be helpful for already embryonated eggs. The present Ph.D. project therefore addressed the above issues by investigating in six separate studies, the ecology and transmission of *A. suum* eggs in organic pig farms, including determination of conditions under which *A. suum* eggs are inactivated.

In the first study (**staining study)**, fluorogenic vital stains namely, 4', 6 diamidino-2-phenylindole (DAPI) and propidium iodide (PI) were successfully tested to distinguish viable and heat killed *A. suum* eggs. A 100-µl egg suspension was incubated with 10 µl each of working solutions of DAPI and PI at 37°C for 3 h. Eggs were washed and examined under an epifluorescence microscope with appropriate filter blocks. Viable eggs excluded both the stains whereas heat killed eggs allowed the stains to enter the eggs. However applicability of these stains in distinguishing live and naturally killed eggs needs to be further investigated.

In the second study (**hatching study**), an egg hatch assay was developed to test the viability of embryonated eggs after modifying and optimizing earlier described methods. A 950-µl decoated *A. suum* eggs suspension in normal saline was incubated with 50 µl pig bile at 38.5°C for 16-17 h in a container filled with carbon dioxide gas which was kept on a mechanical shaker at 150 rpm. The study resulted in highly inconsistent results with a large variation in hatching percentage (12%-99%) between different trials, different eggs batches and different replicates of the same egg batch. The egg hatch assay need to be further optimized to reduce the variability.

In the third study (**paper 1**), the viability of *A. suum* eggs was investigated in separated liquid slurry in comparison with raw slurry during storage. Separate nylon bags with 6000 eggs each, were placed in one litre bottles containing either raw slurry or separated liquid slurry and incubated at 5°C or 25°C for 308 days. At 25°C, viability of eggs declined to zero in both raw slurry and separated liquid fraction by day 308. At that time, 88% and 42% of the eggs were still viable in separated liquid slurry and raw slurry, respectively, at 5°C. Inactivation of eggs at 25°C was correlated with high ammonia content in raw slurry (7·9–22·4mM) and separated liquid fraction (7·3–23·2mM). At 5°C, ammonia content ranged from 3·2–9·5 mM in raw slurry and 2·6–9·5mM in separated liquid slurry. The study demonstrated that slurry if kept at 25°C need to be stored for around 10 months and if kept at 5 °C, need to be stored for even longer to obtain adequate pathogen inactivation.

In the fourth study (**paper 2**), the viability of eggs of the ascarids *A. suum* and *A. galli* was compared at different temperatures and urea levels in pig slurry. Separate nylon bags with 10,000 eggs of each species were placed in 200 ml plastic bottles consisting either of urea-treated (2%) or untreated pig slurry for up to 120 days at 20°C, 6 days at 30°C, 36 h at 40°C and 2h at 50°C. At all temperatures in both the slurry types, *A. galli* eggs were inactivated at a significantly faster rate ($P<0.05$) compared to *A. suum* eggs. For each 10°C increase in temperature from 20°C, T_{50} (time needed to inactivate 50% of eggs) for both types of eggs was reduced markedly. At all temperatures, viability of eggs of both species was significantly higher ($P < 0.05$) in

untreated slurry compared to urea-treated slurry except for *A. galli* eggs at 20°C where no significant difference was detected. In untreated slurry, the levels of pH (6.33-9.08) and ammonia (0.01-1.74 mM) were lower ($p<0.0001$) compared to that of urea-treated slurry (pH: 8.33-9.28 and ammonia 1.13 mM). The study demonstrated that *A. galli* eggs are more sensitive to environmental stress compared to *A. suum* eggs and hence cannot serve as a better hygiene indicator than *A. suum* eggs. Addition of urea markedly reduced the storage duration of slurry needed to inactivate *A. suum* and *A. galli* eggs.

In the fifth study (**paper 3**), physico-chemical conditions (temperature, pH, aqueous ammonia concentration and moisture content) prevailing in deep litter, contamination levels of *A. suum* eggs and development and viability of *A. suum* eggs were investigated at three different defined areas (resting, intermediate and latrine areas) of pig pens and at different depths at four time points from September 2011 to June 2012. Mean temperatures across depths in resting (35°C) and intermediate areas (44°C) were significantly higher compared to latrine areas (30°C) which could be related to higher moisture levels, lower oxygen and less microbial activity (low composting activity) in the latrine area. Moisture content was 79% in latrine areas compared to 43% and 36% in intermediate and resting areas. Ammonia content in intermediate areas (2.6 mM) was significantly higher than that in resting (1.9 mM) and latrine areas (1.0 mM). pH varied significantly with area and samples from intermediate area had the overall highest pH (8.88) followed by resting (8.60) and latrine (8.04) areas. The bedding material in all the three areas, at all depths were contaminated with *A. suum* eggs but the level of contamination was highest in latrine areas (4-40 times more eggs per g DM) compared to intermediate and resting areas. The top most layer contained the highest number of eggs compared to the underlying layers and level of contamination declined significantly as the depth increased. A very small fraction of eggs in resting (4%) and intermediate (0.004%) areas contained fully developed eggs whereas none of the eggs in latrine areas were fully developed. *In-vitro* embryonation of eggs revealed that only 5% of eggs were viable in intermediate areas whereas 17% and 32% of the eggs were viable in resting and latrine areas, respectively. This indicates that intermediate areas create more unfavourable conditions compared to the other areas due to high temperatures associated with microbiological activity and ammonia concentration. In conclusion, deep litter systems in general create unfavourable conditions for development and survival of the majority of *A. suum* eggs. However, transmission of helminth eggs is probably unavoidable as some fully embryonated eggs will be present and further, eggs from the deeper parts of the deep litter bed may resume development if not stored, composted or treated.

The sixth study (**paper 4**) was carried out to investigate the on-farm environmental contamination and transmission by mapping egg contamination levels in pens (young pigs after weaning (starter pigs) and older, finisher pigs (approx. 80-100 kg)) and on pastures (dry sows, lactating sows and starter pigs) and infection levels in animals at four occasions within one year. Three different areas (resting, intermediate and latrine areas) were identified and the upper part of the bedding material (top 10 cm) was sampled regularly in each pen in shallow litter housing systems (three farms) and deep litter housing systems (two farms). All areas of the pens were contaminated and levels were similar in both housing systems but generally higher (5-25 times more eggs per g dry matter (DM) of bedding) in latrines compared to the other two areas. Significantly higher fraction of eggs (44%) started developing in resting areas compared to that of intermediate (33%) and latrine areas (13%). Significantly higher of larvated and infective eggs were found in shallow litter (19 eggs per g DM and 7 eggs per g DM respectively) compared to deep litter (2.5 eggs per g DM and 0.8 eggs per g DM respectively) irrespectively of type of area. Embryonation of *A. suum* eggs from bedding material revealed that 39-97% of the eggs were viable. In the out-door environment, weaning pastures were highly contaminated compared to farrowing pastures (=lactating sow pastures) and dry sow pastures. Averaged across farms and samplings, the prevalence of *A. suum*, in starters, finishers, dry sows and lactating sows was 48%, 64%, 28% and 15%, respectively. In conclusion, it has been shown that organic pigs get infected during the pre-weaning period on farrowing pastures or weaning pastures, pigs at all stages of production are at risk of exposure to infective *A. suum* eggs, resting areas create more favourable conditions for development of eggs and deep litter systems do not pose an additional risk of *A. suum* transmission as compared to shallow litter systems. Strategic use of anthelmintics (e.g. deworming weaned pigs just before shifting them in to indoor pens) combined with thorough cleaning of pens before every new batch of pigs will probably minimize indoor transmission of *A. suum*.

The Ph.D. project has provided the following main findings:

i. Viable and heat killed *A. suum* eggs can be distinguished using fluorogenic vital stains.

ii. *In-vitro* hatching of *A. suum* eggs is a complex process and the study resulted in a large variation in hatching percentage (12%-99%).

iii. Ten months storage of slurry at 25°C (both raw and separated liquid fraction) totally inactivated *A. suum* eggs. At 5°C, 88% and 42% of the eggs were still viable after ten months in separated liquid slurry and raw slurry, respectively.

iv. *Ascaridia galli* eggs are more sensitive to environmental stress compared to *A. suum* eggs and do not serve as better hygiene indicators compared to *A. suum* eggs. Addition of urea markedly reduces the storage time of slurry needed to inactivate *A. suum* and *A. galli* eggs.

v. Deep litter systems provide unfavourable conditions for the development and viability of the majority of *A. suum* eggs. Eggs that are viable can resume development if better conditions are provided and manure and slurry thus need to be properly composted.

vi. In organic production systems, weaning pastures were heavily contaminated with *A. suum* eggs indicating that weaned pigs are mostly at risk of *A. suum* transmission. Grazing rotation for 1-3 year may not be useful in reducing *A. suum* transmission. Deep litter systems do not pose an additional higher risk of *A. suum* transmission as compared to shallow litter systems.

Sammendrag (Danish summary)

Spolorm (*Ascaris suum*) er en meget almindeligt forekommende parasitisk orm hos svin i de fleste produktions-systemer i verden. I de fleste industrialiserede lande er produktionssystemerne intensive og indendørs (lav parasit forekomst) men der er et voksende marked for svin produceret økologisk eller blot udendørs (høj parasit forekomst). I økologisk svineproduktion er der en række forhold i relation til opstaldning, management og generel praksis, der fremmer *A. suum* infektioner. Disse forhold omfatter begrænset anvendelse af ormemidler, sen fravænning af smågrise (tidligst ved 7-ugers alderen) og adgang til udendørs arealer. Tildeling af strøelsesmateriale, der anses for at forbedre de frit-levende parasit stadiers udvikling og overlevelse, er obligatorisk i økologiske produktionssystemer, men mængden og typen af materiale varierer mellem bedrifter og staldsystemer.

Forskelle i udvikling af *A. suum* æg mellem forskellige staldsystemer har været dårligt belyst. Biologisk nedbrydning af organisk materiale i dybstrøelse-systemer påvirker de fysisk-kemiske forhold lokalt, som igen kan påvirke udvikling og overlevelse af *A. suum* æg, men viden på dette område er begrænset. En forståelse af økologiske forhold og dynamikken bag spredningen af *A. suum* er således nødvendig for bedre at forebygge infektionerne. Svinegylle bruges til at gødske afgrøder i økologisk såvel som konventionelt landbrug og kan behandles på forskellige måder for at inaktivere mulige patogener før udkørsel. For at opnå tilstrækkelig inaktivering må gyllen ofte opbevares i tanke i op til et år, hvilket er forbundet med både økonomiske og logistiske problemer. Kemisk behandling af gyllen kan reducere den nødvendige opbevaringstid for at opnå tilstrækkelig inaktivering af patogenerne. Æg af *A. suum* anvendes ofte som "konservativ" indikator for inaktivering af patogener ved behandling af gylle, da disse æg er mere modstandsdygtige overfor miljøstress end bakterier og vira. Æg af spolorm fra fjerkræ, *Ascaridia galli* er også resistente og udbredte i hele verden – også i områder hvor *A. suum* æg ikke findes fordi svinehold ikke praktiseres. Viden om anvendelse af *A. galli* æg som hygiejne indikatorer er imidlertid meget begrænset. For nærværende bestemmes levedygtigheden af *A. suum* æg ved deres evne til at embryonere (udvikles). Dette tager op til 6 uger afhængig af temperatur og der er derfor behov for at udvikle en hurtigere test. Embryonering kan desuden ikke bruges som mål for levedygtighed, hvis æggene allerede er embryonerede og det kunne derfor være relevant at udvikle en kvantitativ klækningstest for allerede embryonerede æg. Ph.D. projektet har undersøgt ovennævnte problemstillinger i 6 separate studier. Studierne er dels gennemført i økologiske svinebesætninger for at undersøge spredningen af *A. suum* infektioner og dels i laboratoriet for at teste hvordan æggene kan inaktiveres.

I det første forsøg (**staining study)**, blev fluorogen vital-farvning med 4', 6 diamidino-2-phenylindole (DAPI) og propidium iodide (PI) med succes anvendt til at skelne mellem levende og varmeinaktiverede *A. suum* æg. En ægsuspension (100-µl) blev inkuberet med en opløsning (10 µl) af henholdsvis DAPI og PI ved 37°C i 3 timer. Æggene blev herefter skyllet and undersøgt med et epifluorescens mikroskop med passende filtre. Farverne kunne ikke trænge ind i de levende æg (eksklusion), mens de inaktiverede æg tillod farverne at komme ind gennem ægskallen. Anvendelse af disse to farvemetoder til at skelne mellem levende og "naturligt" døde æg bør dog undersøges nærmere, før de kan bruges i praksis.

I det andet forsøg (**hatching study**) blev en test til kvantitativ klækning af æg udviklet på basis af eksisterende forskrifter, med henblik på at bestemme levedygtigheden af embryonerede æg. En suspension (950-µl) af "afskallede" *A. suum* æg (den yderste proteinskal var fjernet) i fysiologisk saltvand blev inkuberet med 50 µl svinegalde ved 38,5°C i 16-17 timer i en beholder fyldt med kuldioxid placeret på en mekanisk rystemaskine ved 150 rpm. Forsøget gav en meget varierende klækning (12%-99%) og ringe reproducerbarhed mellem forskellig kørsler, æg-batches og replikater inden for samme æg-batch. Testen bør derfor optimeres yderligere for at reducere variation og øge reproducerbarhed, før den kan bruges i praksis.

I det tredje studie (**paper 1**) blev levedygtigheden af *A. suum* æg undersøgt i forhold til hvor lang tid de blev opbevaret i enten separeret flydende gylle eller almindelig gylle. Nylonposer med 6000 spolormæg blev placeret i 1 liter flasker fyldt med enten almindelig gylle eller separeret flydende gylle. Flaskerne blev inkuberet ved 5°C eller 25°C i 308 dage. Ved 25°C faldt levedygtigheden til nul i begge typer gylle efter 308 dage. Efter same periode ved 5°C var henholdsvis 88% og 42% af æggene fortsat levende i separeret og almindelig gylle. Inaktiveringen af æg ved 25°C faldt sammen med et højt indhold af ammoniak i almindelig gylle (7·9–22·4mM) og i separeret gylle (7·3–23·2mM). Ved 5°C varierede ammoniakkoncentrationen mellem 3·2–9·5 mM i almindelig gylle og mellem 2·6–9·5mM i separeret flydende gylle. Forsøget viste, at for at opnå den ønskede inaktivering af parasitære patogener, bør gylle opbevares ved 25°C i op til 10 måneder og ved 5 °C væsentligt længere.

I det fjerde forsøg (**paper 2**) blev levedygtigheden af æg af spolormene *A. suum* og *A. galli* sammenlignet ved forskellige temperaturer og tilsætninger af urinstof (urea) til svinegylle. Nylonposer med 10,000 æg af hver ormeart blev inkuberet i 200 ml plastikflasker med henholdsvis urea-behandlet (2%) og ubehandlet svinegylle i op til 120 dage ved 20°C, 6 dage ved 30°C, 36 timer ved 40°C og 2 timer ved 50°C. Ved alle temperaturer i begge typer gylle blev æg af *A. galli* inaktiveret signifikant hurtigere end *A. suum* æg

(P<0.05). For hver 10°C temperaturstigning blev T_{50} (tid nødvendig for inaktivering af 50% af æg = halveringstiden) markant reduceret. Ved alle temperaturer var levedygtigheden af både *A. suum* og *A. galli* signifikant bedre (P < 0.05) i ubehandlet gylle i forhold til behandlet gylle med undtagelse af *A. galli* æg ved 20°C, hvor der ikke var en effekt af urea-behandlingen. I ubehandlet gylle var pH (6.33-9.08) og ammoniakkoncentration (0.01-1.74 mM) væsentligt lavere (p<0.0001) end i behandlet gylle (pH: 8.33-9.28 og ammoniakkoncentration 1.13 mM). Forsøget viste, at *A. galli* æg er mere følsomme overfor forskellige typer stress i det eksterne miljø end *A. suum* æg og må derfor anses for at være en dårligere hygiejne-indikatorer end *A. suum.* Tilsætning af urea reducerede markant den opbevaringstid, der er nødvendig for inaktivering af *A. suum* og *A. galli* æg.

I det femte forsøg (**paper 3**) blev antal, udviklingsgrad og levedygtighed af spolormæg undersøgt i svinestier med dybstrøelse og sammenholdt med samtidige målinger af udvalgte fysisk-kemiske forhold (temperatur, pH, ammoniak$_{aq}$-koncentration og vandindhold). Tre veldefinerede områder (hvileområder, latrin, hvor dyrene defækerede, og et mellemliggende område) blev udvalgt i hver sti og undersøgt i forskellige dybder 4 gange mellem september 2011 og juni 2012. Middeltemperaturen i dybstrøelsen på tværs af dybder i hvile- (35°C) og mellemområder (44°C) var signifikant højere i forhold til latrinerne (30°C). Dette hang sandsynligvis sammen med et højere vandindhold, lavere iltindhold og lavere mikrobiologisk aktivitet (lav grad af kompostering) i latrin-områderne, som var kraftigt kontamineret med gødning. Vandindholdet var 79% i latrinerne sammenlignet med 43% og 36% i henholdsvis mellem- og hvileområder. Den gennemsnitlige ammoniakkoncentration i mellemområder (2.6 mM) var signifikant højere end i hvileområder (1.9 mM) og latriner (1.0 mM). pH varierede signifikant mellem områder, og prøver fra mellemområder havde generelt højest pH (8.88), fulgt af hvileområder (8.60) og latriner (8.04). Strøelsesmaterialet i alle 3 områder indeholdt *A. suum* æg i alle dybder, dog således at koncentrationen af æg var størst i latrinområder: 4-40 gange flere æg pr. g DM sammenlignet med de andre områder. Det øverste lag af strøelsen indeholdt den højeste koncentration af æg. Kontaminationsniveauet var således signifikant faldende med stigende dybde. En lille andel af æggene i hvileområder (4%) og i mellemområder (0.004%) var fuldt udviklede frem til infektivitet, mens ingen af æggene i latrinerne var fuldt udviklede. *In-vitro* embryonering af æg fra strøelsen afslørede, at kun 5% af æggene i mellemområder var levedygtige, mens det samme gjaldt for 17% og 32% af æggene i henholdvis hvile- og latrinområder. Dette viser, at forholdene I mellemområderne tilsyneladende er ugunstige for ægudvikling, hvilket kan skyldes høje temperaturer kombineret med en høj mikrobiologisk aktivitet og et højere ammoniakniveau. Det blev konkluderet, at dybstrøelse generelt medfører ugunstige forhold for udvikling og overlevelse af størsteparten af de deponerede *A. suum* æg. Imidlertid tyder undersøgelserne også på, at transmission af

infektionen uundgåeligt vil finde sted, da der hele tiden er nogle fuldt udviklede (infektive) æg til stede. Ikke udviklede æg i de dybere områder af dybstrøelsen vil desuden kunne optage/genoptage udviklingen, hvis ikke strøelsen opbevares i lang tid og komposteres eller på anden måde behandles så æggene dræbes.

Det sjette studie (**paper 4**) blev gennemført for at belyse spredning og miljøsmitte med *A. suum* i 5 økologiske svinebesætninger. Fire gange hen over et år blev smitteniveauet i indendørs svinestier (stier til henholdsvis fravænnede grise og større slagtesvin (80-100 kg)) og på markfolde for goldsøer, diegivende søer og fravænnede grise kortlagt, og infektionsniveauet i dyrene blev undersøgt. Tre forskellige områder i stierne (hvile-, mellem- og latrinområder) blev identificeret og den øverste del af strøelsen (ca. 10 cm´s dybde) blev indsamlet i hver sti i besætninger med henholdsvis hyppig udmugning (tyndt strøelseslag; 3 bedrifter) eller dybstrøelse (2 bedrifter). Strøelsen i alle områderne i stierne var kontamineret med spolormæg, og niveauerne var ens i begge staldsystemer, men kontaminationen var generelt væsentligt højere (5-25 gange flere æg pr. g DM) i latrinområder sammenlignet med de andre områder. En signifikant større andel af æggene i hvileområderne (44%) havde påbegyndt udvikling i forhold til kun 33% og 13% i henholdsvis mellem- og latrinområder. I stier med hyppig udmugning fandtes et signifikant højere niveau af æg med larver og infektive æg (henholdsvis 19 og 7 æg pr. g DM) sammenlignet med strøelse fra dybstrøelsesstierne (henholdsvis 2.5 og 0.8 æg pr. g DM) uafhængigt af område. *In vitro* embryonering af æggene fra strøelsen afslørede, at 39-97% var levedygtige. I det udendørs miljø var fravænningsfoldene stærkt kontaminerede med æg sammenlignet med fare- og goldsofoldene. Beregnet på tværs af besætninger var prævalenserne i fravænnede grise, slagtesvin, goldsøer og lakterende søer på henholdsvis 48%, 64%, 28% and 15%. Det blev konkluderet, at grise i økologiske besætninger med stor sandsynlighed bliver inficeret på farefoldene før fravænning (og på de separate folde til fravænnede, hvor disse bruges). Grise i alle aldersgrupper er eksponerede for infektive *A. suum* æg, og hvileområderne i stierne udgør tilsyneladende det mest gunstige miljø for udvikling af æg. Dybstrøelse indebærer sandsynligvis ikke en øget risiko for spredning i forhold til systemer med hyppig udmugning og mindre strøelse. Ormebehandling af inficerede fravænnede grise i forbindelse med flytning til indendørs stier i kombination med omhyggelig rengøring af stier mellem hold af grise vil minimere indendørs spredning af *A. suum*.

Dette Ph.D. projekt har bidraget med følgende væsentlige nye fund:

i. Levende og varme-inaktiverede *A. suum* æg kan differentieres ved brug af fluorogen vital-farvning.

ii. Ti måneders oplagring af almindelig gylle og separeret, flydende gylle ved 25°C resulterede i total inaktivering af *A. suum* æg. Ved 5°C var en stor andel af æggene derimod fortsat levedygtige efter 10 måneder.

iii. *Ascaridia galli* æg er mere følsomme for ugunstige forhold i miljøet sammenlignet med *A. suum* og kan derfor ikke umiddelbart anbefales som en alternativ hygiejne indikator. Tilsætning af urinstof (urea) medførte, en væsentlig hurtigere inaktivering af *A. suum* og *A. galli* æg, og at den nødvendige opbevaringstid blev tilsvarende kortere.

iv. Dybstrøelse medfører ugunstige forhold for udvikling og levedygtighed for hovedparten af deponerede *A. suum* æg. Æg i strøelsen, der forsat er levende, kan dog optage/genoptage udviklingen, hvis forholdene bliver mere gunstige. Det er derfor væsentligt, at strøelsen komposteres eller behandles på anden måde, der medfører en inaktivering af æggene.

v. I økologiske produktionssystemer var folde til fravænnede grise stærkt kontaminerede med spolormæg, hvilket viser, at fravænnede allerede på dette tidspunkt er inficerede og eksponerede. Rotation af folde med 1-3 årigt intervaller vil med stor sandsynlighed ikke være nok til at reducere *A. suum* transmission.

vi. Systemer med dybstrøelse udgør tilsyneladende ikke en yderligere risiko for smitte med spolorm set i forhold til systemer med hyppig udmugning og mere begrænset anvendelse af strøelse.

Chapter 1: Introduction and objectives of the study

1.1 Introduction

Ascaris suum, commonly known as the large roundworm of pigs, infects both domestic and wild pigs and is distributed throughout the world (Greve, 2012). Though *A. suum* infections are present in all kinds of production systems, they are more prevalent in extensive systems, in particular in organic production systems (Nansen and Roepstorff, 1999; Carstensen et al., 2002; Roepstorff et al. 2011). Organic pig farms need to follow certain regulations (e.g. late weaning, access to pastures and outdoor runs, provision of bedding material and restrictions on prophylactic treatment) laid down by IFOAM (2000) and the EU (Commission Regulation (EC) No 889/2008) which may result in increased risk of *A. suum* transmission.

Provision of bedding material is mandatory in organic production systems and generally the amount of bedding material provided varies greatly; a variety of housing systems ranging from scarce bedding (shallow litter) to deep bedding (deep litter) systems are in operation. Though bedding material is considered as a risk factor for helminth transmission (Carstensen et al 2002), little is known about variation in indoor transmission of *A. suum* between different housing systems of organic production.

Ascaris suum infections in pigs may reduce feed efficiency and weight gain, cause inflammation of lungs and liver condemnation, and reduce vaccination efficiency leading to economic losses (reviewed by Thamsborg et al., 2013). The eggs of *A. suum* are resistant to adverse environmental conditions and can survive for up to 15 years (Kransnonos, 1978). The organic production practices combined with the long term survival capacity of *A. suum* eggs in the environment can make it difficult to control *A. suum* infections in organic production systems. Understanding the ecology and transmission dynamics both in-door and out-door may help in designing effective preventive strategies for *A. suum* infections.

Establishment of larger pig units in Denmark and other parts of the world results in large volumes of pig slurry. New technologies have been introduced to reduce the large volumes of slurry by separation into solid and liquid fractions (Møller et al., 2000). Pathogens in the farm wastes can be inactivated by storage or treatment of farm wastes and several studies have described pathogen (including parasites) inactivation during storage of raw slurry and solid fraction (Ziemer et al., 2010; Bui et al., 2011; Viancelli et al., 2012) but little is known about pathogen survival in the separated liquid fraction.

Eggs of *A. suum* are used as a conservative indicator of inactivation of enteric pathogens in animal manure or sludge during treatment as they are very common and resistant to adverse environmental conditions (Holmqvist and Stenstrom, 2001). Eggs of the poultry ascarid, *Ascaridia galli,* are also considered to be resistant (Anderson, 1992) and ubiquitous (Ramadan and Abouznada, 1992; Skallerup et al., 2005; Dehlawi, 2007; Kaufmann et al. 2011) and thus available in many places where *A. suum* eggs are not available. Though *A. galli* eggs have been used once in sanitation studies (Tønner-Klank et al., 2007), their suitability as a conservative indicator of survival of enteric pathogens has never been studied in detailed.

Hitherto the viability of *A. suum* eggs has been assessed by incubating un-embryonated eggs, and the eggs that reached the fully larvated stage upon incubation were considered as viable (Pecson et al. 2007; Nordin et al. 2009; Mun et al. 2012). Incubation is, however, a time consuming process and cannot be used as a viability test for already larvated eggs. Few studies have reported that the physiological status of *A. suum* eggs can be rapidly assessed by vital staining techniques (Kagei, 1982; Hindiyeh, 1995; de Victorica and Galván, 2003), but viability assessment using conventional vital stains may not be reliable (Kaneshiro and Stern, 1985). Egg hatch assays may also be used for assessing the viability of already larvated (embryonated) eggs. But hitherto described hatching techniques (Fairbairn, 1961; Jaskoski and Colucci, 1964; Han et al., 2000) are either not reproducible or complicated and there is a need to optimise the existing techniques.

The present Ph.D. study aimed to develop a reliable viability test for *A. suum* eggs (**Staining study** and **hatching study**); to investigate the survival of *A. suum* eggs in different fractions of slurry during storage (**Paper 1**); to compare the survival of *A. suum* and *A. galli* eggs in pig slurry at different levels of ammonia and temperature (**Paper 2**); to characterise physico-chemical conditions of bedding material and development and survival of *A. suum* eggs at various locations in a deep litter housing system (**Paper 3**)and to understand the transmission of *A. suum* infections in organic farms (**Paper 4**);

1.2 Objectives of the study

The general objective of this present study was to investigate the ecology and transmission of *A. suum* infections in organic pig farms, including determination of conditions under which *A. suum* eggs are inactivated.

The specific objectives were:

i. To investigate whether the fluorogenic vital stains namely, 4',6 diamidino-2-phenylindole (DAPI) and propidium iodide (PI) can be used to distinguish viable and non-viable *A. suum* eggs and to optimize existing hatching techniques.

ii. To investigate the survival of *A. suum* eggs in raw slurry in comparison with separated liquid slurry stored at two different temperatures over a 10-months period.

iii. To compare the survival of eggs *A. suum* and *A. galli* in pig slurry at different ammonia levels and temperatures, and to investigate whether time needed for inactivation can be reduced by adding urea.

iv. To characterize the temporal physico-chemical parameters prevailing and the development and viability of *A. suum* eggs in different depths of deep litter bedding material in an organic pig farm

v. To study the environmental contamination and transmission of *A. suum* eggs in organic pig farms.

Chapter 2: Background

2.1 Parasitic infections in pigs in relation to production systems

There are a variety of swine production systems with regard to management practices (e.g. intensive indoor, free range and organic) and occurrence of helminth infections in pigs greatly varies with the type of production system (Roepstorff and Nansen, 1994). Intensive indoor production systems are characterized by better hygiene, confinement of pigs indoors and regular use of anthelmintics resulting in eradication of some helminth species and decrease in prevalence and intensity of infection of remaining species (Dangolla et al., 1996; Nansen and Roepstorff, 1999). Over the years the number of pigs raised in organic (which needs to follow certain standards as described in the following paragraph) and free range production systems, though very small compared to conventional production systems, is increasing due to changes in consumer's preferences towards meat products (e.g. better taste, animal welfare, chemical residues in meat). These systems are characterized by poor hygiene, access to outdoor areas which may result in re-emergence of some of the helminth species (e.g. *Toxoplasma gondii*) that are eradicated in intensive indoor production systems (Kijlstra et al., 2004).

Ascaris suum is the most common helminth parasite, though prevalence and intensity of infection vary, in all types of production systems in Denmark (Roepstorff et al., 1992; Carstensen et al., 2002). *A. suum* infections in pigs may reduce feed efficiency and weight gain, cause inflammation of lungs and liver condemnation, and reduce vaccination efficiency leading to economic losses (reviewed by Thamsborg et al., 2013).

2.1.1 Danish organic pig production systems

Organic pig production in Europe must meet the basic principles of the International Federation of Organic Agricultural Movements (IFOAM, 2000). In addition, it should also meet the rules laid down in Commission Regulation (EC) No 889/2008 of 5 September 2008 and respective national legislation with regard to management practices like housing, feeding etc. Each individual nation in Europe has its own certification body whose specifications vary between countries resulting in different organic practices. Some of the existing regulations in Denmark with regard to management are; (1) farrowing sows and piglets should be kept on pastures throughout the year and pregnant sows should be kept on pastures for a minimum of 150 days; (2) the minimum age of piglets at weaning is 7 weeks; (3) pregnant sows, starter pigs (3-4 months age) and finisher pigs (5-6 months age) if kept indoors should be provided with outdoor runs; (4) restriction on preventive medications; (5) provision of bedding material; (6) provision of sprinklers (or wallows on

pasture) for temperature control ; and (7) use of organic feed and provision of roughage in the diet (Anonymous, 2010; Barbara et al., 2011).

2.2 The pig round worm – *Ascaris suum*

2.2.1 Life cycle

Ascaris suum has a direct life cycle and pigs become infected via a faecal-oral route (Roepstorff and Nansen, 1998). The adult worms live in the anterior part of small intestine and each female worm produces up to 2 million eggs per day which enter the environment with faeces (Olsen et al., 1958). Eggs develop from an undifferentiated single cell stage to the multicellular third larval stage (L_3) in the environment under suitable conditions of temperature and moisture (Seamster, 1950). Pigs get infected when they ingest eggs with L_3 inside (infective eggs). Eggs hatch in the small intestine liberating the L_3 which enters the mucosa of the caecum and colon within 24 h and migrate to the liver within 4 days (Murrell et al., 1997). Migration of L_3 in the liver tissue results in pathological lesions commonly known as liver white spots or milk spots (Roneus, 1966). Within 6-8 days post infection, L_3 travel to the lungs through the blood stream and penetrate the capillaries to enter the alveoli after which they pass through the respiratory tract and reach the pharynx. The larvae are then swallowed and reach the small intestine where most of the larvae are expelled (Roepstorff et al. 1997) and the remaining larvae moult twice to L_4 (size: 17-19 mm) (Douvres et al. 1969) and L_5 (size: 22-36 mm) (Pilitt et al. 1981) at around day 10 p.i. and day 23-24 p.i., respectively. L_5 stages finally becomes adults by day 42-49 p.i., and males reach up to 15-25 cm whereas females reach up to 20-35 cm and start producing eggs (Roepstorff et al. 1997). It takes 6-8 weeks from ingestion of infective eggs by pigs to production of eggs by female worms, the so-called pre-patent period (Roepstorff et al. 1997).

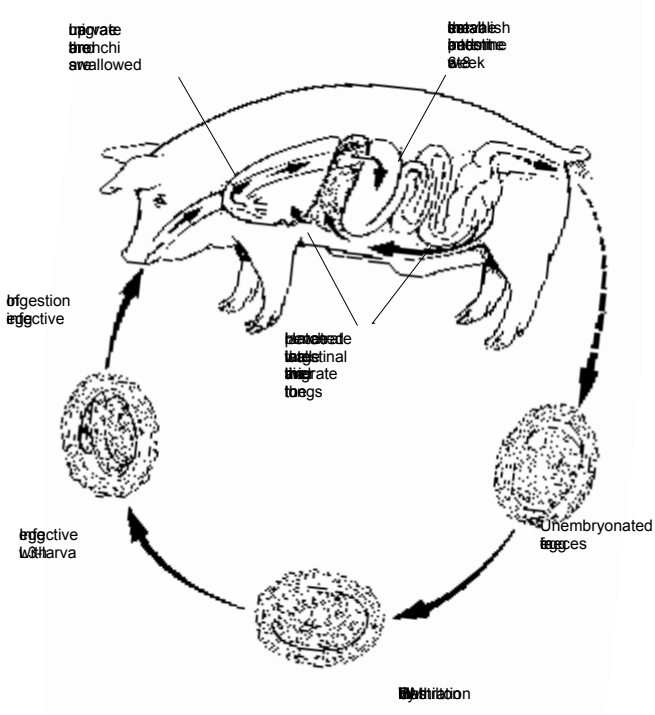

Fig. 2.1 The life cycle of *Ascaris suum* as illustrated by William P. Hamilton (Roepstorff and Nansen, 1998)

2.2.2 Epidemiology

The prevalence of *A. suum* infections depends on various factors like type of production system (organic/indoor/outdoor), management practices (intensive/extensive), age of pigs, season etc. (Thamsborg et al., 2013). Prevalences are generally low in intensive indoor production systems compared to conventional and organic production systems (Roepstorff and Jorsal, 1989; Nansen and Roepstorff, 1999) which might be due to differences in housing and management practices (Roepstorff, 1997). In intensive production systems, slatted floors are used which reduce the risk of exposure of pigs to eggs as the faeces is dropped into underlying pits and bedding material, which is considered as a risk factor for *A. suum* infection (Roepstorff and Nilsson, 1991), is not used. In traditional and organic production systems, pigs are kept either on partially slatted floors or concrete floors and bedding material is provided. Based on faecal egg counts from different age groups of pigs (weaners, fatteners, gilts and sows) in Denmark, Roepstorff (2003) reported prevalences of 10%-50% of pigs in organic farms, 2%-20% of pigs in conventional farms and 0%-15% of pigs in specific pathogen free (SPF) herds.

There is limited age related resistance to *A. suum* infections in pigs (Eriksen et al., 1992b). *Ascaris suum* is moderately immunogenic but repeated exposure of pigs to infective *A. suum* eggs for extended periods of time may result in development of strong immunity (Jungersen, 2002; Miquel et al., 2005), as a consequence of which larvae are expelled from the small intestine either before or after their migration through liver and lungs (Urban et al., 1988; Eriksen et al., 1992a; Helwig and Nansen, 1999; Roepstorff et al., 1997). Generally higher prevalences are observed in younger pigs (starter pigs and finisher pigs) due to a lower level of previous exposure (hence early development of acquired immunity) to *A. suum* infections in conventional and organic production systems (Roepstorff et al., 1992; Roepstorff et al., 1998). Conversely in intensive productions systems low prevalences are observed in younger pigs due to lack of exposures and higher prevalences are observed in older pigs (sows), probably due to first exposure late in life (Roepstorff and Nansen, 1994; Roepstorff et al., 1997). Carstensen et al. (2002) reported *A. suum* prevalence of 28% in weaners, 33% in fatteners and only 4% in sows in Danish organic pig farms and Roepstorff et al. (1998) reported a prevalence of 14% in small fatteners, 21% in fatteners, 13% in dry sows and 10% in lactating sows in Danish conventional farms.

During continuous exposure, already established worms may prevent new larvae from being established (Roepstorff, 2003). Elimination of established worms using anthelmintics may not prevent reinfection if exposed to infective eggs, and often the same pigs are infected as certain pigs are predisposed for *A. suum* infections (Boes et al., 1998; Skallerup et al., 2012).

Under temperate climatic conditions, higher prevalences are observed in late summer and autumn compared to other seasons (Jacobs and Dunn, 1969) as the development of *A. suum* eggs in the environment takes place only during summer (Connan, 1977; Stevenson, 1979).

2.2.3 Characteristics and ecology of eggs

2.2.3.1 Morphology

The eggs of *A. suum* are round to elliptical in shape and measure 56–84 µm × 50–59 µm (Roberts, 1934). The egg shell consists of four layers. The outer mamillated uterine layer consists of muco-polysaccharide and a protein complex and is derived from the uterus of the worm (Wharton, 1980) (Fig. 2.2). The other three layers are derived from the egg. The outer vitelline layer is made up of lipoproteins and the function of this layer is not known. The middle chitinous layer is thick and gives structural strength; damage to of this layer destroys the inner lipid layer and allows harmful chemicals to enter and subsequent inactivation

of the egg (Wharton, 1980). The innermost lipid (ascaroside) layer contains glycolipids and highly impermeable to most of the substances except gases and lipid solvents (Wharton, 1980). The colourless eggs when passing through the intestine of pigs are stabilised by a quinone-tanning process of the outer uterine layer and turn brown and become chemically inert (Wharton, 1980).

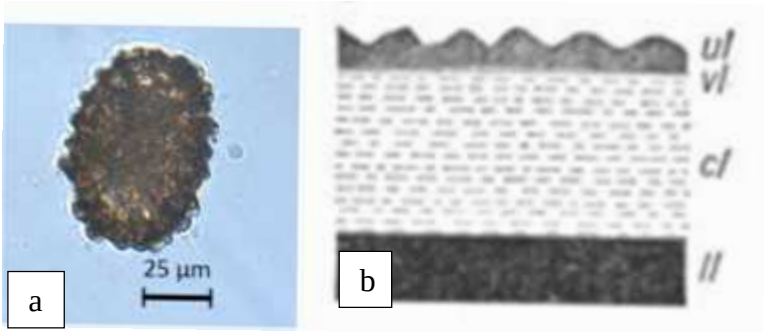

Fig. 2.2 (a) Unembryonated *Ascaris suum* egg. Photo by author. (b) Structure of egg shell of *Ascaris lumbricoides*. Layers: *ul*- uterine, *vl*-vitelline, *cl*-chitinous and *ll*- lipid layer. (Wharton, 1980).

2.2.3.2 Evaluation of egg viability

A number of techniques have been described in the literature for distinguishing viable and non-viable *A. suum* eggs. Some of the techniques include morphological examination (Reimers et al., 1989), dye exclusion test (Meyer et al., 1978; Cacares et al., 1987), incubation technique (Pecson et al. 2007; Nordin et al. 2009;Mun et al. 2011) and infectivity in animal models (Reimers et al., 1989). Using morphological examination, death can be confirmed only if degeneration changes (e.g. vacuolation, cytolysis and shrinkage) are observed, otherwise it is very difficult to distinguish live and dead eggs. Dye exclusion tests are based on the fact that viable cells have intact membranes which exclude certain dyes whereas dead cells have altered membranes which permit the dyes to enter the egg (McCarthy and Evan, 1997). Earlier studies have described some dye exclusion tests for distinguishing viable and non-viable *A. suum* eggs using commonly used vital stains like crystal violet, methylene blue and trypan blue (Kagei, 1982; Shou et al., 1985; Hindiyeh, 1995). Though dye exclusion tests are easy, inexpensive and fast, they may not give accurate and reliable results (Kaneshiro and Stern, 1985). Double staining by two fluorogenic dyes 4', 6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) has been validated and successfully used to distinguish viable and non-viable bacteria and protozoa (Campbell *et al*., 1992). DAPI can enter through both live and dead cells/oocysts whereas PI enters only dead cells/oocysts and $DAPI^{+}/PI^{-}$ and $DAPI^{-}/PI^{-}$

oocysts were considered viable (Jenkins et al., 1997). As DAPI enters both live and dead cells/oocysts, double staining is needed to confirm the live cells/oocysts by observing stained internal structures (e.g. nucleus). At present, most *A. suum* egg inactivation studies use the incubation technique, i.e. the ability of un-embryonated eggs to develop into L_3 larvae upon incubation in a variety of media (H_2SO_4, formalin, distilled water etc.) at 22-28°C as a measure of viability (e.g. Pecson et al. 2007; Nordin et al. 2009; Mun et al. 2011). Though the incubation technique gives accurate results, it takes longer time (up to six weeks depending on temperature) and it cannot be applied to already embryonated eggs. The viability of already embryonated eggs may be assessed by an egg hatch assay. Though some earlier studies have described *in vitro* hatching of *A. suum* eggs (Jaskoski and Colucci, 1964; Han et al., 2000), it is not used as a viability test yet. Infectivity in animal models can be done using *A. suum* naïve pigs. Infectivity of eggs can be monitored by killing the pigs on day 14 p.i. Infectivity testing in animal models is expensive and requires a large number of eggs which may limit its use in routine application (Reimers et al., 1989).

2.2.3.3 Development and survival in the environment

Most *A. suum* eggs (>90%) die within a couple of months after deposition (Kraglund, 1999; Larsen and Roepstorff, 1999) but a considerable number of eggs may still survive and remain infective in the environment for up to 15 years (Kransnonos, 1978). The development of eggs in the environment is mainly influenced by temperature (Seamster, 1950). Development of *A. suum* eggs stops but eggs remain viable for extended periods of time if the temperature is below 14.5°C. Development resumes if the temperature is above this threshold and the rate of egg development increases with increased temperature for up to 37°C and above which the development stops temporarily if exposed for few days (up to 8 days) and permanently if exposed for more than eight days (Seamster, 1950). It takes 37 days at 16.7°C, 28 days at 22°C and 8.3 days at 31.1°C for the eggs to embryonate (i.e. a larva inside) (Seamster, 1950; Oksanen et al., 1990). Newly embryonated eggs presumably need an additional period of time for physiological maturation to become fully infective (Geenen et al., 1999), however, this maturation is poorly defined. Under Danish summer conditions, *A. suum* eggs may develop into the infective stage in 4-6 weeks on pastures (Roepstorff and Murrell, 1997).

Eggs exposed to 38°C may die within 8 days (Seamster, 1950) and above this temperature, the mortality rate increases with increased temperature. Both unembryonated and embryonated eggs are more or less equally susceptible to high temperatures and at 50°C they die within 45 min (Ogata, 1925). Pecson and Nelson (2005) observed a 10% and 70% mortality of eggs within 24 h when incubated at 40°C and 44°C,

respectively, in an ammonia free control solution at a pH of 7. They also observed a 30% and 100% mortality of eggs when incubated for 1 h at 48°C and 52°C.

Development of *A. suum* eggs is independent of the relative humidity (RH) of the surrounding environment but survival of eggs depends on RH and at low RH and high temperature, eggs die at a faster rate due to desiccation (Wharton, 1979). At 76% RH, Wharton, 1979 reported that 100% eggs were collapsed in seven days at 30°C whereas some eggs were alive even after 51 days at 16.5°C.

2.2.3.4 Chemical resistance

The lipid layer of the eggs is impermeable to a wide variety of chemicals making it highly resistant to disinfectants (Wharton, 1979). However some factors like ammonia may reversibly inhibit development of the eggs when these are placed in urine or slurry (Nilsson, 1982). The permeability of the lipid layer may be increased at higher temperatures. Barett (1976) observed a marked increase in the permeability of *A. lumbricoides* eggs to acid fuchsin when incubated for 12 h. Un-ionized ammonia (NH_3) inactivates the eggs, and the inactivation time depends on concentration of NH_3 and temperature (Pecson et al. 2007; Nordin et al 2009). Faster inactivation can be observed at high temperatures and NH_3 levels. Pecson et al. (2007) reported that at 20°C, it took 87 days to inactivate 99% of eggs at 58 mM NH_3 whereas it took only 25 days to achieve the same level of inactivation at 294 mM NH_3. They also reported that at 30°C and 58 mM NH_3, it took only 16 days to inactivate 99% of eggs. A high alkaline pH (>12) in solution also inactivates the eggs when exposed for more than 3 months (Eriksen et al., 1996).

2.3 Pig waste (solid manure, slurry and separated liquid slurry)

Pig farming results in large quantities of pig waste which includes solid manure (bedding material mixed with faeces and urine) and slurry (faeces and urine). In order to reduce the volume of slurry, a variety of slurry separation techniques have been introduced to separate the liquid part of the slurry (= separated liquid slurry) which is then typically used to irrigate the crops. As pig waste contains variable quantities of macro- and micronutrients essential for plant growth and improves physical properties of soil (e.g. enhances nutrient retention, increases water infiltration and improves growth of beneficial organisms) (Zhang, 2002), it is used as a fertilizer in agriculture as an alternative or supplement to chemical fertilizers in many parts of the world. However, pig waste may contain a range of viral, bacterial and parasitic pathogens some of which may be of zoonotic importance like Hepatitis E virus, *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp.,*Yersinia* spp., *Cryptosporidium* spp., and *A. suum* (Guan and Holley,2003;

Hutchison et al. 2005; Ziemer et al. 2010; Bradford et al., 2013) and application of improperly treated manure to agricultural and horticultural crops may contaminate the environment and agricultural products and consumption of raw products (e.g. berries, lettuce) may result in disease occurrence.

Pathogens in pig waste can be inactivated by simple storage, but it takes a long time and inactivation time depends upon various factors like pH, temperature, ammonia content, dry matter, volatile fatty acids, carbon content and nutrient availability (Davis et al. 1999; Nicholson et al. 2005; Nordin et al. 2009). Rapid inactivation of pathogens can be achieved if slurry is subjected to various kinds of treatments like anaerobic digestion, chemical treatment, and ploughing and harrowing of soil immediately after application of slurry (Boes et al., 2005; Bolton et al., 2013; Ottoson et al., 2008). Anaerobic digestion of slurry may not be feasible for small and medium sized farms due to cost constraints, and ploughing and harrowing may not reduce parasitic transmission sufficiently (Mejer and Roepstorff, 2011). Chemical treatment of slurry with ash or lime markedly elevates pH (up to 12) and effectively inactivates pathogens (Eriksen, 1996; Nordin et al., 2009), but to achieve high pH levels large quantities of ash is needed. Addition of ash or lime may reduce the fertilizer value of slurry as at very high pH, all the nitrogen is lost as ammonia gas. Slurry can also be treated with addition of urea (Park and Diez-Gonzalez, 2003; Vinnerås, 2007) or aqueous ammonia (Ottoson et al., 2008; Pecson et al., 2007). Treatment of slurry with urea or aqueous ammonia at low concentrations increases the alkalinity of slurry which favours the equilibrium between NH_4^+ and NH_3 towards NH_3 which is known to have toxic effect on pathogens. Urea treatment is economical and improves the fertilizer value of slurry (Ottoson et al., 2008).

2.3.1 Ammonia production in pig waste

The major source of ammonia in pig waste is rapid degradation of urea in urine into ammonium and carbonate by urease enzyme secreted by faecal bacteria (equation 1) (Mobley and Hausinger, 1989). The other minor source of ammonia is slow degradation of undigested proteins in the slurry during storage (Zeeman, 1991).

$$CO(NH_2)_2 + 3\,H_2O \xrightarrow{\text{Urease}} 2NH_4^+ + HCO_3^- + OH^- \quad \text{(Equation 1)}$$

Total ammonia nitrogen (TAN) is the combination of NH_4^+ and NH_3 which are in constant equilibrium in the aqueous phase (equation 2).

$$NH_4^+{}_{(aq)} \rightleftharpoons NH_{3(aq)} + H^+ \rightleftharpoons NH_{3(gas)} \quad \text{(Equation 2)}$$

pH has a marked influence on the equilibrium (equation 3). Acidic pH favours the equilibrium towards NH_4 and at pH below 7, TAN is largely in NH_4 form and as pH rises above 7, the NH_3 fraction starts to increase and at a pH above 11, TAN is largely in NH_3 from. Temperature influences the equilibrium positively as higher temperature increases the dissociation constant (K_a)(equation 4) which in turn favours NH_3 conversion as shown in equation 3.

$$f = \frac{1}{10^{\,pK_a - pH} + 1} \qquad \text{(Equation 3)}$$

$$pK_a = 0.0901821 + \frac{2729.92}{T_k} \qquad \text{(Equation 4)}$$

T_k= temperature in kelvin degrees (T_k= ^{O}C + 273.2)

$NH_{3\,(aq)}$ is volatile and is converted in to ammonia gas ($NH_{3\,(g)}$) and the conversion is influenced by the concentration of $NH_{3\,(aq)}$ and temperature. Higher levels of $NH_{3\,(aq)}$ and higher temperatures favour volatilization of ammonia (Koerkamp et al., 1998; Monteny and Erisman, 1998).

2.3.2 Toxic effects of ammonia

Most microorganisms can tolerate NH_4^+ even at high concentrations but not NH_3 (Warren, 1962) and several studies have reported the toxic effects of NH_3 on different microorganisms at different concentrations (Ghiglietti et al., 1997;Pecson et al., 2007; Vinnerås, 2007; Ottoson et al., 2008; Nordin et al., 2009; Bolton et al., 2013). Though the actual mechanism of action of ammonia on microorganisms is not known, it is speculated that the smaller size of the NH_3 molecule combined with its solubility both in water and lipids facilitates transportation across cell membranes by simple diffusion. Once it enters the cells, it increases the cytoplasmic pH resulting in an influx of protons to preserve the internal pH which in turn causes the loss of potassium ions vital for cellular mechanisms leading to death of cells (Kadam and Boone, 1996).

2.3.3 Commonly used indicator organisms to measure efficiency of manure treatment

Animal manures need to be treated to reduce pathogen load to acceptable levels before its application as agricultural fertilizers to avoid disease occurrence. It is not practical to assess inactivation of all kinds of pathogens that may be observed in manure due to time and economic constraints. Hence specific indicator organisms can be used to assess the efficacy of a given treatment method in inactivation of pathogens. In order to consider an organism as a good indicator organism, its isolation and quantification methods should

be simple, reliable, definitive and economical (Böhm et al., 1999). Bacteria may not be suitable as indicators of slurry sanitation as they are inactivated very fast and there is a chance of regrowth of some species under favourable conditions (Holmqvist et al., 2002). Viral indicators show some levels of resistance, but current detection methods are not standardized and expensive. Eggs of *A. suum* are therefore generally used as they are ubiquitous and resistant to adverse environmental conditions compared to other enteric pathogens (Holmqvist et al., 2002). Eggs of *A. suum* are also used as indicator organism in human waste as a surrogate to *A. lumbricoides* (Decrey et al., 2011)

2.3.4. *Ascaridia galli* eggs as hygiene indicator

Eggs of the common nematode parasite of poultry *Ascaridia galli* are ubiquitous and can also be found in places where *A. suum* cannot be found due to worldwide consumption of poultry products. The eggs of *A. galli* may remain viable and infective in soil for up to 66 weeks (Farr, 1956). The eggs of *A. galli* are oval and measure 73-92 µm x 45-57 µm (Ackert, 1931). The egg shell consists of three layers, namely an outer albuminous layer, a middle chitinous layer and an inner impermeable lipoid layer (Ackert, 1931; Christenson et al., 1942). These layers might be responsible for long term persistence of eggs in the environment but there is a need for more studies on structure and function these layers, e.g. the exact structure of the inner layer as the previous studies have called this layer as the vitelline layer which in *A. suum* eggs is found as the outer layer (Ackert, 1931).

Fig.2.3 Unembryonated *Ascaridia galli* egg. Photo by author.

Chapter 3: Results and discussion

3.1 Viability testing of *A. suum* eggs

3.1.1 Staining

Three basic vital stains namely Sudan III, Methylene blue and Crystal violet which have been reported to be able to discriminate viable and non-viable *A. suum* eggs (Lillie, 1977; Kagei 1982; Arene 1986), were tested in the **staining study** on live and heat killed (70°C for 20 min) *A. suum* eggs, isolated from pig faeces. No differences in staining pattern between live and heat killed eggs were observed as both kinds of eggs looked similar. It was difficult to decide whether the stain was inside the egg or accumulated on the exterior. This might probably be the reason why basic vital stains have not been used in most of the previous *A. suum* egg viability studies.

Double staining with fluorogenic vital stains DAPI (4', 6-Diamidino-2-Phenylindole) and PI (Propidium Iodide) has been successfully used to distinguish viable and non-viable oocysts of *Cryptosporidium* spp. (Jenkins et al., 1997; Petersen et al, 2012). In the present study, we tested the applicability of double staining to distinguish viable and non-viable *A. suum* eggs. A 10 µl working solution of DAPI (2 mg/mL in absolute methanol) and a 10 µl working solution of PI (1 mg/mL in0.1 M PBS, pH 7.2) were added to a 100-µl egg suspension of either live or heat killed *A. suum* eggs and incubated at 37°C for 3 h. The eggs were then washed with Hank's Balanced Salt Solution (HBSS) and examined using an epifluorescence microscope (Jenkins et al., 1997).

When the eggs were viewed using a green filter block (500-nm excitation, 630-nm emission) for PI (Jenkins et al., 1997), heat killed eggs showed red fluorescence (Fig. 3.1 A)and live eggs did not show any fluorescence. Whereas when eggs were viewed using UV filter block (350-nm excitation, 450-nm emission) for DAPI (Jenkins et al., 1997), heat killed eggs showed blue fluorescence only inside the eggs (Fig. 3.1 B) whereas live eggs showed blue fluorescence all over the eggs (Fig. 3.1 C).

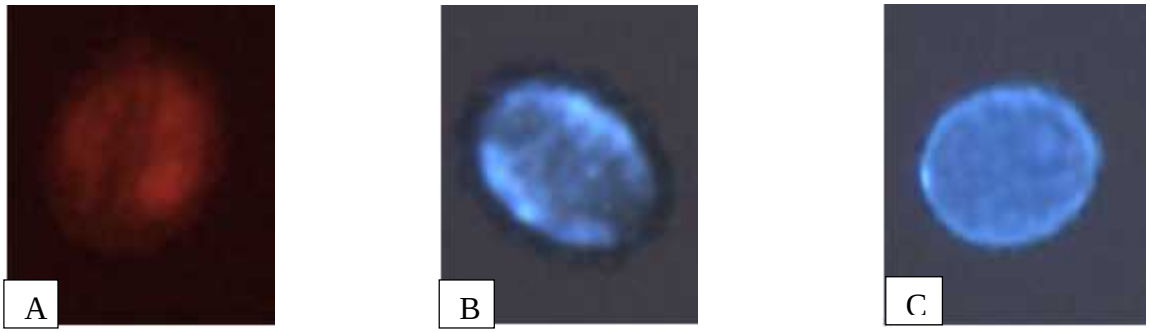

Fig. 3.1. Photomicrographs of (A) heat killed *Ascaris suum* egg stained with PI (Propidium Iodide) showing red fluorescence and (B) heat killed *A. suum* egg stained with DAPI (4', 6-Diamidino-2-Phenylindole) showing blue fluorescence only inside the egg (C) live *A. suum* egg stained with DAPI showing blue fluorescence all over the egg. Photos by Cynthia Dawn Juel.

Heat killed *A. suum* eggs evidently allowed both PI and DAPI stains to enter inside the egg. Live eggs did not permit PI to enter but it was not clear whether live eggs permitted DAPI to enter inside or not as fluorescence was observed all over the egg. Earlier viability studies on cells/oocysts reported that DAPI stain enters both live and dead cells/oocysts. The observed difference between live and heat killed eggs in fluorescence pattern of DAPI might be the presence of microorganisms on the surface of live eggs (fluorescence observed indicating stain passed the microorganisms). Heat might have killed the microorganisms on the surface of heat killed eggs. Fluorogenic vital stains fluoresce when they bind to nucleic acids (DNA and RNA), the property which is absent in basic vital stains and hence the uncertainty of using basic vital stains can be eliminated by using fluorogenic vital stains. The present study gave an indication that fluorogenic vital stains can be used to distinguish viable and non-viable *A. suum* eggs but in order to fully investigate its applicability further studies are needed to examine dead eggs that are killed by factors other than heat (e.g. ammonia, desiccation). As the time allotted for this study was limited we had to stop further investigation on this topic.

3.1.2 Hatching

Earlier described methods for *A. suum* egg hatching (Jaskoski and Colucci, 1964; Eriksen, 1981; Urban et al., 1981; Han et al., 2000) were tested in our laboratory but could not be reproduced consistently. Hence some of the earlier described methods (Jaskoski and Colucci, 1964; Han et al., 2000) were modified by mimicking the environment that *A. suum* eggs are exposed to in the small intestine of pigs by adding bile, creating anaerobic conditions and applying shaking.

Three separate batches of *A. suum* eggs were prepared by isolating eggs from faeces of three different pigs and embryonating in 1N H_2SO_4 (pH 1) at 22°C for six weeks. The eggs were de-coated with 2% NaOCl and washed thrice with normal saline and an egg suspension with a concentration of 5 eggs/ µl was prepared. Four separate hatching trials were conducted on these three egg batches (2-3 replicates) by adding 100 µl of egg suspension and 50 µl pig bile to 850 µl normal saline in a well in 24 wells cell culture plate. The plate was kept in a plastic box and filled with CO_2gas. Shaking was applied (150 rpm) by keeping the box on a mechanical shaker in an incubator at 38.5°C for around 16-17 h.

We only partially succeeded in hatching the eggs as our results were highly inconsistent. We observed a large variation in hatching percentage between different trials, different eggs batches and different replicates of same egg batch (Table 3.1). Even after several trials (data not shown) we could not improve the consistency of the results. The highly inconsistent results obtained indicate that in addition to the conditions provided in the current hatching technique, eggs need an additional factor to stimulate hatching. Hayes et al. (2010) showed that *in vitro* hatching of *Trichuris muris* requires an interaction between the egg and bacteria that are commonly present in mouse caecum (e.g. *Escherichia coli, Staphylococcus aureus*). They have also reported that a structural component of bacteria is responsible for inducing the hatching. We speculate that the observed difference in hatching percentage between different egg batches, different replicates of a same batch and different trials might be presence of unknown bacteria on successful occasions of hatching.

Table 3.1. Mean hatching percentage of different batches of *Ascaris suum* eggs in four repeated trials. Batches represent different sources of eggs. The eggs in batch 1 and 2 were faecal eggs isolated from different pigs whereas batch 3 were uterine eggs collected from uterus of various adult *A. suum* worms pooled together. Values shown in parentheses are individual hatching percentages for each replicate.

	Trial 1	Trial 2	Trial 3	Trial 4
Batch1	89	80	61	56
	(93,88,87)	(88,71)	(55,70,60)	(52,61)
Batch2	90	13	28	12
	(91,100,80)	(23,4)	(44,34,5)	(1,23)
Batch3	29	12	Not tested	Not tested
	(0,0,86)	(0,24)		

3.1.2 Embryonation

In the present study (**Papers 1-4**), *A. suum* eggs were isolated from fresh faeces and embryonated in H2SO4 buffer (pH 1) for six weeks at 22°C and eggs that contained fully developed larva were considered as viable eggs. The viability of these fresh eggs (control) was taken as the golden standard and the viability of *A. suum* eggs in the field samples was assessed through comparison with the control eggs. Viability of control *A. suum* eggs in the present study was 96%.

3.2 Distribution, ecology, development and survival of *A. suum* eggs

3.2.1 On pastures

In the present study (**Paper 4**), not all farms kept starter pigs on pastures and in farms where the starter pigs were kept on pastures, stocking density was very high compared to sows. In general, few sows were infected with *A. suum* and excreted few eggs compared to starter pigs. As a result starter pig pastures were more heavily contaminated ($P<0.0001$) compared to dry and farrowing pastures (Fig. 3.2). Highest contamination of pastures was observed in December 2011 and infective eggs were found during all sampling times.

Despite high faecal egg excretions in September 2011, the observed higher pasture contamination in December 2011 compared to September 2011 might be due to the time needed for dispersion of eggs or degradation of most of the eggs as more eggs die during summer and autumn than winter and spring (Larsen and Roepstorff, 1999). On pastures, many factors like temperature, vegetation, sun light and/or shade, UV radiation and desiccation (Kraglund et al., 1999; Larsen and Roepstorff, 1999; Roepstorff and Murrell, 2001) may influence the development and survival of eggs. *In vitro* studies have reported that biotic factors like nematophagous fungi (e.g. *Pochonia chlamydosporia*) also influence the survival of helminth eggs (Lýsek and Krajcí, 1987; Araújo et al., 2008; Thapa et al., unpublished), however the effect of nematophagous fungi on helminth eggs on pastures have been poorly described.

Development and survival of eggs depend mainly on temperature, and eggs can only develop within a temperature range of 14.5°C to 34.4°C (Seamster, 1950). Although temperatures above 14.5°C can be observed mainly during summer and early autumn, fully developed eggs were observed in all sampling times indicating that some eggs survive from previous years. Temperatures below 14.5°C temporarily stop development of eggs but eggs can stay alive even at freezing temperatures up to -27°C for 30 days and restore development if kept at temperature above 14.5°C (Cram, 1924; Seamster, 1950). However, fully

developed eggs can tolerate extreme low temperatures of -27°C for only 10 days (Cram, 1924). As the average minimum temperatures in peak winter (February) in Denmark never go below -2°C (Danish Meteorological Institute, DMI), any damage to the eggs due to low temperatures may be minimal under Danish winter conditions. However average minimum temperature may not reflect the periods with extreme cold weather.

Direct sunlight may influence the survival of *A. suum* eggs as it increases the temperature of and dries out soil or faecal clumps. Earlier studies have shown that vegetation (long grass cover) or shade protects the free living stages of helminths compared to that of no vegetation, short grass or un-shade areas of soil (Kraglund, 1999; Larsen and Roepstorff, 1999). Probably shade protects eggs or larvae from UV light, heat and drying. Larsen and Roepstorff (1999) recorded high temperatures (up to 51.1°C) in the centre of faecal clumps under Danish summer conditions. These focally high temperatures may be responsible for die-off during summer (Burden and Ginnivan, 1978; Barnard et al., 1987).

3.2.2 In pens

In **Paper 4**, it was shown that the entire indoor area in both starter and finisher pig pens were contaminated with *A. suum* eggs at all farms (Fig.3.2). Inside pens, three different areas namely clean resting, dirty latrine (bedding mostly containing faeces and urine) and an area which was in between these areas (intermediate area; bedding moderately containing faeces and urine) were identified (**paper 3** and **4**). Latrine areas contained the most eggs ($P<0.0001$) as faeces was mostly located in this area followed by the adjacent intermediate area and distant resting area. Eggs of *A. suum* may be very sticky (Gaspard et al., 1994) and movement of pigs all over pens might have resulted in spreading of eggs via the legs. Contamination of bedding material with eggs during different sampling times reflects the egg excretion pattern of pigs and the highest contamination was observed in September 2011 ($P=0.0015$) followed by December 2011, June 2012 and March 2012. The highest egg contamination was observed in finisher pig pens ($P=0.0015$) compared to starter pig pens. Deworming of pigs on some occasions drastically reduced the contamination of pens on subsequent sampling dates.

Inside pens, eggs were exposed to different physico-chemical conditions in different areas (**Paper 3 and 4**). The temperature of the bedding material in shallow litter housing system varied according to the ambient temperature (personal observation and not recorded) whereas bedding material in deep litter housing system was generally warmer, a minimum of 19°C and a maximum of 69°C temperature was observed **(Paper 3)** and it varied greatly between areas (highest in intermediate areas), depths (highest in top layer)

and sampling times (highest in September 2011). The highest moisture content (P<0.0001) of the bedding material was observed in latrine areas (79%) followed by intermediate areas (43%) and resting areas (36%). Bedding material in the intermediate areas had the highest aqueous ammonia ($NH_{3\,(aq)}$) concentration (2.6 mM) followed by that in resting (1.5 mM) and latrine areas (1.0 mM). Though $NH_{3\,(aq)}$ concentration of bedding material in shallow litter housing was not measured one can assume latrine and intermediate areas would have higher concentrations compared to resting areas as they contained faeces and urine (Ni et al., 1999). pH of bedding material in shallow housing systems was not measured. In deep litter housing systems, intermediate areas had the highest pH (8.88) (P<0.0001) followed by resting (8.60) and latrine (8.04) areas.

As described in **paper 4**, irrespective of housing system, the highest percentage of eggs that had started developing were found in resting areas (44%), followed by intermediate (33%) and latrine areas (13%) in top 10 cm of bedding material, indicating that resting areas provide more favourable conditions for the development of eggs compared to the other two areas. However, in terms of absolute numbers, intermediate and latrine areas had higher number of infective eggs (Fig. 3.2). Egg development was more pronounced in September 2011 compared to the other sampling times. This might be due to higher temperatures prior to the sampling in September 2011 as eggs start developing only above 14.5°C (Seamster, 1950). Similarly, Connan (1977) and Stevenson (1979) reported development of eggs in summer and autumn seasons compared to other seasons by *in vitro* embryonation of eggs on farms in temperate regions. In the present study, though the levels varied, eggs at different developmental stages were observed in all the seasons indicating that pens were poorly cleaned between each new batch of pigs resulting in persistence of previously developed eggs or eggs might be using body heat of the pigs when they lie on the bedding. Though eggs started developing in both litter types, significantly higher number of eggs reached the larvated stage (5.1%; 19 eggs/ g dry litter) and infective stage (1.8%; 6.7eggs/ g dry straw) compared to deep litter (larvated stage: 0.3%; 2.5 eggs/ g dry straw and infective stage: 0.1%; 0.8 egg/g dry straw) indicating that deep litter systems provide unfavourable conditions for complete development of eggs. *In vitro* embryonation of eggs revealed that a considerable proportion of eggs (>31%) in all areas in both systems were viable even though they had not completed development in the litter. Our study revealed that deep litter systems which are thought to increase the risk of parasitic infections compared to shallow litter systems actually may not create any additional risk for egg transmission.

In **paper 3**, it was shown that in deep litter housing, in all layers of bedding material, the highest percentage of eggs that had started developing were found in resting areas (21%) compared to intermediate (15%) and

latrine areas (9%). However, the percentage of eggs that reached the larvated stage was very low, and in resting areas it was 4%, in intermediate areas it was 0.04% and in latrine areas none of the eggs reached larvared stage. *In vitro* embryonation revealed that only 5% of eggs in intermediate areas were viable where it was 17% and 32% in resting and latrine areas indicating that intermediate areas create highly unfavourable conditions for the eggs.

3.2.3 In slurry

In the studies reported in **paper 1** and **2**, it is evident that pH and ammonia content of pig slurry varies. Feed ingredients influences the composition of pig slurry and high protein diets results in high pH and ammonia levels (Canh et al., 1998a; Le et al., 2007) and high fermentable carbohydrate diets result in low pH and ammonia levels (Canh et al., 1998a; Canh et al., 1998b; Paul and Beauchamp, 1989; Le et al., 2007). The low initial pH of slurry in **paper 2** (6.46) might be due to high cereal diet while the feed composition of pigs in **paper 1** (initial pH: 7.98) was unknown. pH influences the equilibrium between NH_4^+ and NH_3 and the higher the pH the more NH_4^+ is converted in to NH_3 (Philippe et al., 2011). This may be the reason for the difference in NH_3 concentration of slurry in **paper 1** (8.7mM) and **paper 2** (0.01 mM). Rate of inactivation of eggs in study 1 and 2 are comparable despite the fact that ammonia levels were substantially different. This might be due to the presence of other inhibitory factors like volatile fatty acids which are also known to have inhibiting effect on certain bacteria and viruses (Kunte et al., 1998). NH_3 alters the membrane potential and increases the cytoplasmic pH of microbes resulting in loss of potassium ions (Kadam and Boone, 1996). Similarly, *A. suum* eggs temporarily stop development if exposed to urine and faeces due to the presence of NH_3 (Nilsson 1982). Exposure of eggs to slurry or urine for extended periods permanently inactivates the eggs and time needed for inactivation depends upon NH_3 concentration and temperature (Pecson et al., 2007; Nordin et al., 2009). In **paper 1** and **2**, results revealed that none of the eggs showed any development during storage of slurry. In **paper 1**and **2**, it was also shown that temperature of slurry influenced the time needed for inactivation of eggs during storage and higher temperatures markedly reduced storage time needed for similar level of inactivation. The T_{50} for *A. suum* eggs in pig slurry was 519 days, 242 days, 42 days and 0.08 days at 5°C, 25°C, 30°C and 50°C, respectively.

3.2.4 Comparative evaluation of *Ascarid* eggs as indicator organisms

In **paper 2**, it was shown that T_{50} of *A. galli* eggs was lower at all temperatures both in urea-treated and untreated slurry (Table 3.2) when compared to that of *A. suum* eggs, indicating that *A. galli* eggs are more sensitive and could not be used as conservative hygiene indicator for slurry sanitation. The high sensitivity

of *A. galli* eggs compared to that of *A. suum* eggs might be due to differences in the morphological structure of egg shells. *Ascaridia galli* eggs contain only three layers, an outer proteinaceous layer, a middle chitinous layer and an inner lipoid layer (Ackert, 1931 and Christenson et al., 1942). Whereas *A. suum* eggs contain four layers, an outer proteinaceous layer (uterine-derived), a vitelline layer, a chitinous layer and an innermost lipid layer (Wharton, 1980). Probably the absence of the extra layer in *A. galli* eggs makes them more susceptible to unfavourable conditions compared to *A. suum* eggs.

Table 3.2. Estimated T_{50} (days with 95% lower and upper confidence intervals) of *Ascaris suum* and *Ascaridia galli* eggs at different temperatures in pig slurry with and without urea.

	Urea		No urea	
Temperature	*A. suum*	*A. galli*	*A. suum*	*A. galli*
20°C	42.3 (40.0-44.5)	14.1 (9.5-18.7)	242.6 (167.6-317.6)	24.8(16.7-32.9)
30°C	21.8 (17.2-26.4)	1.22 (0.86-1.58)	41.6 (29.4-53.8)	3.81(3.61-4.01)
40°C	0.83 (0.67-0.99)	0.047(0.028-0.066)	.	0.42(0.32-0.51)
50°C	0.06 (0.05-0.06)	0.009(0.007-0.010)	0.08 (0.07-0.08)	0.039(0.03-0.04)

3.3 Egg transmission

In **Paper 4,** it was shown that the environment in both outdoor (farrowing pastures and weaning pastures) and indoor areas (starter/weaner pens and finisher pens) of organic pig production systems was contaminated with infective *A. suum* eggs (Fig. 3.2) indicating that pigs at all stages of production are at risk of exposure to *A. suum* infections.

3.3.1 On pastures

In **paper 4**, it was shown that 48% of the starter pigs excreted *A. suum* eggs indicating that transmission took place early in their life on farrowing paddocks as the pre-patent period for *A. suum* is 6-8 weeks (Roepstorff et al. 1997). Piglets can get infected with *A. suum* even during the first 1-3 weeks of life due to ingestion of soil on farrowing paddocks and they keep on getting infected until weaning at 7-8 weeks of age if they remain in a contaminated environment (Mejer and Roepstorff, 2006). As there is no age related resistance against *A. suum* infections (Eriksen et al., 1992b) and a similar risk of becoming infected on pastures for both piglets and sows on farrowing paddocks, the observed lower prevalence and intensity of

infection (low egg output) in lactating sows compared to starter pigs might be due to development of immunity by the sows due to a more extended exposure to infective *A. suum* eggs (Jungersen, 2002; Miquel et al., 2005). Maternal exposure to *A. suum* infection may affect the distribution of *A. suum* in piglets (Boes et al 1999), probably due to transfer of maternal antibodies against *A. suum* to piglets (Kelly and Nayak, 1965) but this may not protect against infections in case of prolonged exposure (Mejer and Roepstorff, 2006). **In paper 4**, it was shown that infective eggs were present on weaning /starter pig pastures, indicating that all the farm land was contaminated and that the pigs were continuously exposed to *A. suum* eggs while on pasture.

Earlier experimental studies indicate that pasture rotation may reduce transmission rate of helminth eggs (Larsen and Roepstorff, 1999). But the present results in which pasture rotation was practised for every six months to three years did not show any significant difference in the number of infective eggs indicating possible similar transmission rates in both grazed and un-grazed pastures (**paper 4**). Most likely pasture rotation may not reduce transmission as *A. suum* eggs can survive up to 9 years (Kransnonos, 1978) and remain infective for extended periods of time (more than 7 years) on pastures in Denmark (Mejer et al., 2011).

3.3.2 In pens

The results in **Paper 4** revealed that housing system and sampling time did not influence prevalence of *A. suum* in finishers. The overall prevalence (based on faecal egg counts) of *A. suum* in finisher pigs (64%) was higher than that observed in starter pigs (48%). Prevalences based on liver white spot data obtained in October 2011, which reflects infection during late summer and early autumn, revealed that the prevalence of *A. suum* in finishers in different farms was between 83%-96%. Though there was a difference in number, larvated and infective eggs were observed in both housing systems at all sampling times. Higher prevalences in finishers compared to starters, liver white spots in slaughtered pigs and finding larvated eggs in bedding material (Fig. 3.2) indicate indoor transmission of *A. suum*. However one should be careful in drawing conclusions on indoor transmission based on differences in egg prevalences between finishers and starters. Starters if they become infected late on pastures just before weaning do not excrete eggs until 6-8 weeks after exposure (Roepstorff et al. 1997). Estimation of prevalence during pre-patency will inevitably result in underestimation of transmission rate. Similarly, estimating prevalences based on faecal egg counts may also overestimate transmission as there is higher chance of (up to 36%) false positive results in indoor, group housed pigs due to coprophagia (Boes et al., 1997). Earlier study by Roepstorff (1998) proposed an

arbitrary cut-off value for faecal egg counts (EPG ≤200) to consider as false positive. In study **(paper 4)**, many of the finishers contained EPG values ≤200 and overall prevalence declined to 33% if this criterion for false positive results were taken in to account.

On the other hand one should keep in mind that prevalence (based on faecal egg counts) and worm burdens do not reveal actual transmission rates as only few larvae can establish an infection and become adult worms (Roepstorff and Murrel, 1997; Roepstorff et al., 1997) either due to development of immunity (Miquel et al., 2005) or due to genetic predisposition of host (Boes et al., 1998; Skallerup et al., 2012). Prevalence based on liver white spots may also not reveal actual transmission rates as pigs that are previously exposed to *A. suum* infections may contain few or no liver spots due to development of strong protective immunity resulting in reduction of migration of larvae through the liver (Eriksen et al., 1992a).

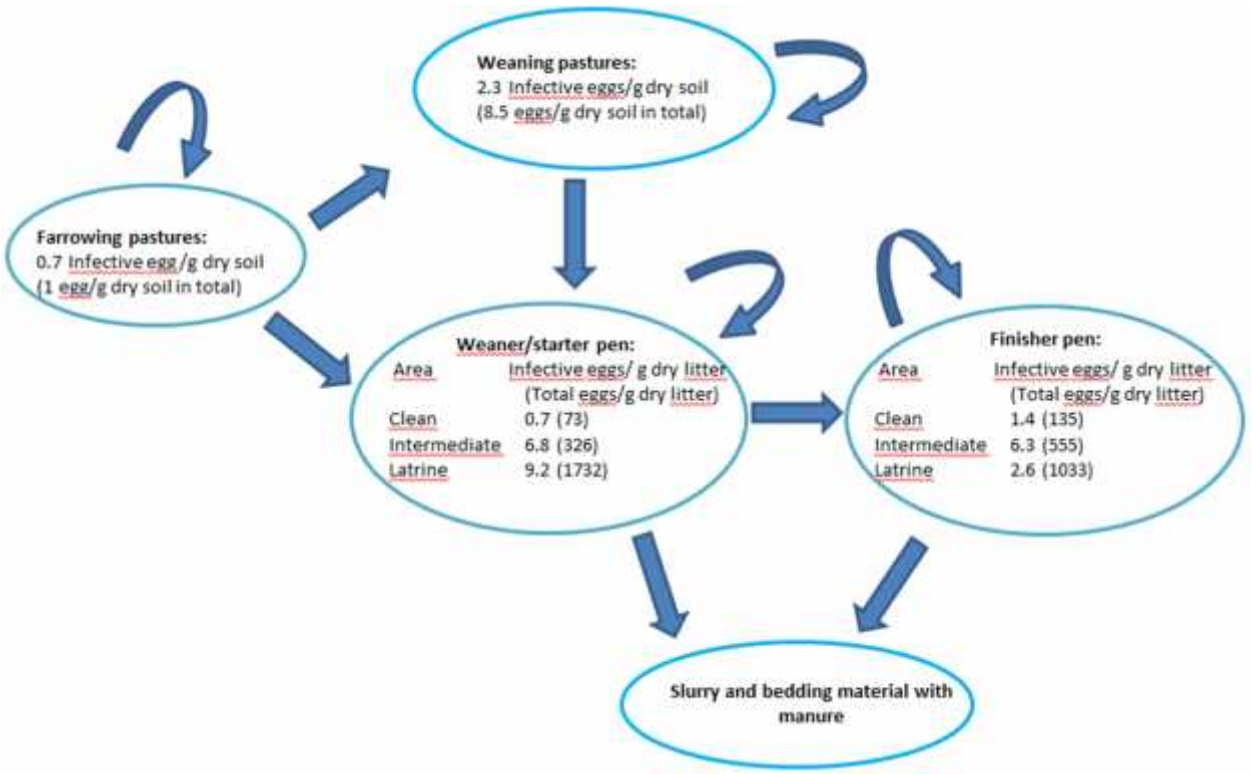

Fig.3.2 Schematic generalized presentation of overall contamination pattern of infective and total *Ascaris suum* eggs in farrowing pastures, weaning pastures, starter pig pens and finisher pig pens and possible transmission routes for *A. suum* infection in pigs. Values = means of five farms.

Chapter 4: Conclusions and Future perspectives

4.1 Conclusions

- Simple storage of raw pig slurry or separated liquid slurry for shorter periods at temperatures that are commonly observed in temperate geographical regions did not inactivate *A. suum* eggs, and the slurry can potentially contaminate crops with pathogens if applied to fields. At 25°C, it took about 10 months to completely inactivate *A. suum* eggs in both the fractions and at 5°C, 42% and 88%of eggs were still viable after 10 months in raw pig slurry and liquid fraction of the slurry, respectively.

- Treatment of pig slurry (either with urea or high temperature or both) drastically reduced the inactivation time of *A. suum* eggs. Addition of urea (2%) raised pH and $NH_{3\,(aq)}$ levels of slurry which resulted in reduction of inactivation time of *A. suum* by 17-76% depending upon storage temperature. For each 10°C increase in temperature from 20°C to 50°C, a significant decrease in T_{50} (time needed to inactivate 50% of eggs) was observed.

- The results from the studied farm (**paper 3**) indicate that deep litter in general created unfavourable conditions for the development and viability of *A. suum* eggs in most parts of the bedding material. The majority (99.99%) of eggs did not fully embryonate in the litter while in the pen, however, a sizeable proportion of eggs (19%) remained viable and could complete embryonation once removed from the litter and manure.

- Significantly fewer eggs were developed in deep litter systems compared to shallow litter systems. However similar infection levels in the pigs were observed in both types of systems. When compared to shallow litter systems, deep litter systems may not create an additional risk for *A. suum* transmission as presumed earlier.

- Weaning pastures were heavily contaminated with *A. suum* eggs indicating that weaned pigs are mostly at risk of *A. suum* transmission. Grazing rotation for 1-3 year may not be useful in reducing A. suum transmission. Deep litter systems do not pose an additional higher risk of *A. suum* transmission as compared to shallow litter systems.

4.2 Future perspectives

- Our study (**staining study**) shows that fluorogenic vital stains can be used to distinguish between viable and heat killed eggs. Further studies are needed to test these stains in distinguishing viable eggs and non-viable eggs that are inactivated by factors other than heat (e.g. ammonia, desiccation).

- Hatching of *A. suum* eggs is a complex process and assays to assess this in a quantitative way seem to yield inconsistent results and cannot be reproduced. However, a study by Hayes et al. (2010), reported an efficient and a consistent *in vitro* hatching of *T. muris* eggs by incubating the eggs with bacterial suspensions. Future *A. suum* hatching studies need to be planned to include and elucidate the role of bacteria.

- Urea treatment of slurry markedly reduces the time needed for inactivation of *A. suum* eggs but the feasibility of its application in the field needs to be investigated.

- Our study shows that the majority (**paper 3** and **4**) of the eggs in bedding material remain viable and further studies are needed to investigate inactivation of eggs by different treatments (e.g. Composting).

- The indoor egg transmission rate was not assessed directly in the current study (**paper 4**). Introducing *A. suum* naïve pigs inside the pens 1-2 days and killing them at day 14 p.i. may provide good estimates of egg transmission rates.

- With restrictions on preventive use of anthelmintics and limited availability of land for long term pasture rotation (up to 8-9 year), it is probably not possible to prevent transmission of *A. suum.* Alternative control strategies like feeding the pigs with bioactive crops (e.g. chicory roots) and development of vaccines have proved to have limited or no effect on control of *A. suum* transmission. Recently larval antigens were produced in rice plants (transgenic rice plants) and it has been shown that feeding mice with these plants elicited antigen-specific antibody response (Matsumoto et al., 2009; Nozoye et al., 2009). The efficacy of these plants needs to be tested in pigs in reducing *A. suum* transmission.

Chapter 5: References

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Chapter 6: Publications and manuscripts

6.1 Paper 1. Viability of Ascaris suum eggs in stored raw and separated liquid slurry

Kiran Kumar Katakam, Allan Roepstorff, Olga Popovic, Niels C. Kyvsgaard, Stig Milan Thamsborg and Anders Dalsgaard

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Viability of *Ascaris suum* eggs in stored raw and separated liquid slurry

KIRAN KUMAR KATAKAM[1], ALLAN ROEPSTORFF[1], OLGA POPOVIC[2], NIELS C. KYVSGAARD[3], STIG MILAN THAMSBORG[1] *and* ANDERS DALSGAARD[1]*

[1] *Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark*
[2] *Department of Agriculture and Ecology, Faculty of Science, University of Copenhagen, Denmark*
[3] *Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark*

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SUMMARY

Separation of pig slurry into solid and liquid fractions is gaining importance as a way to manage increasing volumes of slurry. In contrast to solid manure and slurry, little is known about pathogen survival in separated liquid slurry. The viability of *Ascaris suum* eggs, a conservative indicator of fecal pollution, and its association with ammonia was investigated in separated liquid slurry in comparison with raw slurry. For this purpose nylon bags with 6000 eggs each were placed in 1 litre bottles containing one of the two fractions for 308 days at 5 °C or 25 °C. Initial analysis of helminth eggs in the separated liquid slurry revealed 47 *Ascaris* eggs per gramme. At 25 °C, egg viability declined to zero with a similar trend in both raw slurry and the separated liquid slurry by day 308, a time when at 5 °C 88% and 42% of the eggs were still viable in separated liquid slurry and raw slurry, respectively. The poorer survival at 25 °C was correlated with high ammonia contents in the range of 7·9–22·4 mM in raw slurry and 7·3–23·2 mM in liquid slurry compared to 3·2–9·5 mM in raw slurry and 2·6–9·5 mM in liquid slurry stored at 5 °C. The study demonstrates that at 5 °C, *A. suum* eggs have a higher viability in separated liquid slurry as compared to raw slurry. The hygiene aspect of this needs to be further investigated when separated liquid slurry is used to fertilize pastures or crops.

Key words: raw slurry, separated liquid slurry, viability, *Ascaris suum* eggs, temperature, ammonia.

INTRODUCTION

Establishment of large-scale intensive pig production systems is taking place in many parts of the world to meet increasing demands for pork. Disposal of the large volumes of pig manure generated, mainly as slurry may result in environmental problems like contamination with various pathogens, bad odour, leaching of nitrates and nutrient losses following land application (Watabe *et al*. 2003; Vera *et al*. 2004). Recently, several techniques have been developed for chemical and mechanical separation of slurry into solid and liquid fractions to minimize nutrient losses and to reduce the odour and volume of the slurry. The solid fraction is rich in nutrients and can after storage or various treatments safely be used as fertilizer, e.g. on agricultural land and in horticulture, whereas the liquid fraction is typically used to irrigate field crops and is less polluting compared to the raw slurry because of lower contents of nitrogen and phosphorous (Møller *et al*. 2000; Fangueiro *et al*. 2008).

Pig slurry may contain a variety of viral, bacterial and parasitic pathogens, some of which are zoonotic, e.g. Hepatitis E virus, *Salmonella* spp., *Campylobacter* spp., *Giardia duodenalis*, *Cryptosporidium* spp. and *Ascaris suum* (Hutchison *et al*. 2005; Ziemer *et al*. 2010), and these may potentially contaminate the external environment when applied to fields without any pre-treatment. Survival of pathogens declines when the slurry is stored, depending upon storage conditions and resultant physico-chemical factors like temperature, pH, oxygen tension (aeration), dry matter, unionized ammonia and volatile fatty acids (Ajariyakhajorn *et al*. 1997; Davis *et al*. 1999; Himathongkham and Riemann, 1999; Nicholson *et al*. 2005; Nordin *et al*. 2009). *Ascaris suum* eggs loose the viability if they are exposed to a pH > 12 for more than 3 months (Eriksen *et al*. 1996) or to unionized ammonia at a concentration ~58 mM for 16 days and for 4·8 days at 30 °C at a pH of 12 (Pecson *et al*. 2007). Bacterial pathogens like *Escherichia coli* and *Salmonella* spp. can survive up to 3 months in slurry stored below 20 °C (Pell, 1997; Hutchison *et al*. 2005; Nicholson *et al*. 2005), while 74%, 64% and only 4% of *A. suum* eggs were found to survive in slurry at 4 °C, at room temperature (17–26 °C) and at 42 °C, respectively (Papajova *et al*. 2005).

Compared to raw slurry little is known about the viability of pathogens in the liquid slurry fraction obtained from mechanical and/or chemical separation of raw slurry. Due to increased attention to the

* Corresponding author: Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 15, DK-1870 Frederiksberg C, Denmark. Tel: +45 35332720. Fax: +45 35332757. E-mail: adal@sund.ku.dk

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food-borne diseases and requirement to regulate environmental pollution worldwide, there is a need to assess the food safety and environmental hazards associated with the irrigation of agricultural fields with separated liquid slurry. This should be done by investigating the main chemical factors determining pathogen inactivation like ammonia and pH. *Ascaris suum* eggs are extremely resistant to environmental stress (Roepstorff and Murrell, 1997) and hence viability of *A. suum* eggs is used as a conservative indicator and to assess sanitization of fecally contaminated liquid and solid wastes (O'Lorcain and Holland, 2000). The aim of the present study was to investigate the survival of *A. suum* eggs in raw slurry in comparison with separated liquid slurry stored at 5 °C and 25 °C during a 10-month period.

MATERIALS AND METHODS

Ascaris suum *eggs and slurry*

Ascaris suum eggs were obtained by sequential sieving of feces collected from the large intestine of naturally infected pigs at a Danish slaughterhouse. An egg suspension was prepared by adding deionized water to make a final concentration of 6 eggs/μl of suspension. In total, 180 nylon bags (size 2·5 × 2·5 cm^2) each containing 6000 un-embryonated eggs were prepared by placing 1 ml of egg suspension into nylon bags with mesh size 20 μm (Verseidag-Techfab Gmbh, Geldern, Germany) and then closing the bags with glue. The bags were stored in deionized water at 5 °C until use.

Raw pig slurry and separated liquid slurry were collected from a commercial pig farm where the slurry was separated into solid and liquid fractions by flocculation followed by mechanical separation (Kemira AgroSol™, Kemira Kemwater, Finland). Polyacrylamide polymer (Superfloc C-2260, Kemira Kemwater, Finland) was added so that dissolved and suspended solids were formed into flocs of 2–10 mm size. Flocs were then removed on a belt with 4 mm screen size and further dehydrated by screw press with a 1 mm screen size (Møller *et al*. 2007). Around 150 L of raw slurry was collected from a slurry storage tank connected directly to the animal house, while the same amount of liquid slurry was collected directly from the outlet of the separation unit when operational. Raw slurry and separated liquid slurry were collected in 6 plastic barrels (40–60 L each), transported within 8 h at ambient temperature by car (15–17 °C) to the laboratory and stored overnight at room temperature. After thorough mixing with a rod for 2 min, 700 g samples of raw slurry and separated liquid slurry were transferred into 1-L sterile glass bottles yielding a total of 60 bottles of each slurry type. One nylon bag with *A. suum* eggs was placed in the middle of each bottle by tying it with a thin aluminium wire around the neck of the bottle. The bottle was closed with Parafilm® and fixed with a rubber band creating semi-anaerobic conditions, as initial testing with screw-cap closed bottles increased the pressure to a level where the bottles were at risk of breaking. The concentration of *A. suum* eggs in a sample of liquid slurry used for the experiment was determined by sequential sieving of a 700 g representative sample of liquid fraction followed by counting the number of eggs in six 50 μl replicates of resultant suspension.

Experimental set-up

The *A. suum* egg survival experiment was conducted in triplicate at 5 °C and 25 °C with raw pig slurry and separated liquid slurry. The samples were collected (destructive sampling) at 10 different times (days 7, 14, 21, 28, 42, 56, 112, 168, 238 and 308). Apart from raw slurry and separated liquid slurry, *Ascaris* eggs were also stored in distilled water as unexposed controls at both temperatures for the entire duration of the experiment.

Analysis of pH and aqueous ammonia (NH_3 (aq))

The pH was measured after mixing wet slurry samples with de-ionized water at a ratio of 1:5 (wt:wt) for all samples (Jorgensen and Jensen, 2009). Raw and liquid slurries were centrifuged for 10 min at 4 °C and 10 000 ***g*** and filtered (Advantec TM No. 5). Ammonium was measured in the supernatant by a flow injector analyser system (Lachat Instruments Division, Milwaukee, WI, USA). NH_3 (aq) concentrations were calculated using ammonium concentrations, pH and estimated ionic strength.

Viability testing of A. suum *eggs*

At each sampling time, nylon bags were removed from 3 bottles with raw slurry and separated liquid slurry, respectively and rinsed thoroughly with tap water. The individual nylon bags were opened, rinsed thoroughly with water and the eggs were collected in a 50 ml plastic tube and centrifuged at 253 ***g*** for 7 min. After discarding the supernatant, the sediment was washed 3 times by re-suspension in 0·1 N H_2SO_4 (pH 1) to 45 ml. After the final washing, the supernatant was removed and the sedimented eggs embryonated in 5 ml of sulphuric acid in the same plastic tubes at 22 °C for 6 weeks. Every week the lids were opened for a short period for aeration. Before and after the embryonation at least 100 eggs in each sample were examined under a compound microscope at 10X to determine the developmental stage of the egg. An egg with a fully developed larva after embryonation was considered to be viable and all the other eggs were considered as dead. At day = 0, eggs from 10 nylon bags were embryonated

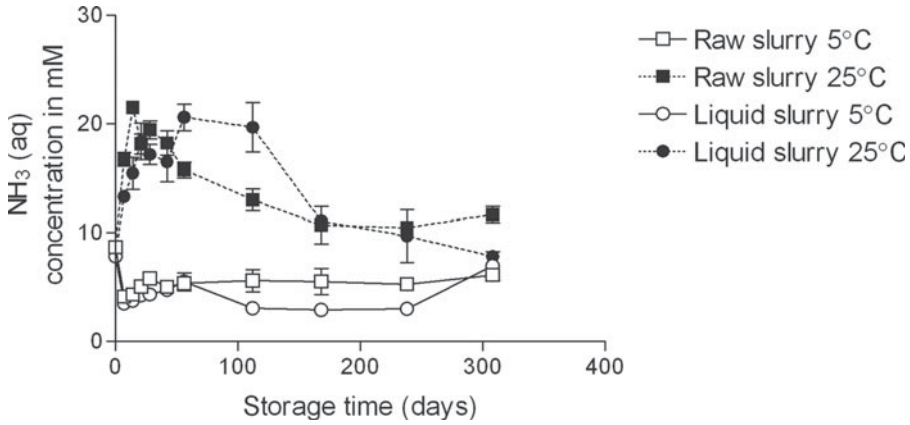

Fig. 1. Changes in aqueous ammonia (NH_3 (aq)) concentration during storage of raw and separated liquid slurry stored at 5 °C and 25 °C.

separately and the initial percentage of viable eggs was estimated by taking the average of all 10 samples.

Statistical analysis

The effect of temperature, slurry type and time on NH_3 (aq) concentrations was analysed in a linear model with temperature and slurry type as categorical variables and time as a continuous variable (Proc mixed, SAS 9.2, SAS institute, Inc; Cary, NC). The percentage of viable eggs in raw slurry and separated liquid slurry stored at each of the two temperatures were compared by fitting the following modified sigmoidal function equation (1), modified from a simple sigmoidal function equation (2), to each of the 4 combinations of temperature and slurry type. This model was chosen as both the rate of decline and lag phase of egg viability can be fitted by the model. Time taken to inactivate 50% of eggs (T_{50}) was calculated from Equation (3) and time taken to inactivate 99% of eggs (T_{99}) calculated from Equation (4).

$$Y_t = Y_0 - \frac{y_0}{1 + e^{-a*(t-c)+b}} \quad \text{(Equation 1)}$$

$$Y_t = \frac{1}{1 + e^{-t}} \quad \text{(Equation 2)}$$

$$T_{50} = c + \frac{b}{a} \quad \text{(Equation 3)}$$

$$T_{99} = \frac{a + c + b - \ln\left(\frac{1}{1 - 0.01} - 1\right)}{a} \quad \text{(Equation 4)}$$

Where

- Y_t is the percent viable eggs at time t
- Y_0 is the percent viable eggs at time 0
- a is a parameter describing the slope of the egg viability curve
- c is a parameter setting a time lag in the decay process of egg viability
- b is an arbitrary parameter, which will displace the curve horizontally

The parameters c and b were estimated in the model, but set to be shared between treatments. Thus only the parameter a had to be fitted for the individual egg survival curve. Statistical differences in egg viability between the two slurry types were declared when the 95% confidence intervals of two a estimates did not overlap. The model was fitted by GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). T_{50} was calculated for comparison of different egg survival curves and T_{99} was calculated as an indicator of inactivation of eggs.

RESULTS

The initial analysis of the liquid slurry collected at the farm showed that the concentration of *A. suum* eggs was 47 eggs per gramme.

Chemical parameters

The pH of raw slurry gradually increased from 7·98 (day 0) to 8·22 and 8·32 (day 308) at 5 °C and 25 °C, respectively, while in separated liquid slurry it gradually increased from 8·00 (day 0) to 8·30 and 8·46 (day 308) at 5 °C and 25 °C, respectively. The NH_3 (aq) concentrations obtained differed between the two temperatures ($P<0·0001$) but there was no significant difference in NH_3 (aq) found between raw and separated liquid slurry ($P=0·16$). The interaction between time and temperature on NH_3 (aq) was statistically significant ($P<0·0001$) as the slope for the decline of NH_3 (aq) over time was close to 0 at 5 °C, but was negative at 25 °C. This means that at 25 °C the NH_3(aq) concentration declined significantly with time starting at 16·7 (±1·1) mM and 13·3 (±0·2) mM at 7 days declining to 11·7 (±1·3) mM and 7·8 (±0·8) mM at 308 days in raw slurry and separated liquid slurry, respectively. In contrast, at 5 °C the NH_3 (aq) concentration ranged between 3·2–9·5 mM and 2·6–9·5 mM in raw slurry and liquid fraction respectively throughout the study (Fig. 1).

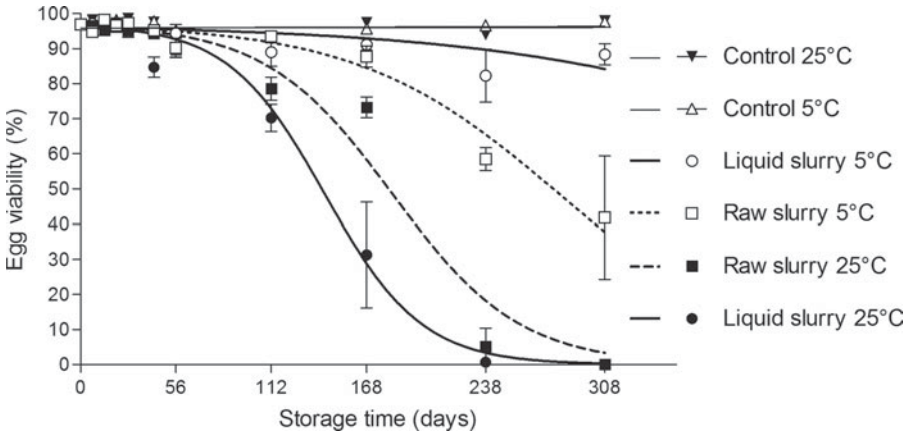

Fig. 2. Viability of *Ascaris suum* eggs in raw and separated liquid slurry stored at 5 °C and 25 °C.

Survival of A. suum *eggs*

None of the *A. suum* eggs that were examined immediately (day 0) after collecting the nylon bags from the stored bottles of raw slurry and separated liquid slurry contained developed larvae. The same was the case for eggs kept as controls in distilled water at 5 °C throughout the study. At 25 °C, 92–99% of the eggs stored in distilled water showed different developmental stages from day 7 to 28, after which the eggs contained fully developed larvae. The viability profiles of the eggs in different treatments after embryonation are shown in Fig. 2. The viability of eggs at the start of the experiment was 97% and the control eggs stored in distilled water continued to have a high viability throughout the study, i.e. 97% ±1% (mean ± S.D.) and 98% ±2% at 5 °C and 25 °C, respectively. The estimates for the slope parameter (a); reduction in percentage egg survival per day, are given in Table 1 together with their 95% CI. There was no significant difference in the viability of eggs between the distilled water controls maintained at 5 °C and 25 °C and between raw and liquid slurry stored at 25 °C, but there was a significant difference in egg viability when the two slurry types were stored at 5 °C and between eggs in distilled water (controls) and separated liquid slurry at 5 °C. The reduction in percentage egg survival per day was ordered in the following sequence: distilled water control 5 °C = distilled water control 25 °C < liquid slurry 5 °C < raw slurry 5 °C < raw slurry 25 °C = liquid slurry 25 °C.

DISCUSSION

The Commission of European Communities (CEC) recommends storing manure for a minimum of 60 days in summer and 90 days in winter (Kelly, 1978; Walton and White, 1981) before application to land. But our results document that even storage for 3 months at 25 °C has little if any effect on the viability of eggs and confirm earlier studies that *A. suum* eggs can survive for more than 100 days in raw pig slurry at temperatures between 10 and 17 °C (Gaasenbeek and Borgsteede, 1998; Papajova *et al.* 2005). The practical implications of our observation is that raw and separated liquid slurries kept at 25 °C can be spread on pastures and crops only after a minimum of 8–10 months storage without contaminating with viable eggs. Raw and liquid slurry stored at 5 °C require even a longer storage time to inactivate helminth eggs and it is not advisable to apply such slurries to pastures e.g. with pigs and ready-to-eat crops as 41·9 (± 30)% and 88·4 (± 5)% of eggs, respectively were still found viable even after 10 months storage.

The chemical-mechanical slurry separation technique used in this study is based on adding flocculant as a pre-treatment followed by mechanical separation. Flocculant is added to aggregate small particles into larger masses (flocs) which are then removed by mechanical separators to the solid fraction. Flocculation followed by treatment in a screw press proved efficient in removing particles from raw slurry to the solid fraction (Hjorth *et al.* 2010; Peters and Jensen, 2011) and the liquid fraction is usually free of particles larger than 250 μm (Popovic *et al.* 2012). As the size of *A. suum* eggs is 56–84 μm × 50–59 μm (Roberts, 1934) the liquid fraction obtained by slurry separation as done in this study will not be devoid of all *A. suum* eggs as documented by our findings and therefore other pathogens may also be present. However, other slurry separation techniques may impact the removal of pathogens differently. Information was not available about the amount of flocculant (polyacrylamide) used for slurry separation in the present study but the optimal dose for efficient total solid suspension (TSS) separation is 0·9–1·8 g/kg total solids (Walker and Kelley, 2003). Any effect of this chemical on the viability of eggs can be ruled out by the observed high viability of eggs in liquid slurry at 5 °C. According to the World Health Organization (2006) guidelines, the concentration of helminth eggs in waste water intended for irrigation

Table 1. Estimation of the slope parameter *a*, T_{50} and T_{99} for different storage conditions of raw and liquid slurry

	Raw slurry 5 °C	Raw slurry 25 °C	Liquid slurry 5 °C	Liquid slurry 25 °C
Mean of *a*[a]	0·0169	0·0267	0·0089	0·0351
95% CI of *a*	0·0145–0·0192	0·0235–0·0300	0·0053–0·0124	0·0293–0·0408
T_{50} (days)	281	183	519	143
T_{99} (days)	553	355	1034	274

[a] The unit for 'a' is reduction in percentage egg survival per day.

should be $\leqslant$1 egg/L of waste water. In the present study where liquid slurry contained more than 40 eggs per gramme, such liquid would not be safe for irrigation of high risk crops like vegetables.

pH was unlikely to be a main determinant of the inactivation of eggs as our observed pH values overall were only slightly alkaline. Eriksen *et al*. (1996) showed that viability of *A. suum* eggs in lime-treated sewage sludge was effected only at a pH of >12 for at least 3 months while Gantzer *et al*. (2001) showed that sludge should be exposed to a pH above 11·5 for 6 months to produce sanitized sludge. Thus the inhibiting effect of raw and separated liquid slurry in the present study should be attributed to other physico-chemical factors. One such factor is NH_3 (aq) which has been documented to impact the viability of helminth eggs when these are stored in ammonia-treated wastewater sludge (Ghiglietti *et al*. 1997; Pecson *et al*. 2007) or exposed to ammonia in urine and feces (Nordin *et al*. 2009).

Increased temperature increases the permeability of the helminth egg shell. Barrett (1976) studied the permeability of *A. lumbricoides* to acid fuchsin by incubating eggs for 12 h at different temperatures and reported a marked increase in the egg permeability at 44 °C. Although the highest temperature in the present study was 25 °C, prolonged exposure to such a temperature might have caused the lipid membrane of *A. suum* egg, which acts as a barrier to toxic chemicals, to lose its integrity, allowing the toxic ammonia to penetrate the egg, resulting in death of 100% of the eggs in both slurry types after 8–10 months storage. Thus, inactivation of helminth eggs and pathogens in general is higher at high temperatures corresponding to summer conditions or tropical climates, than at low temperatures, e.g. seen during winter in temperate climates, which corroborate findings from studies of egg inactivation conducted under natural conditions (Larsen and Roepstorff, 1999).

NH_3 (aq) concentration was similar in both slurry types and depended on temperature with higher concentrations found at 25 °C than at 5 °C and a significant decline seen at 25 °C when the study was terminated (Fig. 1). The reason being that at high temperature and alkaline pH the equilibrium between nontoxic NH_4^+ (aq) and toxic NH_3 (aq) shifts towards NH_3 (aq) which is volatile and may evaporate as NH_3 gas.

At 25 °C, we found that both high NH_3 (aq) concentration and poor survival were associated, thus NH_3 (aq) could be the causative factor, but this can't be proven directly from this experiment. Nordin *et al*. (2009) studied the inactivation of *A. suum* eggs in human feces and urine at 24 °C; a temperature comparable to our 25 °C experiment. At 24 °C, an NH_3 (aq) concentration of 20 mM was measured on day 35 and a mean 99% inactivation time (T_{99}) of 74 days was predicted. Though we observed similar NH_3 (aq) concentrations at 25 °C (16·5–20·6 mM during 21–56 days storage of liquid slurry and 18·2 mM – 21·5 mM when raw slurry was stored between 14 and 42 days), total inactivation was observed at 238 days and 308 days in liquid and raw slurries respectively. The faster inactivation reported by Nordin *et al*. (2009) might be due to the use of eggs obtained from the uterus of adult female *A. suum* worms which had an initial viability of only 62%. We assume helminth eggs need exposure to the pig intestine environment to fully mature and become resistant to unfavourable conditions in the external environment as suggested by Fairbairn (1957). The relative low percentage of initial egg viability (Nordin *et al*. 2009) indicates that the eggs used were not fully matured therefore likely to be more susceptible to unfavourable conditions in the external environment. Pecson *et al*. (2007) reported a T_{99} of 87 days at 1000 mg/l ($\sim$ 58 mM) ammonia and 25 days at 5000 mg/l ($\sim$ 294 mM) ammonia at pH 12 and 20 °C. The low ammonia concentrations found in the present study, i.e. 7·9–22·4 mM in raw slurry and 7·3–23·2 mM in liquid slurry, might explain the longer duration (238–308 days) needed for a total inactivation of the *A. suum* eggs. It should be noted that our bottles with slurry were closed with Parafilm® during storage and it is unknown to what extent NH_3 gas evaporated from the bottles.

Although there was no significant difference in the NH_3 (aq) concentrations between the two slurry types at 5 °C, the viability of *A. suum* eggs in liquid slurry was higher (88·4%) even after prolonged storage as compared to raw slurry (41·9%) indicating that there might be other physico-chemical factors than toxic ammonia in raw as opposed to liquid slurry

responsible for the increased inactivation of eggs in stored raw slurry.

The present study found no embryonation or indication of development while the eggs were in the slurry under any storage conditions, which clearly indicates that the *A. suum* eggs may not be infective when present in different types of slurry. It is well known that embryonation of eggs would not take place at 5 °C as *A. suum* eggs require >16 °C to embryonate under controlled laboratory conditions (Seamster, 1950) and eggs only develop during the summer months in temperate climates (Stevenson, 1979; Nilsson, 1982). NH_3 (aq) in slurry might also reversibly inhibit embryonation in a similar way as temperature, as is suggested by findings of non-embryonated eggs only following storage in urine (Nilsson, 1982). Biological factors like the presence of ovicidal fungi may also play a role in inactivation of *A. suum* eggs. Lýsek and Krajcí (1987) reported that fungal species, *Verticillium chlamydosporium* penetrate and destroy *A. lumbricoides* eggs. Araújo *et al*. (2008) evaluated *in vitro* the effect of different nematophagous fungi on *A. suum* eggs and reported an ovicidal effect of *Pochonia chlamydosporia*. Little is known about the presence and survival of ovicidal fungi upon storage of pig slurry and their impact on egg survival warrants further investigation.

Earlier studies on *A. suum* egg viability have also used the ability of un-embryonated eggs to develop into fully larvated eggs upon embryonation as a criterion for viability (e.g., Pecson *et al*. 2007, Nordin *et al*. 2009, Mun *et al*. 2011). The actual physiological status of the larvae is, however, not known and the eggs might be non-viable after reaching the fully larvated stage or have lost their infectivity. In addition, embryonation of eggs into fully larvated eggs cannot be used as a criterion for eggs already larvated prior to incubation. An egg hatch assay would presumably give a more valid status of viability and the results of such an assay might have provided different results in the present study, but none of the hatching techniques hitherto described (Fairbairn 1961; Han *et al*. 2000) have worked consistently in our laboratory (unpublished data) and there is a clear need to develop a validated egg hatch assay.

Several non-linear regression models have been used to model microbial survival curves as a function of time (Membré *et al*. 1997; Pecson *et al*. 2007). The advantage of our model was that only a single parameter was needed to describe the difference between the survival curves (i.e. the slope parameter *a*). The estimated survival functions described the observed egg survival curves well for 3 of the 4 datasets, whereas the fit was poorer for the dataset representing raw slurry at 25 °C.

In conclusion, our study demonstrated that under laboratory conditions at 5 °C, storage for 10 months does not affect the viability of *A. suum* eggs in liquid slurry and has a limited effect on viability of eggs in raw slurry, whereas at 25 °C all eggs lost their viability within this period of time irrespective of slurry type. Further studies are needed to investigate whether treatment of raw and liquid slurries (e.g. addition of lime or urea) may reduce the viability of helminth eggs and other pathogens. Recommendations are needed for safe application of liquid slurry to fertilize different crops used for animal and human consumption.

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6.2 Paper 2. Survival of *Ascaris suum* and *Ascaridia galli* eggs in liquid manure at different ammonia concentrations and temperatures

Kiran Kumar Katakam, Helena Mejer, Anders Dalsgaard, Niels Christian Kyvsgaard and Stig Milan Thamsborg

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Survival of *Ascaris suum* and *Ascaridia galli* eggs in liquid manure at different ammonia concentrations and temperatures

Kiran Kumar Katakam[1], Helena Mejer[1]*, Anders Dalsgaard[1], Niels Christian Kyvsgaard[2] and Stig Milan Thamsborg[1]

[1]Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
[2]Section for Veterinary Medicine, Danish Health and Medicines Authority, Copenhagen, Denmark

* Corresponding author:
Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences,
University of Copenhagen, Dyrlægevej 100,
DK- 1870 Frederiksberg C, Denmark.
Tel: +45 35332789, Fax: +45 35331433
E-mail: hem@sund.ku.dk

Abstract

Eggs of *Ascaris suum* from pigs are highly resistant and commonly used as a conservative indicator of pathogen inactivation during slurry storage. Eggs of *Ascaridia galli*, the poultry ascarid, are also known to be highly resistant but the suitability as an indicator of pathogen inactivation has never been tested. Pig slurry has to be stored for several months to inactivate pathogens but chemical treatment of slurry may reduce this time. The suitability of *A. galli* as an indicator of slurry sanitation was tested by comparing the survival of eggs of *A. suum* and *A. galli* in pig slurry. In addition, the effect of urea treatment on storage time was also tested. Nylon bags with 10,000 eggs of either species were placed in 200 ml plastic bottles containing either urea-treated (2%) or untreated pig slurry for up to 120 days at 20°C, 6 days at 30°C, 36h at 40°C or 2h at 50°C. At all the temperatures in both slurry types, *A. galli* eggs were inactivated at a significantly faster rate ($P<0.05$) compared to *A. suum* eggs. For each 10°C raise in temperature from 20°C, t_{50} (time needed to inactivate 50% of eggs) for both types of eggs was reduced markedly. At all temperatures, viability of eggs of both species was significantly higher ($P < 0.05$) in untreated slurry compared to urea-treated slurry except *A. galli* eggs at 20°C where no significant difference was detected. In untreated slurry, the levels of pH (6.33-9.08) and ammonia (0.01-1.74 mM) were lower ($p<0.0001$) compared to that of urea-treated slurry (pH: 8.33-9.28 and ammonia 1-13 mM). The study demonstrated that *A. galli* eggs are more sensitive to unfavourable conditions compared to *A. suum* eggs. The use of *A. galli* eggs as hygiene indicator may thus be suitable to assess inactivation of pathogens that are less susceptible than *A. galli* eggs. Addition of urea may markedly reduce the storage time of slurry needed to inactivate *A. suum* and *A. galli* eggs.

Keywords: slurry; viability; *Ascaris suum* eggs; *Ascaridia galli* eggs; temperature; ammonia

1. Introduction

Eggs of ascarid helminths (nematodes) are known to be highly resistant and can withstand highly unfavourable environmental conditions (Barette, 1976; Wharton, 1983). The pig ascarid *Ascaris suum* is ubiquitous and the eggs can be easily processed and enumerated in the laboratory. The viability of these eggs is therefore often used as an indicator for assessing whether animal manure and human waste have been sufficiently sanitized under mesophilic conditions (Holmqvist and Stenström, 2001). At temperatures > 50°C, *A. suum* eggs die off rapidly (Burden and Ginnivan, 1978; Barnard et al., 1987) and may not be suitable to use as hygiene indicators compared to other indicators like bacteriophages. The eggs of the chicken ascarid, *Ascaridia galli* are also considered to be very resistant to adverse conditions. The infection is non-zoonotic and eggs are easily obtainable. *Ascaridia galli* could thus be of use in areas where *A. suum* eggs are not available. Though *A. galli* eggs have been used in one sanitation study (Tønner-Klank et al., 2007), their suitability as a hygiene indicator has not been fully investigated.

Intensive pig production results in large quantities of slurry and solid manure which are commonly used as soil fertilizers after a period of storage. The manure may contain a wide variety of pathogens including those of zoonotic potential (Bradford et al., 2013). Application of stored slurry and solid manure to agricultural and horticultural crops can therefore lead to contamination of the produce as well as water sources through surface run-off (Mawdsley, 1995). Human consumption of contaminated produce, e.g. berries, lettuce and other crops consumed raw, may subsequently result in disease (Hanning et al., 2009). On an average, storage capacity of slurry storage tanks in Europe is sufficient for six months (Menzi, 2002) indicating the maximum storage time. During storage of farm waste the pathogens may be inactivated due to exposure to unfavourable conditions, e.g. high temperatures due to composting processes (Grewal et al., 2006). Inactivation time during storage also depends upon factors like pH, aeration, ammonia, carbonate ions and volatile fatty acids (VFA) concentrations, dry matter content and pathogen type, and different studies have reported different storage times (Ghiglietti, 1997; Gantzeret al*.,* 2001; Brewster et al., 2003; Nordinet al., 2009; Katakam et al*.,* 2013) for inactivation of *A. suum* eggs.

Pig production and the associated application of slurry on fields may cause leakage of nitrogen and eutrophication of recipient water bodies. The European Commission (IPPC Directive 96/61/EC) recommends therefore that the best available practices (e.g. low protein diets, housing design) should be used to reduce such environmental pollution. It has been attempted to reduce the ammonia emissions from slurry by modifying dietary composition e.g. by adding fermentable carbohydrates (Canh et al., 1998).

However, slurry with reduced ammonia levels may require longer storage time to ensure effective inactivation of pathogens and this may be undesirable for economic and logistic reasons. Faster reduction of pathogens in sludge, slurry or faeces and thus more efficient sanitation may be achieved at higher temperatures, pH or ammonia concentrations (Eriksen et al., 1996; Pecson et al., 2007; Nordin et al., 2009 and Bolton et al., 2013), and knowledge on how to manipulate these parameters may help reduce effective storage time.

The primary source of ammonia in slurry is rapid degradation of urea to ammonium and carbonate ions by urease secreted by faecal bacteria (Mobley and Hausinger, 1989). A minor source of ammonia is slow degradation of undigested proteins in the slurry during storage (Zeeman, 1991). In the aqueous phase, ammonium (NH_4^+) and ammonia (NH_3) are in an equilibrium which is influenced by temperature and pH. Though high temperatures shift the equilibrium towards NH_3, pH has a much stronger influence on equilibrium; at pH below 7 the total ammonia nitrogen (TAN) is mostly in NH_4^+ form and above 11 it is mostly in NH_3 form (Philippe et al., 2011). Though the exact mechanism of action is not known, ammonia (in NH_3 form) has been shown to have toxic effect on many microbes (Ghiglietti et al., 1997; Pecson et al., 2007; Bolton et al., 2013).

The objective of the present study was to compare the survival of eggs of ascarids (*A. suum* and *A. galli*) in pig slurry at different levels of ammonia and temperatures and to investigate how egg survival can be reduced by adding urea.

2. Materials and methods

2.1. Experimental design

Ascaris suum and *Ascaridia galli* eggs were incubated in triplicate in water (control), urea-treated or untreated pig slurry at 20°C, 30°C, 40°C and 50°C under laboratory conditions. Samples stored at 20°C, 30°C and 40°C were kept in incubators whereas those stored at 50°C were kept in a water bath. pH, aqueous ammonia ($NH_{3\,(aq)}$), ascarid egg development and viability were monitored at regular time intervals depending on incubation temperature and expected inactivation time (Table 1).

2.2. Ascarid eggs

Faeces were collected from the large intestines of pigs at a Danish slaughter house and fresh chicken faeces were collected from the floor at a Danish poultry farm. Both *A. suum* eggs and *A. galli* eggs were isolated from the faeces by sequential sieving and washing through a series of sieves with mesh sizes 500 µm, 212 µm, and 90 µm followed by collection on a 36 µm sieve. Eggs were isolated from the material retained on

the 36 µm mesh size sieve by flotation as described by Larsen and Roepstorff (1999). Nylon bags (size 2.5 x 2.5 cm^2) with a mesh size of 20 µm (Sefar AG, Heiden, Switzerland) were prepared each containing 10,000 fresh un-embryonated *A. suum* or *A. galli* eggs and were sealed with a glue gun.

2.3. Pig slurry

Fresh pig slurry was collected from an organic pig farm, where pigs were mainly fed cereals (barley), and transported to the laboratory within 4 h and stored at 5°C for 5 days before setting up the experiment. Before the start of experiment the slurry was mixed thoroughly for 2 min and divided into two portions. Urea (Urea Technical grade, Applichem GmbH) was added to one of the portions to ensure a final urea concentration of 2% w/w. Approximately 150 g of slurry with or without urea was transferred to 200 ml plastic bottles. Two nylon bags containing either *A. suum* or *A. galli* eggs were then submerged in the slurry of each bottle. The bottle was only partially closed with a lid, allowing some ventilation as the bottle would else burst due to generation of gases from the slurry during storage.

2.4. Egg development and viability

At each sampling, the two nylon bags were recovered from a given bottle and rinsed thoroughly with tap water. Each bag was gently pulled a part and the eggs washed off into separate tubes and washed three times with 0.1M H_2SO_4 buffer by centrifugation at 253 x *g* for 7 min and discarding the supernatant. After the final wash, the supernatant was discarded and H_2SO_4 was added to a total volume of 5 ml. The eggs were then embryonated at 22°C for six weeks (Oksanen et al. 1990). Eggs were aerated every week by opening the lids for 2-3 min. A minimum of 100 eggs were examined microscopically before and after embryonation to determine the developmental stage of the eggs. After embryonation, eggs containing a fully developed larva were considered to be viable whereas all other stages of development were considered to be non-viable. Initial viability of eggs was measured by embryonating eggs from 10 nylon bags for each species at the start of the experiment.

2.5. Measurements of pH and aqueous ammonia ($NH_{3\,(aq)}$)

The pH was measured in all slurry samples after mixing with de-ionized water at a ratio of 1:5 (w:w) (Jorgensen and Jensen, 2009). Slurry samples had initially been stored at -20°C. Total ammonia nitrogen (TAN) was then later extracted from 5 grams of thawed and homogenized slurry suspended in 1 M KCl to a total volume of 100 mL and subjected to end-over-end shaking for 45 min. The extracts were then filtered through filter papers (Advantec TM No. 5A, Advantec MFS, Inc., Dublin, CA, USA) and once more frozen at

20°C. TAN was later measured in the thawed extracts by a flow injector analyser system (Lachat Instruments Division, Milwaukee, WI, USA). $NH_{3\,(aq)}$ concentrations were calculated using the TAN, pH and dissociation constant (pKa) values as described by Armstrong et al. (1978).

2.6. Statistical analysis

For each temperature, the effect of slurry type (+/- urea), time and their interaction on pH and $NH_{3\,(aq)}$ concentration was analysed using a linear model with slurry type as a categorical variable and time as a continuous variable (Proc GLM, SAS 9.2, SAS institute, Inc; Cary, NC). The percentage of viable eggs in slurry and water controls stored at each temperature were compared by fitting the following Boltzmann sigmoidal function equation using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). This model was selected as it has fixed top and bottom constants and a variable slope and the time needed to inactivate 50% of the eggs (T_{50}) can be calculated to compare the egg viability curves.

$$Y_t = b + \frac{a - b}{1 + \exp(\frac{T_{50} - t}{slope})}$$

Y_t = percent viable eggs time t
a = initial viability percent (top constant)
b = zero viability percent (bottom constant)
T_{50}= time needed for reduction in 50% viability
t = time

Two estimates of T_{50} were considered significantly different ($P<0.05$) if their respective 95% confidence intervals did not overlap. This is a cautious approach, as (smaller) overlaps of confidence intervals can be found even if two estimates are significantly different (Knezevic, 2008).

3. Results

3.1. pH and aqueous ammonia ($NH_{3\,(aq)}$)

The pH of untreated slurry was lower compared to that of urea-treated slurry at all temperatures (*P*<0.0001) (Fig.1). The pH of both slurry types at 20°C and 30°C was slightly lower than that at 40°C and 50°C. The pH of untreated slurry stored at 20°C, 30°C and 40°C steadily increased with storage time (*P*<0.001) whereas at 50°C, it did not change over time. pH of urea-treated slurry did not show any significant change over time at any of the temperatures.

The initial $NH_{3\,(aq)}$ concentrations in the urea-treated slurry were higher at higher temperatures (from 5.8 mM to 13 mM) and levels decreased at varying degrees over time at 30°C-50°C (*P*=0.03) but remained fairly stable at 20°C (Fig.2).In untreated slurry, the $NH_{3\,(aq)}$ concentration remained very low (below 0.1 mM) throughout the study period at 30°C-50°C and slightly increased over time at 20°C (*P*=0.03). The $NH_{3\,(aq)}$ concentration in untreated slurry thus remained much lower than in the urea-treated slurry at all temperatures.

3.2. Egg development and viability

None of the *A. suum* and *A. galli* eggs showed any sign of development during incubation in the slurry irrespective of type and temperature. None of the control eggs in water developed at 40°C and 50°C whereas control eggs kept at 30°C showed different stages of development at days 1 to 6. Fully developed control eggs were observed at 20°C after day 30.

Temporal changes in viability of *A. suum* and *A. galli* eggs at the different temperatures are shown in Figure3. Mean initial viability of *A. suum* and *A. galli* eggs was estimated to be 98% and 91%, respectively. At all temperatures, viability of both egg species was significantly higher ($P< 0.05$) in untreated slurry compared to urea-treated slurry except for *A. galli* eggs stored at 20°C for which no significant difference was detected. At 40°C, there was no decline in viability of *A. suum* eggs in untreated slurry within the study period of 36 h and hence T_{50} could not be calculated (Table 2). The viability of *A. suum* control eggs kept at 20°C, 30°C and 40°C remained consistently high throughout the study period whereas those stored at 50°C showed decreased viability after 60 min. The viability of *A. galli* control eggs kept at 20°C and 30°C was also constantly high throughout the study period, but *A. galli* eggs kept at 40°C and 50°C showed decreased viability before or at a rate similar to those in untreated slurry (Fig. 3).

In urea-treated slurry kept at 20°C, total inactivation of *A. suum* and *A. galli* eggs occurred after 90 days and 105 days, respectively. At this time 95% and 6% of *A. suum* and *A. galli* eggs were still viable in untreated slurry (Fig. 3). At 30°C, after day 6, the viability of *A. suum* and *A. galli* eggs in urea-treated slurry was 76% and 5%, respectively whereas it was 91% and 19% for *A. suum* and *A. galli* eggs in untreated slurry. At 40°C, after 36 h, all *A. suum* and *A. galli* eggs were dead while 96% of *A. suum* eggs and 6% of *A. galli* eggs were still viable in untreated slurry. At 50°C, 1% and 0% of *A. suum* and *A. galli* eggs were viable, respectively after 120 min, a time when 43% and 1% of *A. suum* and *A. galli* eggs, respectively were viable in untreated slurry (Fig. 3).

Overall in the present study, higher $NH_{3\,(aq)}$ concentrations were associated with lower viability of eggs. For both types of eggs and in both slurry types, the estimated T_{50}decreased drastically as the temperature increased from 20°C to 50°C. Estimates of T_{50} were lower in urea-treated slurry compared to that for untreated slurry with the exception of *A. galli* eggs at 20°C. The estimates of T_{50} for *A. suum* were higher compared to that of *A. galli* eggs (Table 2).

4. Discussion

The present study compared the survival of *Ascaridia galli* eggs with those from *Ascaris suum* with the aim to assess whether the former could be used as an alternative to *A. suum* as a conservative hygiene indicator for slurry. The results revealed that *A. galli* eggs are less suitable as indicators for the hygienic quality of treated pig slurry as they died faster than *A. suum* eggs.

The difference in inactivation times between two species might be due to the structural differences of the egg shells. The *A. suum* shell contains four layers, namely an outer proteinaceous layer (uterine-derived), a vitelline layer, a chitinous layer and an innermost highly impermeable lipid layer (ascaroside layer) (Wharton, 1980). The *A. galli* shell contains only three layers, namely an outer proteinaceous layer, a middle chitinous layer and an inner lipoid layer (Ackert, 1931 and Christenson et al., 1942). The presence of an extra layer may make *A. suum* eggs more heat tolerant and resistant to other unfavourable conditions (e.g. high $NH_{3\,(aq)}$ levels) compared to *A. galli* eggs. However, it remains to be further investigated exactly how this as well as the other layers protects against environmental stress.

Survival of specific microbial indicators is generally used to estimate the effectiveness of a treatment method to inactivate pathogens in slurry, sewage sludge or other types of organic wastes. Helminth eggs are well-known for their high resistance to environmental stress, e.g. they may survive for years in agricultural soil (Kransnonos, 1978). Several studies have evaluated different viral, bacterial and parasitic species as hygienic indicators (Placha et al., 2001; Ottoson et al., 2008; Jensen et al., 2010; Bøtner et al., 2012; Katakam et al., 2013) depending upon purpose of the study. To consider an organism as a good hygiene indicator, isolation and quantification methods should be simple, reliable, definitive, cost-effective and the indicator should show higher survival rates as compared to other pathogens (Böhm et al., 1999). Bacterial, e.g. *E. coli* and enterococci, and phages, e.g. *E. coli* phage are used as indicators of bacterial and viral pathogens, respectively, but these indicators show a much faster die-off than parasites, in particular helminth parasites. Enterococci may under certain conditions even show regrowth (Holmqvist et al., 2002). Thus, helminth eggs are excellent hygienic indicators as their slower die-off ensures that both bacterial and viral pathogens are fully inactivated.

The initial pH (6.46) of the slurry used in the present study was relatively low compared to most previous studies citing pH values of fresh pig slurry from 6.31 to 8.65 (Kirchmann and Witter, 1992; Canh et al*.,* 1998a; Sutton, 1999; Le et al*.,* 2009; Katakam et al., 2013). The pH value of fresh slurry may depend upon the feed ingredients, and high protein diets generally result in higher pH and ammonia levels (Canh et al., 1998a and Le et al*.,* 2007) whereas high fermentable carbohydrate diets result in high levels of volatile fatty acids (VFA), thereby reducing the pH (Paul and Beauchamp, 1989 and Canh et al*.,* 1998b). In the present study, slurry was obtained from a farm where the pigs were mainly fed on cereals which might have resulted in low pH levels. In the present study, addition of urea to the fresh slurry raised the pH by 2 units, which may have been due to enzymatic conversion of the added urea to ammonium (NH_4^+) by urease secreted by faecal bacteria present in the slurry (Muck and Steenhuis, 1981).

Variation of pH levels is likely a main factor behind the observed differences in $NH_{3\,(aq)}$ levels between the two slurry types as at pH below 7, TAN will be mostly in NH_4^+ form whereas above pH 7, increase in pH dramatically increases the conversion NH_4^+ to NH_3 (Philippe et al., 2011). The increase in $NH_{3\,(aq)}$ levels with increase in storage temperature in urea-treated slurry might thus be due to a temperature mediated shift in the equilibrium between NH_4^+ and NH_3 towards NH_3 (Philippe et al., 2011). The decrease in level of $NH_{3\,(aq)}$ over time at 30°C-50°C might be due to volatilization of NH_3 due to partial closure of bottles. The slight increase in $NH_{3\,(aq)}$ levels over time in untreated slurry at 20°C might be due to slow degradation of organic nitrogen during storage (Zeeman, 1991).

The present results confirm earlier studies that increased temperature and $NH_{3\,(aq)}$ concentration may increase the inactivation of helminth eggs (Pecson et al., 2007; Nordin et al*.,* 2009). At 50°C, both *A. galli* and *A. suum* eggs were inactivated at similar rates in untreated slurry and water, but at a slower rate compared to urea-treated slurry. This indicates that temperature was the main contributing factor in egg mortality for the two former treatments, whereas addition of urea helped increase $NH_{3\,(aq)}$ concentrations and thereby further increased the mortality rate. The *A. galli* eggs in water controls at 40°C and 50°C were inactivated at a faster rate compared to the eggs in untreated slurry. It is possible that the organic matter in the slurry protected the eggs as speculated by Popat et al*.* (2010) who studied the thermal inactivation of *A. suum* eggs at 51°C-55°C and concluded that anionic detergents and amino acids in sludge may provide substantial protection against thermal inactivation *A. suum* eggs.

At ≤40°C and ≤30°C *A. suum* and *A. galli* eggs, respectively, temperature alone did not affect survival of eggs in water. At each temperature, egg mortality increased with increased storage in both urea-treated and untreated slurry. Permeability of the egg shell of another *Ascaris* spp. (*A. lumbricoides*) increases markedly at temperatures above 44°C (Barett, 1976) and the observed faster inactivation of eggs at 50°C might be due to increased permeability of eggs to $NH_{3\,(aq)}$. Slower inactivation at lower temperatures might be due to prolonged time needed to increase the permeability of the egg shell to $NH_{3\,(aq)}$ in combination with lower development of ammonia at lower temperatures. Several other studies have also showed the toxic effect of ammonia (in NH_3 form) on many other microbes (Ghiglietti et al., 1997; Pecson et al., 2007; Vinnerås, 2007; Ottoson et al., 2008; Nordin et al., 2009; Bolton et al., 2013) and the mechanism of inactivation of microbes by ammonia might be due to alteration of membrane potential, increase in cytoplasmic pH and/or loss of potassium ions (Kadam and Boone, 1996; Bujozek, 2001).

Though there was a large increase in $NH_{3\,(aq)}$ levels in urea-treated slurry, the levels were apparently too low to inactivate the eggs at 20°C. In the absence of other inhibiting factors, at 20°C and at a pH 12, Pecson et al. (2007) observed 99% inactivation of *A. suum* eggs within 87 days at around 58 mM $NH_{3\,(aq)}$ and within 25 days at 294 mM. In the present study, at 20°C, all the eggs were inactivated in 90 days though the $NH_{3\,(aq)}$ concentration ranged from 2-4 mM in urea-treated slurry. Inactivation of *A. suum* eggs even at seemingly very low levels of ammonia in the present study indicates that, along with ammonia, other factors not measured in the study may also have been responsible. They might be carbonate ions or volatile fatty acids (VFA) which are also known for pathogen inactivation. Diez-Gonzalez et al. (2000) and Park and Diez-Gonzalez (2003) reported the effect of carbonate ions in inactivation of food borne pathogens like *Escherichia coli* and *Salmonella typhimurium* in cattle manure. Similarly Kunte et al. (2000; 2004) reported the effect of VFA inactivation of some enteric bacteria and viruses. Little is known about effect of carbonate ions and VFA on helminth eggs and further studies are needed.

The effect of urea-addition could have practical implications at normal storage temperatures. At 20°C prolonged survival of *A. suum* was observed for the untreated slurry but inactivation within days was achieved with urea-addition. Animal slurry should be stored for a certain period before it is spread on the fields (Sommer et al., 2009). In Europe, pig slurry is generally stored for around 6 months (Burton and Turner, 2003), but several studies have reported that *A. suum* eggs may survive in pig slurry beyond 6 months (Gaasenbeek and Borgsteede, 1998; Papajova et al., 2005; Katakam et al., 2013). This is confirmed by the present study which also demonstrated that addition of urea can alter the physico-chemical balance

in the slurry so that all *A. galli* and *A. suum* eggs can be inactivated to maximum extent by day 60 and day 90, respectively when stored at 20°C.

Several studies reported significant inactivation of some of the zoonotic pathogens when slurry is subjected to anaerobic digestion, treated with urea, ammonia and lime, or when soils are ploughed and harrowed immediately after slurry application (Boes et al., 2005, Bolton et al., 2013 and Ottoson et al., 2008). Ploughing the soil may not be effective in inactivating pathogens like helminth eggs. Mejer and Roepstorff (2011), who studied the transmission of *A. suum* and *Trichuris suis* reported that ploughing may transfer the eggs deeper in to soil but this does not promote inactivation. Though thermophilic anaerobic digestion efficiently inactivates *A. suum* eggs, mesophilic anaerobic digestion may take longer to inactivate the eggs. Plachy et al. (1997) observed 76% viable *A. suum* eggs even after 30 days of mesophilic anaerobic digestion whereas total inactivation of *A. suum* eggs within 10 min of thermophilic anaerobic digestion. Anaerobic digestion may be useful for few big farms only and may not be feasible for small and medium sized farms due to economic reasons (Parsons, 1986). Treatment of slurry with lime may effectively inactivate *A. suum* eggs (Eriksen, 1996; Nordin et al., 2009) but it contains no nutrients and has no value as a fertilizer. Treatment of slurry with urea at low concentrations is economical and improves the fertilizer value of the slurry (Ottoson et al., 2008).

In conclusion, the present study has demonstrated that *A. galli* eggs are more sensitive to unfavourable conditions compared to *A. suum* eggs, and hence their use as hygiene indicators may be limited to assess inactivation of other susceptible pathogens. Addition of 2% urea may markedly reduce (by 17-76% for *A. suum* eggs and 11-57% for *A. galli* eggs depending upon storage temperature) the storage time of slurry needed to inactivate *A. suum* and *A. galli* eggs. Addition of urea may markedly reduce the storage time of slurry needed to inactivate *A. suum* and *A. galli* eggs.

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Table 1. Experimental design

Temperature	Storage time units	Storage time	
		Slurry with urea	Slurry without urea
20°C	days	30,60,75,90,105,120,150	30,60,75,90,105,120,135,150
30°C	days	1,2,3,4,5,6,	2,4,6,12,18, 24
40°C	hours	2,4,6,8,10,12,36	2,6,12,18,24,30,36
50°C	minutes	20,40,60,80,100,120	20,40,60,80,100,120

Table 2. Estimated T_{50} in days with 95% lower and upper confidence intervals of *Ascaris suum* and *Ascaridia galli* eggs at different temperatures in pig slurry with and without urea.

	Urea		No urea	
Temperature	*A. suum*	*A. galli*	*A. suum*	*A. galli*
20°C	42.3 (40.0-44.5)	14.1 (9.5-18.7)	242.6 (167.6-317.6)	24.8(16.7-32.9)
30°C	21.8 (17.2-26.4)	1.22 (0.86-1.58)	41.6 (29.4-53.8)	3.81(3.61-4.01)
40°C	0.83 (0.67-0.99)	0.047(0.028-0.066)	.	0.42(0.32-0.51)
50°C	0.06 (0.05-0.06)	0.009(0.007-0.010)	0.08 (0.07-0.08)	0.039(0.03-0.04)

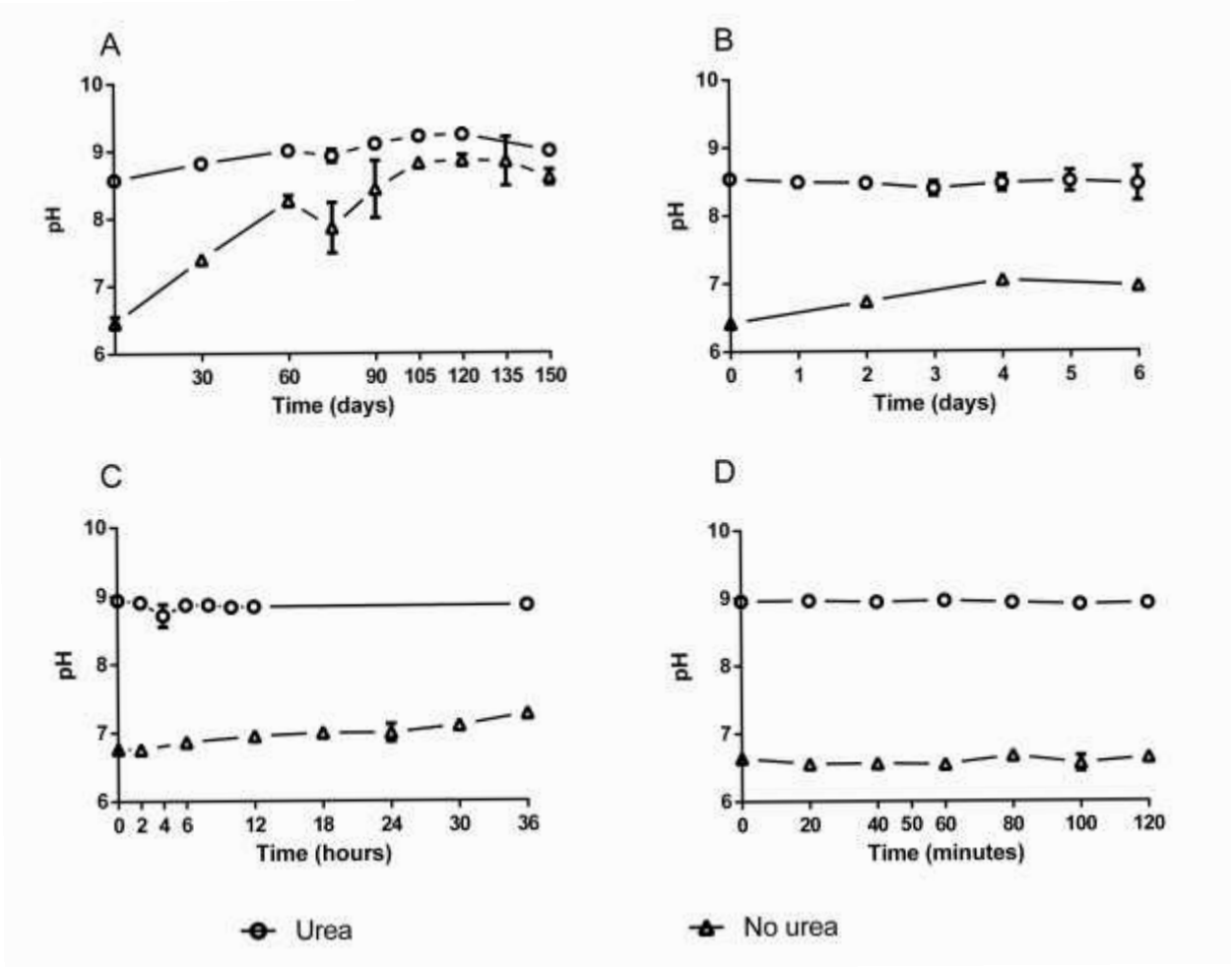

Figure 1.Changes in mean (± SD) pH values during storage of urea-treated and untreated pig slurry at 20°C (A), 30°C (B), 40°C (C) and 50°C (D). All measurements were means of three replicates. Please note that horizontal axes have different scales.

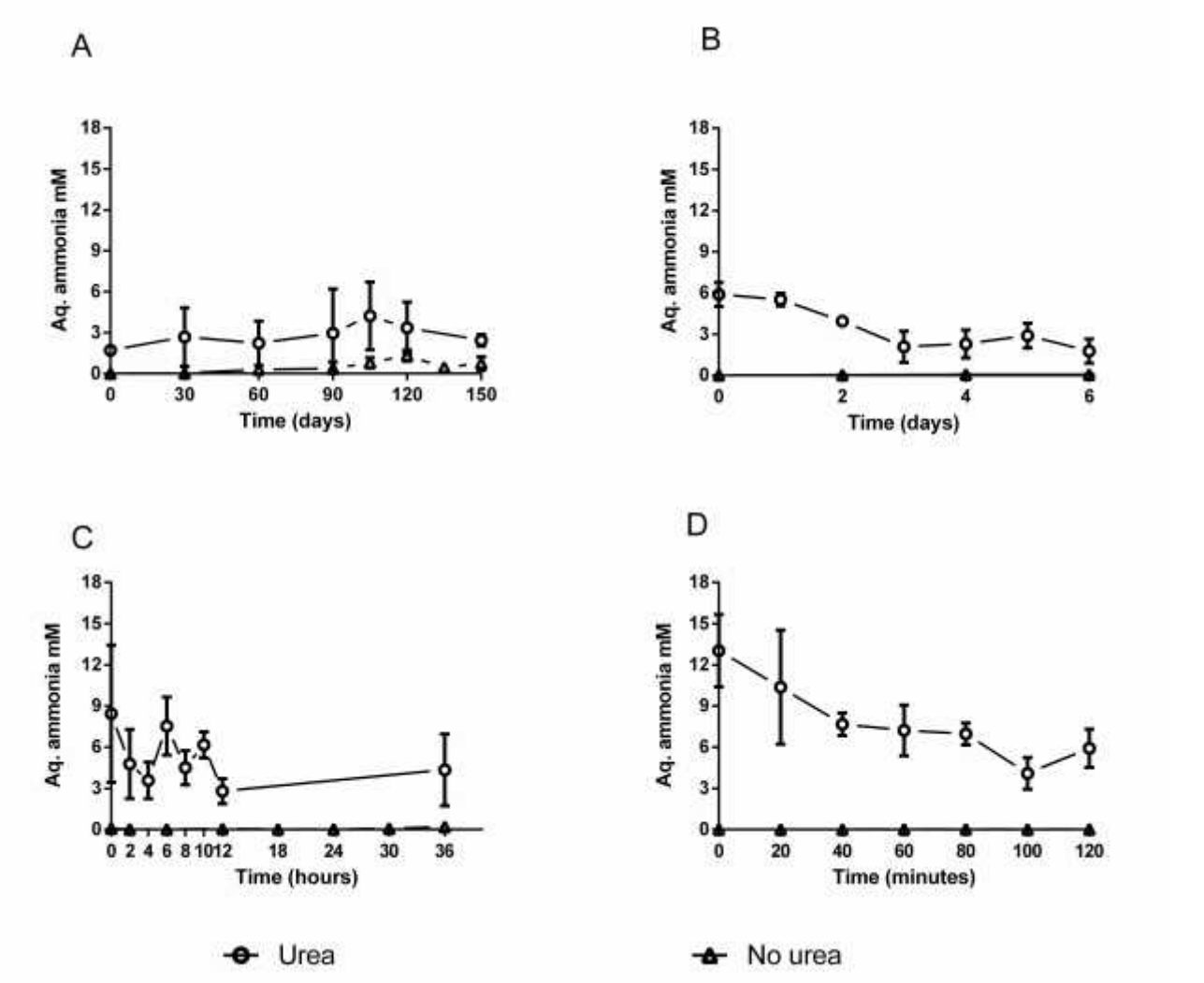

Figure 2. Changes in mean (± SD) aqueous ammonia ($NH_{3\,(aq)}$) concentrations during storage of urea-treated and untreated pig slurry at 20°C (A), 30°C (B), 40°C (C) and 50°C (D). All measurements were means of three replicates. Please note that horizontal axes have different scales.

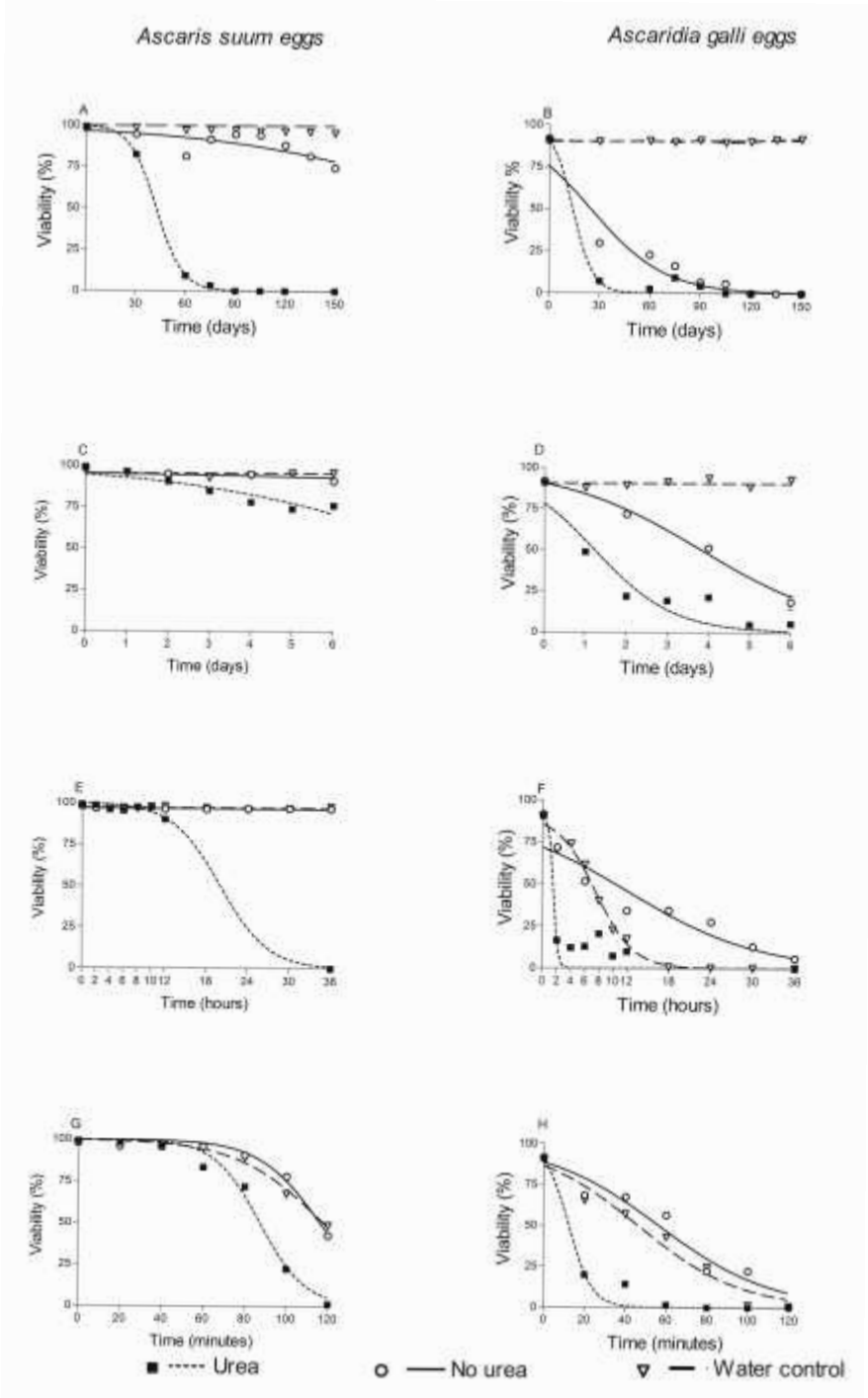

Figure 3. Mean viability of *Ascaris suum* and *Ascaridia galli* eggs in pig slurry with and without urea and in water controls stored at 20°C (A and B), 30°C (C and D), 40°C (E and F) and 50°C (G and H) respectively. All measurements were means of three replicates. Please note that horizontal axes have different scales.

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6.3 Paper 3. Development and survival of *Ascaris suum* eggs in deep litter of pigs

Kiran Kumar Katakam, Stig Milan Thamsborg, Niels Christian Kyvsgaard, Anders Dalsgaard, and Helena Mejer

Submitted to **Parasitology**

Development and survival of *Ascaris suum* eggs in deep litter of pigs

KIRAN KUMAR KATAKAM, STIG MILAN THAMSBORG, NIELS CHRISTIAN KYVSGAARD, ANDERS DALSGAARD and HELENA MEJER*

[1]Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
[2]Section for Veterinary Medicine, Danish Health and Medicines Authority, Copenhagen, Denmark

* Corresponding author:
Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences,
University of Copenhagen, Dyrlægevej 100,
DK- 1870 Frederiksberg C, Denmark.
Tel: +45 35332789, Fax: +45 35331433
E-mail: hem@sund.ku.dk

SUMMARY

Indoor transmission of *Ascaris suum* partly depends on the physico-chemical conditions in bedding material. Temperature, pH, aqueous ammonia, moisture, occurrence and development of *A. suum* eggs were therefore compared in different areas (resting, intermediate and latrine) of two deep litter pens on an organic farm in four seasons. There was some variation, but mean ammonia levels were generally very low (1.0-2.6 mM) and pH levels were moderate (8.04-8.88) in all three areas. Relatively, resting areas were characterized by overall moderate moisture (36%) and moderately high temperature (35.7°C) levels. The area contained few eggs (50 eggs/g DM) of which 17% were viable, and though only 4% were larvated and 0.7% appeared infective, it was more than in the other areas. Intermediate areas had moderate moisture (43%) and high temperature (43.6°C) levels. There were many eggs (523 eggs/g DM), but overall viability was very low (5%) and few eggs were larvated (0.004%) or even infective (0.002%). Latrines typically had high moisture (79%) and moderate temperature (30°C) levels. The concentration of eggs was very high (1444 egg/g DM) and though 32% were viable, none had developed larval stages. The large majority of *A. suum* eggs appear to die and only few become infective while in the deep litter. However, a large fraction of eggs may remain viable for some time and could thus contaminate agricultural land and develop to infectivity, if the manure is not composted appropriately.

Keywords: deep litter, bedding material, *Ascaris suum* eggs, development, viability, temperature, ammonia, pH, moisture

INTRODUCTION

The pig roundworm, *Ascaris suum* is the most common nematode parasite in pigs reared in any kind of management system (Roepstorff *et al.* 1992; Roepstorff, 1997 Roepstorff *et al.* 1998). The eggs of *A. suum* are highly resistant to adverse environmental conditions and to a range of chemicals (Barrett *et al*., 1976; Krishnaswami and Post, 1968; Roepstorff and Murrell, 1997). This makes *A. suum* infections difficult to control, but allows the eggs to be used as indicators of the level of hygienisation taking place in compost material, including deep litter bedding.

Concern for animal welfare has resulted in new regulations for housing of pigs. EU Council Directive 2001/93/EU suggests the provision of suitable rooting and manipulation materials such as straw, sawdust, wood or peat in order to satisfy the exploratory behaviour of pigs raised indoor, the lack of which may lead to adverse activities like tail biting and aggression (Guy and Edwards, 2006). As a result, there is an increased focus on alternative housing and production systems, including systems with deep litter pens (Petit and van der Werf, 2003; Honeyman *et al*. 2005). Deep litter pens may vary in their design, e.g. a typical deep litter pen in a Danish organic pig fattening farm is characterized by an indoor area covered with barley, wheat or oat straw and an outdoor area with a partially slatted concrete floor. If pigs defecate indoor, the bedding material is continuously mixed with faeces and urine. Fresh straw is added regularly resulting in the build-up of a litter-bed. Removal of deep litter generally depends upon season, number of pigs and the convenience of the farmer. During this period, the organic matter in the litter may, depending on the availability of oxygen, biodegrade through composting, resulting in generation of heat as well as production of ammonia and volatile fatty acids (Kirchmann and Witter, 1989) which are toxic to most pathogens (Kunte *et al*. 2004; Nordin *et al*. 2009). To what extent this takes place under farming conditions is largely unknown. Most of the studies on deep litter housing address issues on animal welfare, pig management, pig production and ammonia emission (Groenestein and VanFaassen, 1996; Hill *et al*. 1998; Beattiea *et al*. 2000; Gentry *et al*. 2004; Morrison *et al*. 2007) while few studies have investigated risk of pathogen survival and transmission, e.g. parasites, in animals kept in deep litter bedding (Holmgren and Nilsson, 1998). None of the studies hitherto have focused on pathogen survival and described the actual habitat conditions like temperature, moisture level and ammonia concentration in deep litter in different parts of pens. Thus, it is unknown whether deep litter bedding constitutes a risk factor in the epidemiology of *A. suum* infections.

The aim of the present study was to determine the distribution of *A. suum* eggs and impact of the different physico-chemical conditions, prevailing in deep litter bedding material in an organic pig farm, on development and viability of eggs.

MATERIALS AND METHODS

Study design

The study was conducted as a repeated cross-sectional investigation in a Danish organic pig farm with 200 sows where finishers were kept on deep litter in a barn. It was evident from preliminary farm visits that parts of the pens were used for different purposes by the pigs and could be designated as a clean resting area, a dirty defaecation (latrine area) and an intermediate area (transition zone) covering around 40%, 40%, and 20% of the surface of the pen. Duplicate samples of bedding material were collected in September 2011, December 2011, March 2012 and June 2012 from different depths of the deep litter in the three areas in each of two pens with finishers of approx. 5-6 months of age. The bedding material was examined for the total number, development and viability of *A. suum* eggs in relation to temperature, moisture content, pH and ammonia content of the bedding material.

Farm and pen description

Farrowing took place outside on pasture and piglets were moved indoors after weaning at 7-8 weeks of age to a stable unit with shallow litter (10-20 cm deep). At the approx. age of 12-16weeks the pigs were then moved into the finishing stable with deep litter (up to 85 cm deep). This stable contained a total of 28 pens of equal size, 14 pens on each side of a wide aisle. Each pen housed 20-35 finishers and consisted of an indoor area (10m x 3.9m) with a concrete floor which was covered with barley straw, and access to an outdoor area (10m x 3.9m) with a partially slatted concrete floor. Automatic feeders, drinkers and a sprinkler system were all located in the outdoor area. The indoor and outdoor areas were connected by a small opening covered with rubber sheets, which allowed pigs to move freely while reducing influx of cold air to the indoor area. Pigs defaecated both inside and outside. Inside the pens, the pigs defaecated in the part of the pen facing the aisle, creating a latrine area where the straw was heavily mixed with urine and faeces. The adjacent area was characterized by some contamination with urine and faeces, but to a lesser extent compared to the latrine, and was considered as the intermediate area. The part of the pen closest to the outdoor area contained straw that appeared dry and clean, and thus with no apparent contamination with urine and faeces, was designated as the resting area. Fresh straw was spread on top of the latrine area once or twice a week. This led to a high build-up of material in the latrine area with a gradual decline in thickness towards the resting area.

Faecal samples

At each visit, rectal faecal samples were collected from 10 finisher pigs in each of the two pens. Faecal egg counts were estimated by a concentration McMaster technique (Roepstorff and Nansen, 1998) with a sensitivity of 20 eggs per g (epg) faeces using a flotation fluid of a saturated NaCl solution with 500 g glucose/L (specific gravity 1.27 g/mL).

Collection of bedding material

In each area, a rectangular pit (approx. 20 x 40 cm) was dug all the way down to the concrete floor. The depth of the pit varied for different areas, pens and seasons. Starting from the surface, the deep litter was divided into layer 1 (0-10 cm), layer 2 (20-25 cm), layer 3 (30-45 cm), layer 4 (50-65 cm) and layer 5 (70-85 cm). The temperature was measured in each layer at either side of the pit by inserting a sensor (NavTMP, Forston Labs, USA) horizontally approx. 10 cm into the solid litter. Air temperature of the pens was also measured using the same sensor once at the time of sampling during each study period. Samples of approx. 500 g bedding material were collected from each of the layers at either side of the pit. A representative sub-sample of each of the samples was immediately placed in an air tight plastic bag and stored at -20°C for later estimation of ammonia content. The remaining bedding material was homogenized by cutting it into pieces of up to 5 cm length that was thoroughly mixed before sub-sampling. Sub-samples were then stored at 5°C for later measurement of moisture content, measurement of pH and isolation of eggs.

Measurement of physico-chemical parameters

The frozen sub-samples were thawed and homogenized by cutting it into pieces of up to 5 cm length and mixing thoroughly. Total ammonia nitrogen (TAN) was extracted from 5 g the sample suspended in 1 M KCl to a total volume of 100 mL and subjected to end-over-end shaking for 45 min. The extracts were then filtered through filter papers (Advantec TM No. 5A) and stored at -20°C. The TAN concentration was later measured in the thawed extracts by a flow injector analyser system (Lachat Instruments Division, Milwaukee, WI, USA). $NH_{3\,(aq)}$ concentrations were calculated using TAN, pH and dissociation constant (pK_a) values as described by Armstrong *et al*. (1978).

Moisture content was measured by drying a 5 g refrigerated sample at 105°C for 24 h. pH was measured with a pH sensor (NavPHA, Forston Labs, USA) in a 5 g sample diluted 1:15 in deionized water.

Isolation of eggs and quantification of different developmental stages

Eggs from 5 g of a refrigerated litter sample were isolated by soaking the material in 1M NaOH for 16-18 h as described by (Larsen and Roepstorff, 1999). The material was then washed through a 212 µm mesh size sieve on to a 20 µm sieve and the collected residue was transferred to a 50 ml centrifuge tube using tap water (total volume of sediment 10 mL). Flotation fluid (see above) was then added to a total volume of 50 ml and centrifuged for 7 min at 253 g. The supernatant was transferred to a container and the pellet was re-suspended with flotation fluid after which centrifugation and collection of the supernatant were repeated. The supernatant was washed with tap water on a 20 µm sieve and the residue was collected in a centrifuge tube. The sample was centrifuged for seven min at 253 g, the supernatant was removed and the pellet was re-suspended by adding flotation fluid (1:8). For samples with low numbers of eggs, the entire sample was transferred to McMaster chambers and all the eggs were counted. For samples containing large quantities of eggs, only 20% of the sample material was examined to estimate the total number of eggs. For each sample, up to 50 eggs were examined microscopically (200x) to differentiate different developmental stages and the findings were extrapolated to obtain total counts. Eggs with no development were considered as undeveloped (unembryonated) eggs; eggs which had displayed early development (a single condensed cell in the middle to multicellular stages) as pre-larval stage eggs; eggs at late development (thick early larva to a slender and fully developed larva) as larvated eggs; and eggs with vacuolization of the cytoplasm and irregular shape or structure as non-viable eggs.

Viability of unembryonated A. suum *eggs*

Additional eggs were isolated from each straw sample by soaking 10-60 g of litter material in tap water for 16-18 h, followed by washing and flotation as described above. Viability of seemingly normal but yet un-embryonated or partially embryonated eggs was assessed by their ability to embryonate. The isolated eggs were kept in H_2SO_4 buffer (pH1) for 100 days at 22°C. Eggs with fully developed larvae were considered as viable and the percentage of viable eggs was estimated by evaluating up to 50 eggs microscopically. All eggs were examined in samples where the recovered number of eggs was below 50.

Statistical analysis and calculations

Different physico-chemical parameters (temperature, $NH_{3\,(aq)}$, pH and moisture content), level of litter contamination with *A. suum* eggs (total number of eggs/g dry litter) across different pen areas, depths and study periods were compared by analysis of variance (ANOVA) using PROC GLM (SAS version 9.2, SAS institute Inc., 2000-2008). Area, depths and study period were categorical variables and the measurements of the different physico-chemical parameters and number of eggs/g dry litter as response variables. Due to

non-normal distribution of data, transformations were carried out to temperature, $NH_{3\,(aq)}$ and number of eggs (total eggs and viable eggs) data for a better fit to model assumption. For analysing development and viability of eggs, temperature measurements were classified as low or high if the temperature was ≤ 28°C or > 28°C, respectively. Moisture measurements were classified as low (≤ 40%), medium (40% to 80%) or high (>80%). pH was classified as low (≤8) or high (>8). $NH_{3\,(aq)}$ measurements were classified as low (≤ 30 mg/L) or high (>30 mg/L).

Percentage of developing of eggs, number of viable eggs/g litter and percentage of viable eggs were compared by ANOVA using the *lm* package in R (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) by using area, depth, season and all the physico-chemical parameters as fixed effects. The number of viable eggs was normalized by log (x+1) transformation whereas percent eggs with different development stages and percent viable eggs were normalized by square root transformation.

The number of larvated eggs (before laboratory embryonation) was compared pairwise between different pen areas, layers of litter and study periods by the Kruskal-Wallis test using PROC NPAR1WAY in SAS. Spearman correlation for percentage egg viability with $NH_{3\,(aq)}$ and temperature was calculated using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). The level of significance for all the analyses was $P < 0.05$.

The total number of eggs of different types (total eggs, larvated eggs, eggs with fully developed larva and viable eggs) per g straw (wet weight) was determined and used for estimating the approximate content in the total bedding material in each area. The density of bedding material was estimated by measuring the volume occupied by five g of bedding material from each area. The total volume of bedding material in each area was determined by measuring the length, width of respective areas and the height of the bedding material in that area. The total mass (m) of bedding material was calculated by using the volume occupied by it in a given area and density (d) of respective bedding material (m=v*d).

RESULTS

General observations

The height of the bedding material ranged from 30-65 cm in the resting areas, 30-55 cm in the intermediate areas and 40-85 cm in the latrine areas during different study periods. The straw in the latrine areas was moist, strongly interlaced, highly compacted, difficult to dig into and heavily mixed with faeces and urine. The straw in the resting area was drier (except the bottom layer sampled in December 2011 and March

2012), looser, easier to sample and the pigs rooted in it more often than the other areas. The conditions of the straw found in the intermediate areas were similar to the latrine areas, but there was less faeces and urine.

The prevalence of *A. suum* in the finisher pigs was 100%, 95%, 75% and 75% and the mean egg excretion was 5000, 2026, 1116 and 787 epg in September 2011, December 2011, March 2012 and June 2012, respectively.

Temperature

The air temperature of the pens was 18.8°C, 1.2°C, 5.2°C and 16.8°C during September 2011, December 2011, March 2012 and June 2012, respectively. The mean temperature (min-max) of the litter across depths was 35.7°C (23.2-69.0°C), 43.6°C (33.8-57.0°C) and 30°C (18.9-53.8°C) in the resting, intermediate and latrine areas, respectively, and there was an overall significant difference in temperature between the areas ($P<0.0001$) (Fig. 1A). In general, the September 2011 temperatures were higher in all areas compared to the other time points ($P<0.0001$). As the depth of the deep litter increased, the temperature declined in all the areas ($P<0.0001$), but the decline was very sharp in September 2011 compared to the other time points indicating a significant interaction between study period and depth ($P=0.034$). In general, the temperature in the different layers of the three areas did not vary much between the different time points except in the latrine area where the deep litter in September 2011 was warmer at all the depths indicating a significant interaction of study period and area ($P<0.0001$). The majority of samples (92%) in intermediate area and some of the samples in resting area (25%) and latrine areas (13%) showed temperatures ≥38°C which is considered as higher threshold for development of eggs (Seamster, 1950) at the time of sampling.

Aqueous ammonia ($NH_{3\,(aq)}$)

The $NH_{3\,(aq)}$ concentration in the bedding material varied significantly with pen area ($P<0.0001$). Of the three areas, samples from the intermediate areas had significantly higher $NH_{3\,(aq)}$, concentrations followed by those from the resting and latrine areas (Fig. 1B) with mean (min-max) $NH_{3\,(aq)}$ concentrations of 2.6 mM (0.04-5.8), 1.5 mM (0.006-17.3) and 1.0mM (0.01-6.7), respectively. The $NH_{3\,(aq)}$ concentration also varied with study period ($P<0.0001$) and was highest in September 2011 and lowest in June 2012. Overall, the depth of the deep litter also had an effect on the $NH_{3\,(aq)}$ concentration ($P<0.0001$) as the concentration decreased with increased depth. This was prominent in resting areas compared to the other two areas indicating a significant interaction of depth and area type ($P=0.002$).

Moisture content

The bedding material in the latrine area had the highest moisture content, irrespectively of depth, as pigs defaecated and urinated in this area, followed by the intermediate and resting areas with means (min-max) of 79% (54-88%), 43% (14-86%) and 36% (14-84%), respectively (P<0.0001) (Fig. 1C). Samples from March 2012 had significantly higher moisture content compared to those in other time points (P<0.0001). Urine had accumulated in the bottom bedding material layers of all three pen areas in December 2011 and March 2012 and hence samples from these layers had significantly higher moisture content compared to the top layers (P<0.0001).

pH

The pH varied significantly with area (P<0.0001) (Fig. 1D), study period (P=0.0347) and depth (P<0.0001). Samples from the intermediate areas (mean (min-max): 8.88 (6.82-9.31)) had the overall highest pH followed by the resting (8.60 (6.57-9.36)) and latrine areas (8.04 (6.50-8.95)). Though statistical analysis revealed significant effect of study period and depth on pH, no clear pattern was observed.

Contamination levels of A. suum *in litter*

The total number of eggs per g dry straw varied significantly with area (P<0.0001), study period (P<0.0001) and depth (P<0.0001). Latrine areas contained the highest number of eggs followed by the intermediate and resting areas (Fig.2) which was reflected in the estimated total numbers of eggs in the entire volume of bedding material in the various areas (Table 1). The mean (min-max) number of eggs per g dry straw in latrine, intermediate and resting areas was 1444 (37-11,317), 523 (0-7,791) and 50 (1-434), respectively. Except for the resting areas, the highest numbers of eggs were found in September2011 followed by December 2011 whereas egg counts were lowest, but comparable, in March 2012 and June 2012. As the depth of the litter increased, the number of eggs decreased and the decrease was overall more prominent in latrine areas indicating an interaction of depth and area on egg numbers (P=0.012).

Development of eggs within the litter

Sampling time had a highly significant effect on the development of eggs while they were in the litter (P<0.0001) and the highest proportion of eggs, which had started developing was detected in December2011 (22%) followed by March 2012 (17%), September 2011 (12%) and June 2012 (8%). The pen area also had a significant effect on development (P=0.036) in that the resting area seemed to be the most favourable (Fig. 3) with around 21% of the eggs at some stage of development compared to the intermediate (15%) and latrine areas (9%). Depth of the deep litter also had a significant affect (P=0.037)

and 20% of the eggs had started developing in layer 1 as compared with13%-15% in the underlying layers. Of the physico-chemical parameters measured, only pH significantly influenced egg development (P=0.006) and at the low pH level (≤8)20% of eggs had started to develop whereas this was the case for only 10% of the eggs at the high pH level (>8). The proportion of larvated eggs (before laboratory embryonation) were significantly higher (P<0.0001) in resting areas (4%) compared to intermediate (0.004%) and latrine areas (0%). Similarly, the proportion of fully developed (presumably infective) eggs was higher in resting areas (0.7%) compared to intermediate (0.002%) and latrine areas (0%). This corresponds to the estimated total number of larvated and fully developed eggs in Table 1. Combined across areas, a significantly higher (P=0.03) proportion of larvated eggs (0.1%) was observed during December 2011 than other study periods (September 2011=0.05%, June 2012=0.04% and March 2012=0.02%).

Total number of viable eggs

Following embryonation in the laboratory, the total number of viable eggs varied significantly with pen area (P=0.0001), depth (P=0.0001), study period (P=0.0001), temperature (P=0.0014), pH (P=0.0001) and moisture (P=0.019). The highest numbers of viable eggs/g dry straw were found in the latrine areas followed by the resting and intermediate pen areas which correspond to the estimated total number of viable eggs in the entire bedding material (Table 1). Layer 1 generally contained the most viable eggs as the number of viable eggs decreased as the depth increased. Overall viability was highest in March 2012 followed by December 2011, June 2012 and September 2011. Low temperatures and low pH seemed to enhance viability, and though high moisture seemed to have the same effect, the effect of medium and low moisture levels was less clear. There was an interaction of season and temperature (P=0.0089) and the lowest viability was observed in March 2012 and at high temperature (> 28°C).

Percentage of viable eggs

The overall proportion of viable *A. suum* eggs was significantly influenced by pen area (P<0.0001), depth (P=0.0002) and study period (P=0.0002) (Fig.4). The overall percentage of viable *A. suum* eggs was thus highest in latrine areas (32%) followed by resting (17%) and intermediate pen areas (5%). Viability of eggs was overall highest in layer 1 (30%) as 11%-28% of eggs in the underlying layers were found viable. Viability of eggs was highest in December 2011 (25%) followed by March 2012 (23%), June 2012 (18%) and September 2011 (11%). Viability was also significantly influenced by temperature (P<0.0001), pH (P<0.0001) and $NH_{3\,(aq)}$ (P=0.008) in that high levels negatively influenced the viability. At low temperatures overall viability was 42% whereas at high temperatures it was 13%. Similarly, viability was 37% and 22% at low levels of pH and $NH_{3\,(aq)}$ and only 13% and 7% at higher levels of pH and $NH_{3\,(aq)}$, respectively. Effect of

moisture was significant (P=0.049) and lower viabilities were observed at low (15%) and medium (18%) levels of moisture when compared to that of high (31%) levels of moisture. The Spearman's correlation (r) for viability of eggs with temperatures was -0.53 whereas with $NH_{3\,(aq)}$ was -0.55.

DISCUSSION

The present study determined the development and viability of *A. suum* eggs in relation to selected physico-chemical conditions in deep litter bedding material in an organic pig farm. The study also documented that different areas of a given pen can provide different environments and that the development and viability of *A. suum* eggs may differ accordingly. The majority (99.99%) of eggs did not fully embryonate while in the litter in the pen, however, a sizeable proportion of eggs (19%) remained viable and could complete embryonation (i.e. to fully developed larvae) once removed from the litter and manure. The study further revealed that though *A. suum* eggs could be detected throughout the deep litter, the concentration of eggs in a given area generally reflected the level of faecal contamination of the straw. The eggs recovered from the apparently clean resting areas were probably due to contamination of eggs through passive transfer via the movement and rooting behaviour of the pigs.

The seasonality of the *A. suum* infections in the current study are in agreement with earlier studies (Martini *et al*. 1988; Menzies, 1994) with high prevalence and egg excretion levels seen in late summer-early winter (September 2011 and December 2011). This probably indicates a higher level of transmission of *A. suum* during the summer and early autumn due to high temperatures favourable for the development of eggs both on pastures and in pens (Connan, 1977; Stevenson, 1979; Roepstorff and Murrel, 1997). It can only be speculated if the patent infections were picked up in the farrowing pastures, weaner pens, or the finisher pens. Both weaner and finisher pens might have been contaminated by previous batches of pigs and not cleaned properly or the finisher pens could have been contaminated directly after introduction of the examined finisher pigs. Embryonation of *A. suum* eggs require at least 4-5 weeks at constant ideal temperature (Oksanen *et al*. 1990) and the pre-patent period is 6-8weeks (Roepstorff *et al*. 1997). As most pigs had been in the finisher pens for 2-3 months before the sampling, it is potentially possible that the first eggs excreted in the pen could have become infective and transmission might have taken place. However, the population dynamics of *A. suum* are complex in that protective immunity against migrating larvae (pre-hepatic barrier) and reduced establishment of new incoming larvae due to already established adult worms due to previous exposure (e.g. on farrowing pastures) may have reduced the chance of acquiring patent infections while in the finisher pens (Eriksen *et al*. 1992; Jungersen *et al*. 1999; Mejer and Roepstorff, 2006). Despite finding approximately 10 billion eggs in the bedding material of the deep litter pens, only 0.1

million eggs were fully developed. Thus, the level of transmission of *A. suum* may have been low in the pens due to a low probability of picking these few eggs spread across the deep litter combined with probable acquired immunity due to previous exposure on farrowing pastures.

Though the temperature varied substantially among the different pen areas, it was generally high compared to ambient temperatures, e.g. maximum temperatures above 50°C irrespective on the height of the litter. Similar findings were reported by Sommer and Moller (2000) who observed temperatures of up to 70°C in deep litter of fattening pigs. The high temperatures in the current deep litter indicate that composting took place due to aerobic microbial activity, which may result in the production of heat, $NH_{3\,(aq)}$, CO_2, H_2O and organic acids (Bernal *et al*. 2009). The observed variation in temperature among the three pen areas indicates differences in composting activity, which was found to be higher in the intermediate areas followed by the resting and latrine areas. Several factors like temperature, pH, moisture, porosity (oxygen), C/N ratio and nutrient availability may affect the composting process (Bernal *et al*. 2009). For effective composting, the initial temperature of the material should ideally be 20°C or more (Mosher and Anderson, 1977), the pH 5.5-8 and moisture content 50-70%(de Bertoldi *et al*. 1983; Kalyuzhnyi *et al*. 1999; Petric *et al*. 2009). The composting process is slowed down if the moisture content either drops below 20% due to reduced microbial activity (EPA 1994) or exceeds 70% due to reduced aeration (Bernal *et al*. 2009).

Both excess and limited porosity has a negative effect on the composting process due to loss of heat and creation of anaerobic conditions, respectively (Bernal *et al*. 2009). Though we did not measure porosity of the bedding material, one can assume a high porosity in resting areas as the bedding material was loose and contained no or hardly any faeces and urine. In contrast, porosity was low in latrine areas as the bedding material was highly compacted and heavily mixed with faeces and urine, leaving no pockets of air and thus creating anaerobic conditions. The porosity of bedding material in the intermediate areas was likely to have been more optimal due to the moderate levels of faeces and urine, thus favouring microbial composting which caused the temperature to rise. The combination of different sub-optimal conditions like low temperature, high moisture and low aeration in latrine areas and similarly low moisture and limited availability of nutrients in resting areas might have resulted in low levels of composting as indicated by the low observed temperatures. Higher levels of composting activity in latrine areas in September 2011 might have been due to higher ambient temperatures up to the time of sampling. The decline in temperature with increasing depth might be due to reduced composting activity due to anaerobic conditions as a result of compaction of bedding material and/or high moisture.

Recovery of eggs from latrine areas in September 2011 were different from the other three time points as practically no viable eggs were present which might be due to high temperatures. Lack of or low level development while in the litter and presence of larvated eggs upon *in vitro* embryonation of eggs from latrines area at the other three time points revealed that the general conditions prevailing in latrine areas may temporarily arrest the development of eggs. But once removed from the latrine 23% of the previously unembryonated eggs were still viable and able to complete development. In contrast, the conditions prevailing (e.g. high temperatures) in the intermediate areas inactivated almost all eggs (99.6%). When compared to the other two areas, the resting area created favourable conditions for the development of eggs as it had the highest overall percentage of eggs which had started developing (21%) and a considerable proportion of eggs managed to reach early to late larval stages (4%) in this area. Nevertheless, only 0.7% of the eggs contained fully developed larva. As only 50 eggs were differentiated in each sample irrespective of the number of eggs present, the number of fully developed eggs may have been underestimated in samples with large number of eggs.

Development of *A. suum* eggs depends upon temperature (Seamster, 1950; Arene, 1986; Katakam *et al*., unpublished) and below 14.5°C they stop developing but remain viable for extended periods of time (Seamster, 1950). Higher temperatures adversely affect the eggs either by inhibition of physiological processes, desiccation in the absence of moisture or by increasing the permeability of eggs to toxic substances in the presence of moisture (Seamster, 1950, Barrett, 1976; Barnard *et al*. 1987). The eggs thus stop developing at 38°C and can only remain viable for only eight days at this temperature, after which they permanently lose the capacity to develop (Seamster, 1950). At 50°C, Barnard *et al*. (1987) reported a t_{99} (time needed for inactivation of 99% of eggs) of about 6 h in water for *A. suum* eggs whereas Pecson *et al*. (2007) reported a t_{99} of about 110 min in sludge. The very high proportion of samples with high temperatures ≥38 °C in the intermediate areas might explain the high degree of inactivation of eggs in those areas. In the latrine areas, the temperatures were mostly much lower than 38°C and still only few percent of the eggs started developing and very few percent reached larval stages. This supports that the presumed very low porosity (thus low oxygen) may have been a major inhibiting factor in preventing eggs from developing.

Above the minimum threshold temperature, the rate of development increases with an increase in temperature; at 22°C it takes 28 days to fully develop whereas at 31°C it takes only 8 days (Seamster, 1950). Though higher temperatures increases the rate of development, infectivity of the eggs may be reduced as Arene (1986) reported that eggs embryonated at ≥28°C resulted in larvae with limited ability to migrate *in*

vitro compared to those embryonated at lower temperatures. In the present study, it was mainly eggs in the resting areas that contained larval stages and the majority of samples (90%) in the resting areas had a temperature of ≥28°C indicating that infectivity to pigs may have been low even for fully developed eggs.

$NH_{3\,(aq)}$ levels were highest in intermediate areas and resting areas. This might have been due to overall higher of temperatures and pH in these areas shifting the equilibrium between NH_4^+ and NH_3 towards NH_3 (Philippe *et al*. 2011). The decrease in $NH_{3\,(aq)}$ concentration as the depth of the litter in the latrine areas increased might have been due to volatilization or a decrease in temperature pushing the equilibrium towards $NH_4^+{}_{(aq)}$, the toxicity of which has never been described for microbes. $NH_{3\,(aq)}$ generally has an inhibiting effect on development and viability of *A. suum* eggs (Nilsson, 1982; Katakam *et al*. 2012) and the time needed to inactivate eggs depends on the level of $NH_{3\,(aq)}$. Based on *in vitro* experiments in closed containers, Pecson *et al*. (2007) reported a t_{99} of 87 days at a 58 mM $NH_{3\,(aq)}$ and 25 days at 294 mM at 20°C and at a pH 12 in sewage sludge. Nordin *et al*. (2009) reported a T_{99} of 74 days at a concentration of 58 mM and only 13 days at 220 mM at 24°C in faeces. The observed much lower mean $NH_{3(aq)}$ levels in the present study might be due to evaporation as higher temperatures of bedding material favour conversion of $NH_{3\,(aq)}$ to $NH_{3\,(gas)}$ (Philippe *et al*. 2011). However, it is not known if higher $NH_{3\,(aq)}$ levels may have been present and affected the eggs at some point in time in the microenvironment in the current study. Though the present study revealed a negative correlation between viability and temperature and $NH_{3\,(aq)}$, the exact contribution of each parameter to the inactivation of *A. suum* eggs cannot be ascertained from the present study as the age of the eggs and duration of their exposure to a specific level of each parameter was not known.

In order to have an inhibitory effect on the development and viability of *A. suum* eggs, pH should be above 11.5 (Eriksen *et al.* 1996; Gantzer *et al*. 2001). Though the observed pH was slightly alkaline, the significant effect of pH on development and viability of eggs in the present study might have been indirect as pH affects the equilibrium between $NH4^+$ and NH_3 (Philippe *et al*. 2011).

Moisture in itself plays an important role in the development and viability of *A. suum* eggs and continuous exposure of eggs to low moisture content may lead to desiccation. Seamster (1950) who exposed eggs to relative humidities (RH) of 80%, 85% 95% and 100% at 31.1°C reported that eggs that were kept at 100% RH developed whereas those kept at the lower RH were desiccated. In the present study, moisture may have been a limiting factor for development and viability in resting areas as the average observed moisture contents of the litter was only 36%. Sanguinetti *et al*. (2005), who measured viability as

described in the present study, observed a reduction in viability of eggs when the humidity dropped below 40%.

A previous study by Holmgren and Nilsson (1998) reported that deep litter systems pose a higher risk for helminth infections compared to shallow litter systems, but the present results revealed that most of the eggs were inactivated within the deep litter indicating that this may not be as risky as previously presumed. The study thus demonstrated that the deeper parts of the bedding material in the pens create unfavourable conditions for the development of eggs to infectivity although a significant proportion remains viable. Transmission is, however, unavoidable because eggs become infective in the upper layers of the cleaner parts of the pen. Application of used bedding material (spent litter) to crops without proper treatment may potentially contaminate the fields with *A. suum* eggs. A thorough composting of the bedding material before application to fields that may at some point be used for animals is therefore imperative.

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Table 1. Mean (min-max) number of total eggs, larvated eggs, fully developed eggs and viable eggs (in millions) in bedding material of different areas of a pen during four seasons. Min-max denotes variation between seasons.

Area	Total eggs	Larvated eggs	Fully developed eggs	Viable eggs
Resting	19 (1-56)	0.7 (0.02-1.67)	0.11 (0.06-0.39)	1.0 (0.04-11)
Intermediate	58 (4-215)	0.004(0-0-01)	-	0.2 (0.05-2)
Latrine	10,900 (700-40,730)	-	-	103(18-820)

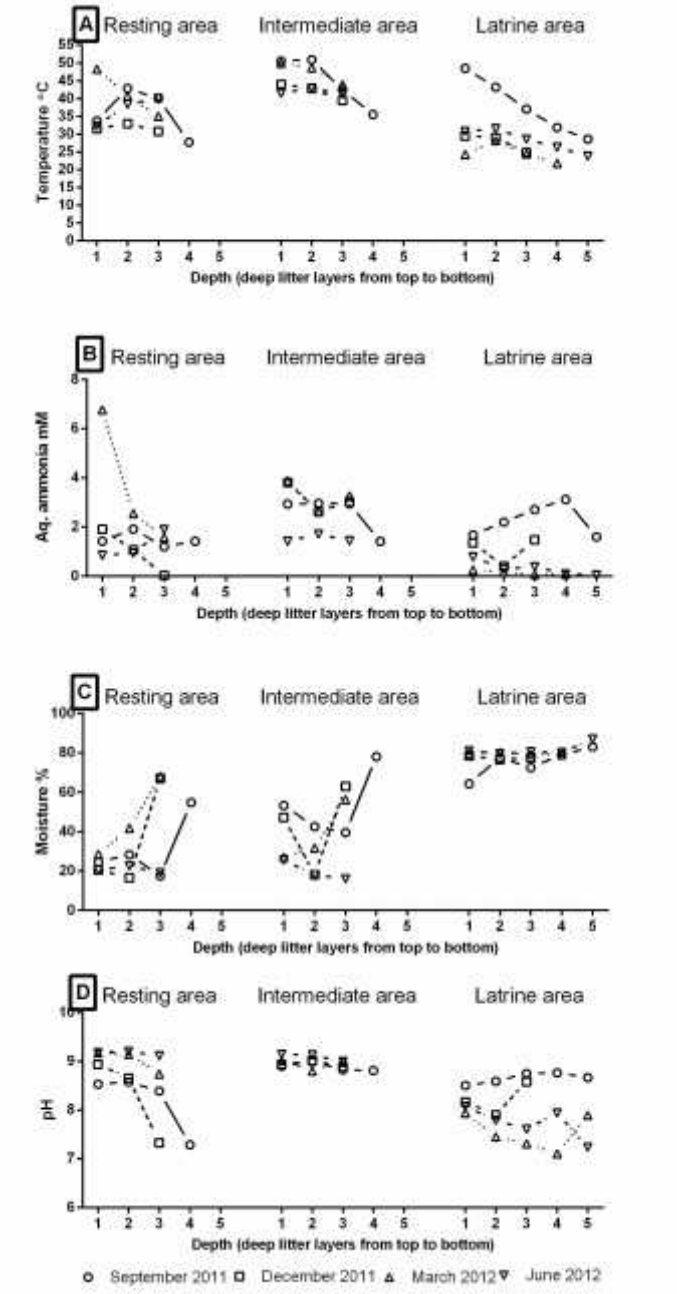

Fig. 1 (A) Mean temperature levels (°C). (B) Mean concentration of aqueous ammonia ($NH_{3\,(aq)}$, mM). (C) Mean moisture content (%) in different layers of litter in resting, intermediate and latrine areas of two finisher pig pens with deep litter at different seasons. Sample depths were: layer 1: 0-10 cm, layer 2: 20-25 cm, layer 3: 30-45cm, layer 4: 50-65cm and layer 5: 70-85cm.

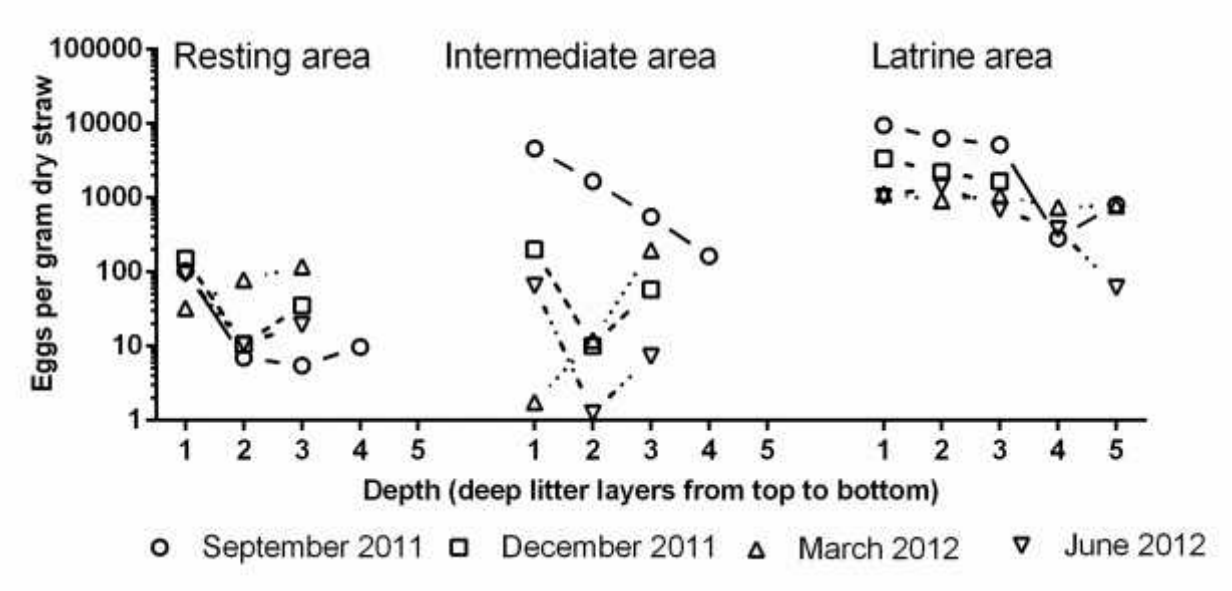

Fig. 2 Mean number of *Ascaris suum* eggs/g dry straw in different layers of litter in the resting, intermediate and latrine areas of two finisher pig pens with deep litter at different seasons. Sample depths were: layer 1: 0-10 cm, layer 2: 20-25 cm, layer 3: 30-45cm, layer 4: 50-65cm and layer 5: 70-85cm.

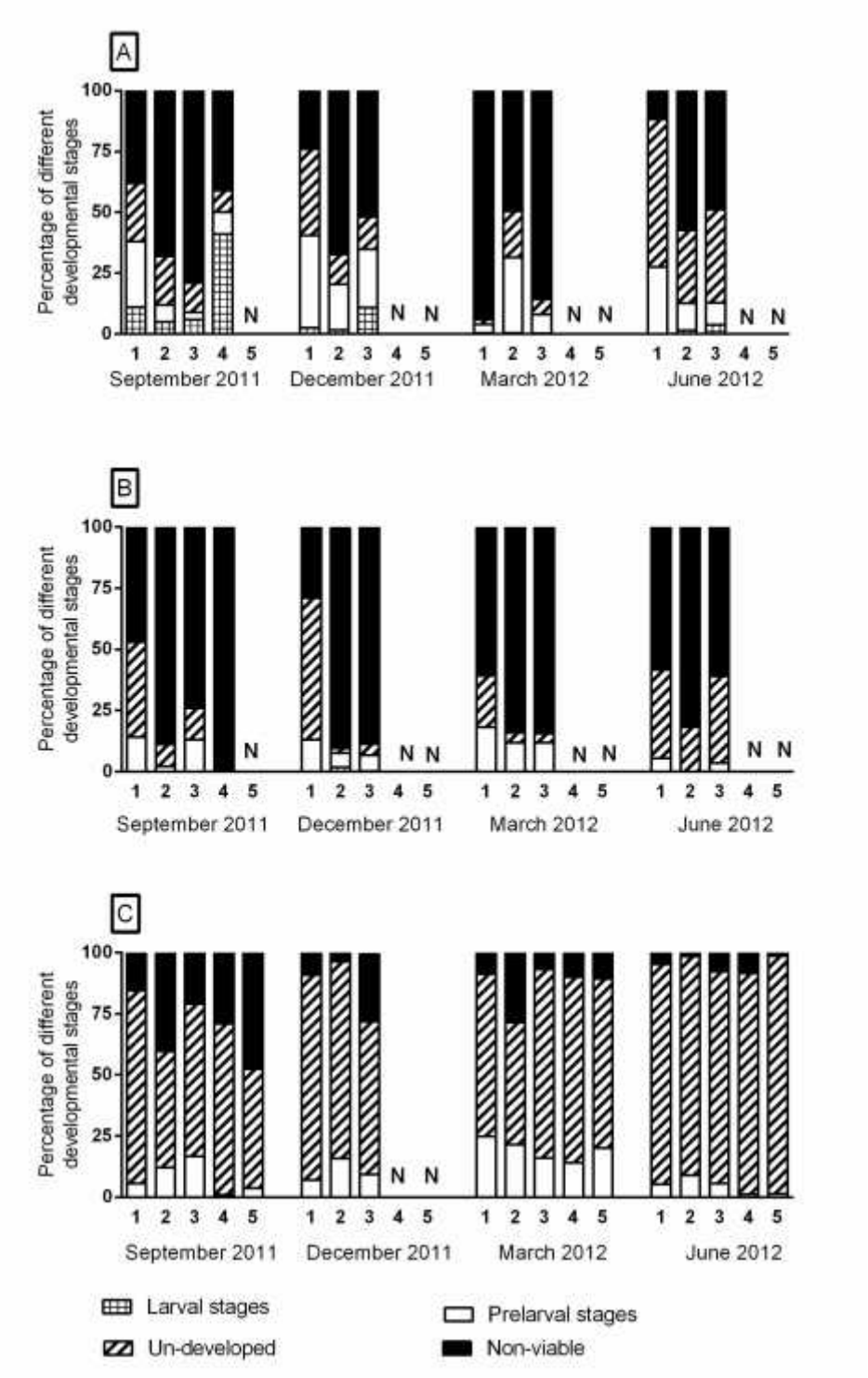

Fig.3 Mean percentage of different developmental stage of *Ascaris suum* eggs in different layers of litter in (A) resting, (B) intermediate and (C) latrine areas of two finisher pig pens with deep litter at different seasons. Sample depths were: layer 1: 0-10cm, layer 2: 20-25 cm, layer 3: 30-45cm, layer 4: 50-65cm and layer 5: 70-85cm. N denotes absence of a given layer in that particular area and season.

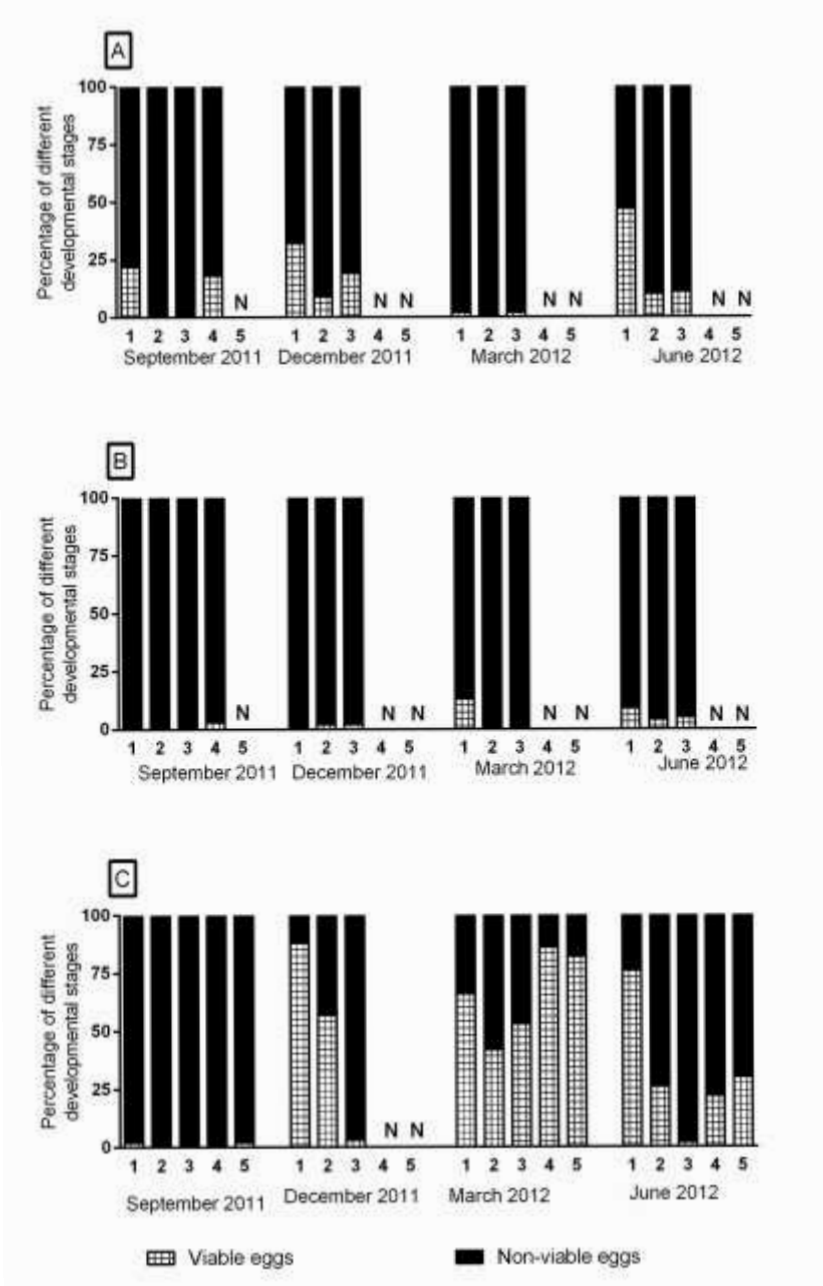

Fig.4 Mean percent viable *Ascaris suum* eggs in different layers of litter in resting (A), intermediate (B) and latrine areas (C) of two finisher pig pens with deep litter at different seasons. Viability was a measure of ability of previously unembryonated eggs to develop in to fully larvated eggs upon *in-vitro* embryonation. Sample depths were: layer 1:0-10 cm, layer 2: 20-25 cm, layer 3: 30-45cm, layer 4: 50-65cm and layer 5: 70-85cm. N denotes absence of specific layer in that particular area and season.

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6.4 Paper 4. Environmental contamination and transmission of *Ascaris suum* in Danish organic pig farms

Kiran Kumar Katakam, Stig Milan Thamsborg, Anders Dalsgaard, Niels Christian Kyvsgaard and Helena Mejer

Manuscript in preparation

Environmental contamination and transmission of *Ascaris suum* in Danish organic pig farms

Kiran Kumar Katakam[1], Stig Milan Thamsborg[1], Anders Dalsgaard[1]and Helena Mejer[1]*

[1]Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

* Corresponding author:
Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences,
University of Copenhagen, Dyrlægevej 100,
DK- 1870 Frederiksberg C, Denmark.
Tel: +45 35332789, Fax: +45 35331433
E-mail: hem@sund.ku.dk

SUMMARY

Although *Ascaris suum* is the most common nematode in pig farms, the on-farm transmission dynamics is not well described. Hence we performed a 1-year field study on organic pig farms, mapping egg contamination levels in pens and on pastures (dry sows, lactating sows and starters) and infection levels in animals. Three different areas were identified and bedding material (top 10 cm) was sampled in each pen (resting, intermediate and latrine areas) in shallow litter housing systems (three farms) and deep litter housing systems (two farms). All areas of the pens were contaminated and levels were similar in both housing systems but generally higher (5-25 times) in latrines compared to the other two areas. Development of eggs was highest in resting areas (44%) compared to intermediate (33%) and latrine areas (13%). Significantly higher numbers of larvated and infective eggs were found in shallow litter (5.1% and 1.8% respectively) compared to deep litter (0.3% and 0.1% respectively; $P<0.0001$). Embryonation of *A. suum* eggs from bedding material revealed that 39-97% of the eggs were viable. The majority of pastures contained infective *A. suum* eggs. Averaged across farms and samplings, the prevalence of *A. suum*, in starters, finishers, dry sows and lactating sows was 48%, 64%, 28% and 15%, respectively. In conclusion, eggs were developing indoors irrespective of housing system, but the development of eggs was limited in deep litter systems compared to shallow litter systems. Hence deep litter systems may not pose an increased risk for *A. suum* infections compared to shallow litter systems. Finding larvated eggs both indoors and on pastures indicates that pigs are under continuous exposure to *A. suum* and transmission is likely happening in both places on organic farms. Strategic use of anthelmintics (e.g. deworming weaned pigs just before shifting them in to indoor pens) combined with thorough cleaning of pens before every new batch of pigs will probably minimize indoor transmission of *A. suum*. Highly infective bedding material should be stored, composted or treated before spreading on crops and never used for pastures.

Key words: *Ascaris suum*, transmission dynamics, viability, deep litter, shallow litter, development of eggs, pastures, bedding material.

INTRODUCTION

In typical Danish outdoor pig production systems, including organic farms, breeding sows and piglets are kept outdoors on pastures while weaned pigs are moved indoors and maintained either in group pens with deep litter (straw added continuously) or in shallow litter pens (less straw and regular removal of bedding material) until finishing. Several studies have shown that such systems may result in an increased risk of parasite infections as compared to intensive indoor production (Roepstorff et al., 1999; Sanchez-Vazquez et al., 2010).

Ascaris suum is the most common nematode of pigs in organic farming (Carstensen *et al.* 2002; Eijck and Borgsteede, 2005) and may cause production losses due to reduced meatiness, reduced weight gain, increased fodder consumption and liver condemnation (reviewed by Thamsborg et al., 2013). A single *A. suum* female worm may produce around 200,000 eggs per day (Olsen *et al.* 1958) and though the large majority of eggs die very quickly outdoors (Larsen and Roepstorff, 1999; Kraglund, 1999), many eggs may remain viable in the environment up to nine years (Kransnonos, 1978).On farms with limited pastures this complicates traditional pasture rotation schemes to control parasite transmission. As *A. suum* eggs need a minimum temperature of 14.5°C for development (Seamster, 1950), eggs can develop only during spring and summer seasons in Northern Europe (Roepstorff and Murrell, 1997; Mejer *et al.* 2000).

With the aim to minimize the use of anthelmintics in organic livestock production and the risk of developing anthelmintic resistance as seen in conventional production systems (Dangolla *et al.*1996) if used too frequently, there is an obvious need to control infections by other means than repeated administration of drugs. Understanding the transmission dynamics of *A. suum* is a prerequisite for the development of alternative strategies in organic and outdoor farming. An earlier study has reported that young pigs can pick up *A. suum* infections on pastures before weaning (Mejer and Roepstorff, 2006) and may thus bring the infection indoors when moved.

The overall aim of the present study was therefore to assess on-farm environmental contamination and transmission of *A. suum* by systematic seasonal investigation of its prevalence in different age groups of pigs and by mapping the level of egg contamination and infectivity of bedding material in different well-defined areas of shallow and deep litter pens and in the soil on pastures.

MATERIALS AND METHODS

Study design

The study was conducted as a non-interventional and repeated, cross-sectional investigation in five Danish organic pig farms: three farms with shallow litter (A, B and C) and two farms with deep litter (D and E) (Table 1). The farms were selected based on a known history of *A. suum* infections and visited four times (September 2011, December 2011, March 2012 and June 2012). Four categories of pigs namely; starter pigs (12-16 weeks of age), finisher pigs (20-24 weeks), dry sows and lactating sows, were selected for the study. Faecal, soil and bedding material samples were collected at each visit and examined for *A. suum* eggs.

Pastures

The farrowing pastures consisted of large areas with smaller paddocks for 1-5 sows separated by a single wire electrical fence that allowed piglets free access to the entire farrowing area. Rotation schemes varied from 9 months to 3 years according to the availability of land (Table 1). In farms (B, C and D) with paddocks for starter pigs (introduced at weaning), the paddock areas were overall semi-permanent, ensuring 6 to 12 month rotation schemes, but fences were at times removed between groups of pigs. The same paddocks could therefore not be identified and sampled each time and there were no starter pig paddocks in use in farm C (June 2012) and farm D (September 2011 and June 2012). In all five farms, slurry and solid manure were used to fertilise land used for agricultural crops and not pastures for pigs.

Stables

In the deep litter farms, bedding material was removed once it reached the height of approx. 80-100 cm whereas it was generally removed prior to introducing a new batch of pigs in the shallow litter farms. New bedding material was added to areas with defaecation (= latrine) once or twice in deep litter farm resulting in high build-up of bedding in these areas with a gradual decline in thickness towards the resting area. Most of the stable facilities were fully closed with access to outdoor concrete runs with partial roofs and partially slatted floors (Farm A, B, D and E (finishers only)), but Farm E had a semi-open stable for starters with outdoor runs. Farm C had a combined semi-open stable for all age groups in which pens only had three walls and the roof only covered two-thirds of the pens so that there was no clear distinction between indoor and outdoor areas. Sprinklers were positioned on the roof on the edge. On each day of sampling, the indoor area of each pen was divided into a resting, intermediate and latrine area based on the level of contamination of bedding material with urine and faeces (Katakam et al., unpublished). The resting area appeared clean, dry and minimally contaminated with urine and faeces, whereas the latrine was wet and heavily contaminated. The zone, bridging the resting and latrine areas, with more moderate contamination

level was defined as the intermediate area. The relative size of each area varied markedly depending on pen, farm and season. In general, resting, intermediate and latrine areas comprised approx. 50-70%, 10-20% and 0-40% of the total pen area, respectively.

Faecal samples

At each visit, rectal faecal samples were collected from 10 starter pigs and 10 finisher pigs in each of two pens. Faecal samples were also collected from 10 dry and 10 lactating sows. All samples were stored at 5°C until processing. Faecal eggs counts were estimated using a concentration McMaster technique (Roepstorff and Nansen, 1998) with a sensitivity of 20 eggs/g and a flotation fluid of saturated NaCl solution with 500 g glucose/L (specific gravity 1.27 g/mL).

Soil samples

At each occasion, soil was sampled from the pastures used for dry and lactating sows on all farms whether or not they contained animals at the time of sampling. Soil was also sampled from starter pastures on farm B, C and D whenever pastures were present. A soil sample was obtained by walking along a 'W' route through a given pasture, collecting approx. 50-80 subsamples (depending on size) of 5-10 g of soil from the top 5 cm. This was repeated along a second route and the two replicate samples per pasture were stored at 5°C until processing. Each soil sample was homogenized by thorough mixing for 30 minutes by hand. Isolation and estimation of the number of eggs in a 5 g subsample of soil per sample was done as described by Larsen and Roepstorff (1999). Dry weight of all soil samples was estimated by drying a 5 g subsample at 105°C for 24 h. All recovered eggs were counted and examined microscopically (200x). Eggs with diffuse dark content were considered as undeveloped whereas eggs with a single condensed cell to multicellular stages as pre-larval. Eggs with an early to late larva were designated as larvated. The latter types, i.e. eggs with a late, slender larva were considered fully developed and thus infective. Lastly, eggs with vacuolization of the cytoplasm and an irregular shape or structure as non-viable while eggs which could not be categorized into any of the above categories were considered as un-identifiable eggs.

Litter samples (bedding material)

Bedding material was collected from all the three different areas from two pens for both starter and finisher pigs. Two replicate samples were collected by walking along two separate 'W' routes in each area and collecting approx. 20 subsamples from the uppermost 10 cm. Samples were stored at 5°C until processing.

The bedding material was homogenized by cutting it into pieces of approx. max 2-5 cm and thoroughly mixing it. A 5 g subsample was then soaked in 0.5 M NaOH for 16-18 hours. Each sample was then washed thoroughly using tap water on a212 µm sieve placed on top a20 µm sieve. The retained material containing eggs in the 20 µm sieve was then transferred to a 50 ml tube to a total volume of 10ml. Flotation fluid was then added to a total volume of 50 ml and isolation and estimation of the number of eggs in the retained material was then done as described by Larsen and Roepstorff (1999). The total number of eggs was counted for most samples, but for samples with large quantities of eggs, the total egg count was calculated from a 20 % subsample. For each sample,50 eggs were examined microscopically (200x) to determine their stage of development as described above and the finding extrapolated to the total counts. The dry matter content of all litter samples was estimated as above.

Estimation of viability

Additional eggs from all the litter samples were isolated by soaking 10-60 g of each sample in tap water and processing it as described above. Control eggs were collected from pigs with high faecal egg counts for each farm. The eggs were isolated by sequential sieving (through sieves of sizes 500 µm, 212 µm, and 90 µm) of the faeces using tap water, followed by collection on a 36 µm sieve. Eggs were isolated from the retained material on the 36 µm mesh size sieve by flotation (Larsen and Roepstorff, 1999).Viability was then estimated by incubating the eggs from bedding material and faeces in H_2SO_4 buffer (pH1) for 100 days at 22°C followed by microscopic assessment of the proportion of non-larvated and larvated eggs in a subsample of minimum 50 eggs. Viability was not examined for eggs from the soil due to the very low numbers.

Liver white spots

In October 2012, 15 – 25 livers (105 in total) were randomly selected from batches of finisher pigs sent to the slaughter house on a given day for each of the farms. The total number of superficial liver white spots were enumerated for each liver irrespective of whether they were of the diffuse granulation-tissue type or lymphonodular type(Roneus, 1966).

Statistical analysis

Statistical analysis of *A. suum* prevalences (not adjusted for false positives) was conducted by PROC GLIMMIX (SAS version 9.2, SAS institute Inc., 2000-2008). The model used binomial distribution of *A. suum* prevalence (dependent variable) and the effects and interaction of pig age group (dry sows, lactating sows, finisher pigs and starter pigs), litter type (shallow litter vs. deep litter) and season (sampling days) as

independent variables. Farms were nested within litter type. Soil contamination levels (total and infective *A. suum* eggs)of pastures for dry sows, lactating sows and starter pigs during different seasons were compared after normalizing the data (log(x+1) transformation)by analysis of variance (ANOVA) using PROC GLM in SAS.

Total number, number of larvated and number of infective eggs were normalized by log(x+1) transformation. Effect of pen area, season and litter type (farms were nested within litter type) on total number of eggs and % of eggs with development was analysed by an ANOVA using PROC MIXED in SAS. Faecal eggs counts in different age groups, in different seasons, numbers of larvated and infective *A. suum* eggs in two litter types were compared by the Kruskal-Wallis test using PROC NPAR1WAY in SAS. The level of significance for all analyses was $P<0.05$.

RESULTS

General observations

Starter pigs generally completely destroyed the grass cover irrespective of season. Pastures for dry and lactating sows had good grass cover during spring to summer, whereas it was sparser and the soil wet and muddy during late autumn to winter. Faeces were deposited over the majority of the pasture areas. Pens in farm A contained no latrine areas as pigs mostly defaecated in the outdoor run with slatted floors and faeces found indoors was removed on a daily basis. On farm B, most of the pigs also defaecated outdoors so that only a few pens contained a small latrine and intermediate areas on occasional samplings. The faeces indoors were not removed manually. On farm C, pigs defaecated in the area between the innermost roofed resting area and the outermost unroofed part of the pen to the extent that they created a large latrine area covering the width of the pen. Pigs on deep litter farms D and E defecated both outdoors and indoors, converting approx. 25-40% of the indoor areas into a latrine.

Faecal egg counts

Pigs on all farms harboured *A. suum* eggs. The overall prevalence was higher in finishers (64%) and starters (48%) compared to dry sows (28%) and lactating sows (15%) (Fig. 1).However, if pigs with faecal egg counts ≤ 200 epg are considered as false positives (coprophagia), the *A. suum* prevalence is reduced to 19%, 33%, 13% and 12% in starters, finishers, dry sows and lactating sows, respectively. *Trichuris suis* eggs, strongyle eggs and coccidian oocysts were also found in all farms.

Statistical analysis revealed an overall significant effect of age group (P<0.0001) and an interaction between age group and season on *A. suum* prevalence (P<0.0001). This may reflect that finishers, dry and lactating sows showed similar prevalences throughout the study whereas starter pigs had overall higher prevalences in September. Age group and litter type also had a combined significant effect (P=0.0005) in that starters had similar prevalences in both litter systems whereas all other groups had slightly higher prevalences in the deep litter systems compared to the shallow litter systems. Statistical analysis revealed interaction of age group, litter type and season (0.0042) on prevalence but no clear pattern of interaction was observed. However, there was no significant combined effect of season and litter type.

Similarly, the overall trend in mean *A. suum* faecal egg counts across farms and seasons differed between age groups (P<0.0001) with highest egg counts in finishers followed by starters, dry sows and lactating sows (Table 2). The mean faecal egg counts across seasons and age groups were generally highest in September 2011 (P=0.02) followed by December 2011 and the counts in March 2012 and June 2012 were lowest and comparable.

Egg contamination of soil

In all farms, un-grazed and grazed parts of the pastures showed similar trends over time within a given farm in the number of total and infective eggs and results were therefore combined (Table 3). *Ascaris suum* pasture contamination levels varied significantly with farm (P=0.0006), season (P=0.0124) and pasture type (P<0.0001) and there was a significant interaction of farm and pasture type (P=0.0003). Overall, soil contamination was highest in September 2011 and lowest in March 2012. Pastures for starter pigs were generally the most contaminated (2-30 fold more than those for sows), although the combined dry and lactating sow pastures of farm E was also very heavily contaminated (Table 3). Only a few infective eggs (farms B: 8% and D: 5%) were observed in starter pig pastures with the exception of farm C (53%). The number of infective eggs significantly varied with farm (P<0.0001) and season (P=0.0011) but not with pasture type. Overall, few infective eggs were found in farm A compared to the other farms.

Egg contamination of top bedding material

Contamination of bedding material significantly differed (P<0.0001) (Fig. 2 and 3)between the three areas with latrines having the highest density of eggs followed by intermediate and resting areas (Table 4). The level of contamination was significantly lower (P=0.0015) in starter pig pens compared to finisher pig pens, corresponding to the egg excretion levels of the two age groups. On farm A, the contamination level was drastically reduced in March 2012 and June 2012 in starter and finisher pens as a result of deworming the

starter pigs January to April 2012 (Fig. 2.1). On farm D, the pigs were dewormed two weeks before the start of the study and contamination levels were thus initially low both in starter and finisher pig pens, but rose before the sampling in December (Fig. 3.1 and 3.3). Litter type and season did not play any effect on level of contamination but interaction between litter type and season (P= 0.0103) had a significant effect on the total number of eggs with highest contamination of deep litter systems in September 2011 compared to shallow litter systems.

There was no effect of litter type on the proportion of developing eggs, but age group (P=0.0014), area (P<0.0001) and season (P<0.0001) had a significant effect. The highest percentage of developing eggs was observed in finisher pig pens (27%) compared to starter pig pens (15%) and in the resting areas (44%) followed by the intermediate (33%) and latrine (13%) areas. Development was more advanced in September followed by March, June and December (Fig. 4). However, litter type had an effect on the number of larvated and infective eggs (P<0.0001). Shallow litter farms contained higher percentage of larvated (5.1%) and infective eggs (1.8%) compared to that of deep litter farms (0.3% larvated and 0.1% infective eggs).

Viability of eggs in bedding material

The viability of the control eggs isolated from fresh faeces ranged from 94-98% depending on the farm. Compared to the controls, eggs isolated from bedding material showed a reduced viability, but overall 31%to 98% of the eggs were still viable (Table 5). Area (P=0.0008), season (P=0.0039) and age group (P=0.0001) had a significant effect on viability. In general, eggs isolated from the latrines had the highest viability followed by the intermediate and resting areas. Viability was lowest in September 2012 whereas the viability in other seasons was relatively uniform. Viability of eggs in starter pig pens was higher compared to that in finisher pig pens. Litter type did not appear to affect viability.

Liver white spots

In total, 92 of 105 livers from finisher pigs had fresh liver white spots (diffuse and lymphonodular). The percentage of pigs with liver white spots was 83%, 87%, 86%, 84% and 96% for farm A (n=18), B (n=15), C (n=19), D (n=25) and E (n=25), respectively. In addition, most livers had low numbers of partially healed older lesions.

DISCUSSION

The present study has investigated the exposure levels of pigs to *A. suum* eggs in organic pig production systems in relation to faecal egg counts as indicators of the on farm transmission dynamics in different age groups. Weaned and starter pigs were seen to bring the infection from the farrowing and weaning pastures into the stables, which were moderately to heavily contaminated. Though the majority of eggs in the bedding material were not infective, transmission did occur. However, deep litter did not seem to pose an increased risk of *A. suum* transmission compared to shallow litter system as previously suggested.

Our results showed that farrowing pastures were contaminated with a considerable number of infective *A. suum* eggs indicating that piglets in organic farms are exposed to eggs early in their life. As piglets move freely on pastures, there is a high possibility of picking up infection due to ingestion of soil accidentally or intentionally. As the pre-patent period of *A. suum* is 6-8 weeks it is difficult to prove on-pasture transmission in piglets based on faecal egg counts. However, based on liver white spot data and presence of intestinal larvae upon necropsy of pigs, earlier studies have shown that piglets get infected on pastures even before three weeks of age (Mejer and Roepstorff, 2006). Observing faecal egg excretion in 48% of starter pigs and contamination of weaning pastures and starter pens in the present study indicate that piglets picked up infective eggs on farrowing pastures and brought the infection to weaning pastures and starter pens.

In general, contamination of farrowing pastures with *A. suum* eggs was lower compared to that of weaning pastures. The observed low contamination of farrowing pastures might be due to low prevalence and low faecal egg excretion of lactating sows as a result of acquired immunity (Jungersen, 2002; Miquel et al., 2005) combined with low animal density (few animals over a large area). In the present study, though higher faecal excretion was observed in September 2011, pasture contamination at that time was lower compared to December 2011 and June 2012 which might be due to degradation of eggs due to UV light, heat or desiccation (Kraglund et al., 1999; Larsen and Roepstorff, 1999; Roepstorff and Murrel, 2001) or time needed for dispersion of eggs. Though eggs can only develop during summer season in northern temperate regions (Larsen and Roepstorff, 1999), infective eggs were observed in all the sampling times indicating persistence of eggs that were developed during summer season. Carstensen et al. (2002) found only 14% of the soil samples from organic sow pastures were positive for *A. suum* eggs which is markedly less than the present study (75%). This might be due to the fact that the farms they chose had started outdoor production only a few years prior to the study whereas in the present study except one farm all others were producing pigs for more than 10 years.

In the present study farmers were practicing grazing rotation of sows for every six months to three years between two areas, except in farm E where three year strip grazing was practiced. The present findings revealed that there is no difference between pastures currently grazed or un-grazed in relation to the level of contamination (total eggs) and the number of infective eggs. This might be due to the survival capacity of *A. suum* eggs which can survive up to 9 years (Kransnonos, 1978). Mejer et al. (2011) reported pastures contaminated with *A. suum* eggs were infective even after 7 years in Denmark. Limited availability of land makes it highly impractical for the farmers in Denmark to follow a grazing rotation with an interval of more than 7-8 years, in fact, making pasture spelling impossible as a means of eliminating *A. suum* eggs on pastures.

In the present study, weaning pastures contained infective eggs in all sampling times indicating the risk of possible transmission during all seasons if starter pigs are kept on it. In contrast, indoor pens contained infective eggs mainly in September 2011. Moving pigs indoors directly at weaning instead of a shorter or longer stay on weaning pastures may prevent picking up of new infections (except during summer and early autumn) besides preventing further contamination of pastures in temperate regions like Denmark. Finding fresh liver white spots along with scars (resolved white spots) in finishers in the present study indicates continuous exposure of pigs to infective eggs in the pens. Similar findings were reported in pigs on continuous pastures by Roepstorff and Murrell (1997). However, in the present study liver white spot data were collected only once (October) which represents eggs deposited in the bedding material in late summer and early autumn and hence, no inferences can be made for other seasons. Sanchez-Vazquez et al. (2012) who monitored prevalence of *A. suum* based on liver white spots in abattoirs in England reported a seasonal variation of prevalence with peaks in summer and early autumn.

Though considerable numbers of *A. suum* eggs were present indoors, overall few had started to embryonate and very few had become infective. The highest degree of development was observed in September which is similar to the findings by Connan (1977) and Stevenson (1979) who studied the *in vitro* development of *A. suum* eggs on the surface of 2% aqueous agar under farm conditions in England. The higher ambient temperatures during summer and early autumn (above the threshold of 14.5°C) ensure better conditions for development compared to other seasons (Seamster, 1950). Presence of few larvated and infective eggs at other sampling times might be due to poor or lack of washing of pens between each new batch of pigs resulting in persistence of previously developed eggs or eggs might be using body heat of the pigs when they lie on the bedding.

In the current study only 0.8% of all the eggs were considered to be infective, whereas Connan (1977) and Stevenson (1979) demonstrated development of 68-94% of eggs to infectivity *in vitro* under farm conditions. They only studied the effect of temperature on the development of eggs and all the eggs were exposed to the similar conditions. In reality on-farm, the microclimate of egg habitats vary greatly from area to area and eggs are exposed to a variety of determinants (e.g. different temperatures, moisture levels and ammonia concentrations) as development of *A. suum* eggs is influenced not only by temperature but also by moisture (Seamster, 1950), ammonia (Pecson et al. 2007; Nordin et al. 2009; Katakam et al. 2013) and pH (Eriksen et al. 1996). The high occurrence of eggs in latrine and intermediate areas reflect the defaecation pattern of the pigs. As there was no apparent faeces in the resting areas it is likely that the *A. suum* eggs present there disseminated from other areas through the movement of pigs. Absence of faeces in resting areas seems to provide favourable conditions for development of eggs compared to intermediate and latrine areas as a higher fraction of eggs was developing in this area. Presence of faeces and urine in intermediate and latrine areas might have inhibited the development of eggs (Nilsson, 1982).

Earlier studies by Holmgren and Nilsson (1998) who studied the occurrence of *Oesophagostomum* spp. larvae in litter samples reported 92.3% of deep litter samples to be positive for *Oesophagostomum* spp. larvae whereas only 42.7% of shallow litter samples were positive. However the present study showed contrasting results with *A. suum* eggs. The relative poorer development of eggs in deep litter systems compared to shallow litter systems in the present study might be due to composting activity in the former systems (Katakam et al., unpublished), resulting in the production of heat, ammonia, CO2, H2O and organic acids (Bernal et al., 2009). Despite of the differences in development of eggs, the observed similar high prevalences in both litter types indicates that pigs probably got infected on outdoor pastures or second, the availability of infective eggs indoors was sufficiently high to ensure infection. As only surface layer (top 10 cm) of bedding material was analysed and only 50 eggs were differentiated irrespective of the number of eggs present in a sample, the number of fully developed eggs overall may have been underestimated.

Estimation of transmission based on liver white spot data also may be a poor indicator of overall exposure level throughout life as pigs exposed to infection may develop strong immunity and show few or no liver white spots due to reduced migration of larvae through liver (Eriksen et al., 1991). Introducing *A. suum* naïve pigs inside the pens 1-2 days and killing at day 14 p.i. may give a good estimate of transmission rate (Reimers et al., 1989).

The reduced viability of eggs (as assessed by embryonation rate) in bedding material compared to the control eggs might be due to desiccation in resting areas (Seamster, 1950) or exposure to ammonia in other areas (Pecson et al. 2007; Nordin et al. 2009; Katakam et al. 2013). The slightly higher inactivation in September 2011 compared to other sampling times might be due to high temperatures during summer and autumn resulting in loss of moisture in resting areas and conversion of less toxic ammonium to toxic ammonia in latrine and intermediate areas (Philippe et al., 2011). As the majority of eggs were still viable, application of bedding material to agricultural crops as fertilizer without storage or composting may pose a greater risk of contamination as there is a possibility of contamination of pig pastures from contaminated agricultural crops due to movement of vehicles or workers (via the tyres or legs).

In the present study deworming of pigs on few instances was shown to drastically reduce the contamination of bedding material with *A. suum* eggs. As it is not possible to prevent the pigs from picking *A. suum* infection on contaminated pastures, deworming weaned pigs just before shifting them to indoor pens may prevent/reduce contamination and thus the risk of indoor transmission. Repeated use of anthelmintics may lead to development of anthelmintic resistance (Nansen and Roepstorff, 1999). Thorough cleaning of pens before each new batch of pigs will probably minimize indoor transmission of *A. suum.*

In conclusion it has been shown in organic production systems, though the environment is heavily contaminated only a very small fraction of eggs are infective in stables, resting areas create favourable conditions for the development of eggs and clearly have higher fraction of infective eggs. Deep litter systems do not pose an additional risk of *A. suum* transmission as compared to shallow litter systems. Though majority of pigs gets infected at weaner/starter pig stage during the pre-weaning period on pasture or on separate paddocks for weaners, pigs at all stages of production are at risk of exposure to infective *A. suum* eggs. As a direct outcome of the study, we recommend composting of all bedding material. To assess the importance of in-door transmission with certainty, we need to look at infection levels in weaner pigs brought in from paddocks.

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Table 1.Farm characteristics of participating farms (A-E).

	A	B	C	D	E
Bedding material (litter type)	Shallow	Shallow	Shallow	Deep	Deep
Depth of bedding material (cm)	≤5 cm	≤25 cm	≤20 cm	≤100 cm	≤100 cm
Stable system	Closed	Closed	Semi-open	Closed	Closed/Semi open
Total number of sows	180		190	150	
Number of starters per pen	20-25	85-90	40-62	55-98	58-65
Number of finishers per pen	13-21	11-24	7-24	30-40	35-40
Size of starter pen (m^2)	27	37- 40	48	60	39
Size of finisher pen (m^2)	14	37-40	48	60	39
Starter pigs	Stable	Pasture/stable	Pasture/stable	Pasture/stable	Stable
Finishers	Stable	Stable	Stable	Stable	Stable
Dry sows	Pasture	Pasture	Pasture/stable	Pasture/stable	Pasture
Lactating sows	Pasture	Pasture	Pasture	Pasture	Pasture
Lactating sows – pasture rotation	One year	One year	Six months		3 year strip grazing scheme*
Dry sows - pasture rotation	One year	One year	Nine months		3 year strip grazing scheme*
Lactating sow pastures (hectare)	1.8		0.8	2	
Dry (weaned) sow pastures (hectare))	1.8		0.8	3	
Breed of sows					

*Dry and lactating sows were kept on the same pasture area

Table 2. Mean *Ascaris suum* faecal egg counts (epg) (min-max) across four seasons in different groups of pigs on five farms (A-E). Min-max denotes variation between seasons for each farm.

Farm	Litter type	Starters (epg)	Finishers (epg)	Dry sows (epg)	Lactating sows (epg)
A	Shallow	280 (41-868)	388 (0-977)	58 (2-146)	3(0-10)
B	Shallow	985 (43-1075)	716 (165-1557)	211 (50-384)	99 (24-234)
C	Shallow	1081 (59-2876)	2232 (787-5000)	183 (0-684)	549 (62-1076)
D	Deep	270 (2-259)	976 (76-1870)	379 (62-904)	30 (0-86)
E	Deep	468 (125-1132)	372 (254-592)	200 (40-300)	0

Table 3. Mean (min-max) number of infective and total *Ascaris suum* eggs in soil (eggs/kg dry soil) from pastures used for starter pigs, dry sows and lactating sows across four seasons. On farm A and E, pigs were always moved directly indoors at weaning. Min-max denotes variation between seasons for each farm.

		Starters		Dry sows		Lactating sows	
Farm	Litter type	Infective eggs	Total eggs	Infective eggs	Total eggs	Infective eggs	Total eggs
A	Shallow	No Starter pigs	No starter pigs	185 (0-377)	725(361-1316)	78 (0-239)	508(188-659)
B	Shallow	290 (0-869)	3656 (1033-2073)	60 (0-112)	123 (60-171)	1710 (353-3264)	2106 (527-3778)
C	Shallow	5397 (842-15758)	10109 (1405-20455)	246 (63-327)	485 (252-927)	261 (0-669)	484 (188-1060)
D	Deep	398 (0-795)	7787 (341-15232)	597 (116-1316)	876 (116-1711)	949 (119-1778)	1556 (357-3333)
E	Deep	No starter pigs	No starter pigs	1000(0-1673)*	4288 (655-9923)*	As dry sows	As dry sows

*Data for dry and lactating sows are identical as they were kept on the same pasture area.

Table 4. Mean (min-max) number of *Ascaris suum* eggs/g dry straw in the uppermost 10 cm of three different areas of pens for starter and finisher pig pens across four seasons on three farms with shallow litter (A, B and C) and two farms with deep litter (D and E). Min-max denotes variation between seasons for each farm.

Farm	Litter type	Starter pens			Finisher pens		
		Resting (eggs/g dry straw)	Intermediate (eggs/g dry straw)	Latrine (eggs/g dry straw)	Resting (eggs/g dry straw)	Intermediate (eggs/g dry straw)	Latrine (eggs/g dry straw)
A	Shallow	23 (2-70)	94 (6-283)	No latrine area	207 (26-549)	668 (44-1538)	No latrine area
B	Shallow	170 (92-280)	351	1965 (1060-2869)	48 (19-80)	93 (22-203)	344(217-521)
C	Shallow	101 (11-204)	589 (94-1056)	1191 (69-3447)	60 (18-111)	391(110-717)	550 (213-1141)
D	Deep	12 (5-19)	343 (48-639)	2936 (11-8681)	40(2-97)	327 (2-589)	1197 (1-2750)
E	Deep	79 (21-173)	364 (38-1122)	348 (90-873)	91 (22-214)	824 (47-2430)	1665 (1392-1869)

Table 5.Mean percent viable (min-max) *Ascaris suum* eggs from three different areas of pig pens on three farms with shallow litter (A, B and C) and two farms with deep litter (D and E) across four seasons. Viability was defined as the ability of eggs to fully embryonate under laboratory conditions after isolation from the bedding material. Min-max denotes variation between seasons for each farm.

		Area types in starter pens			Area types in finisher pens			
Farm	Litter type	Resting (%)	Intermediate (%)	Latrine (%)	Resting (%)	Intermediate (%)	Latrine (%)	Controls (%)
A	Shallow	82(79-86)	87 (82-94)	No latrine	69 (43-87)	84 (80-89)	No latrine	94
B	Shallow	92 (90-95)	92	94 (90-98)	65 (47-84)	66(41-91)	75(50-92)	98
C	Shallow	77 (64-95)	78 (70-82)	86 (82-90)	81 (77-84)	88 (83-90)	90 (86-92)	95
D	Deep	78 (64-93)	81 (70-91)	81 (67-98)	66 (45-78)	68 (31-84)	77 (64-89)	98
E	Deep	77 (47-97)	86 (77-90)	91 (86-95)	51 (39-54)	50 (41-64)	87 (84-89)	97

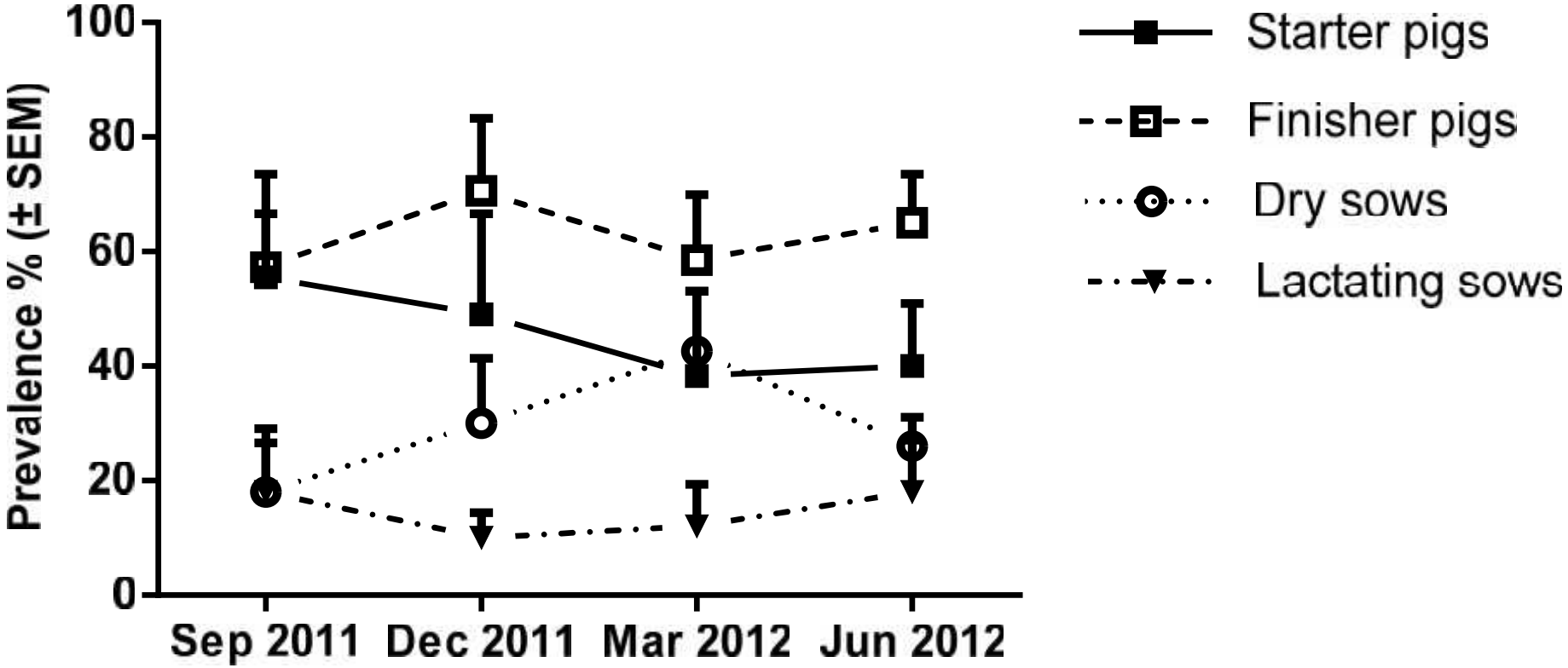

Fig. 1 Prevalence of *Ascaris suum* (% positive faecal samples) in different age groups of pigs across five farms during different seasons

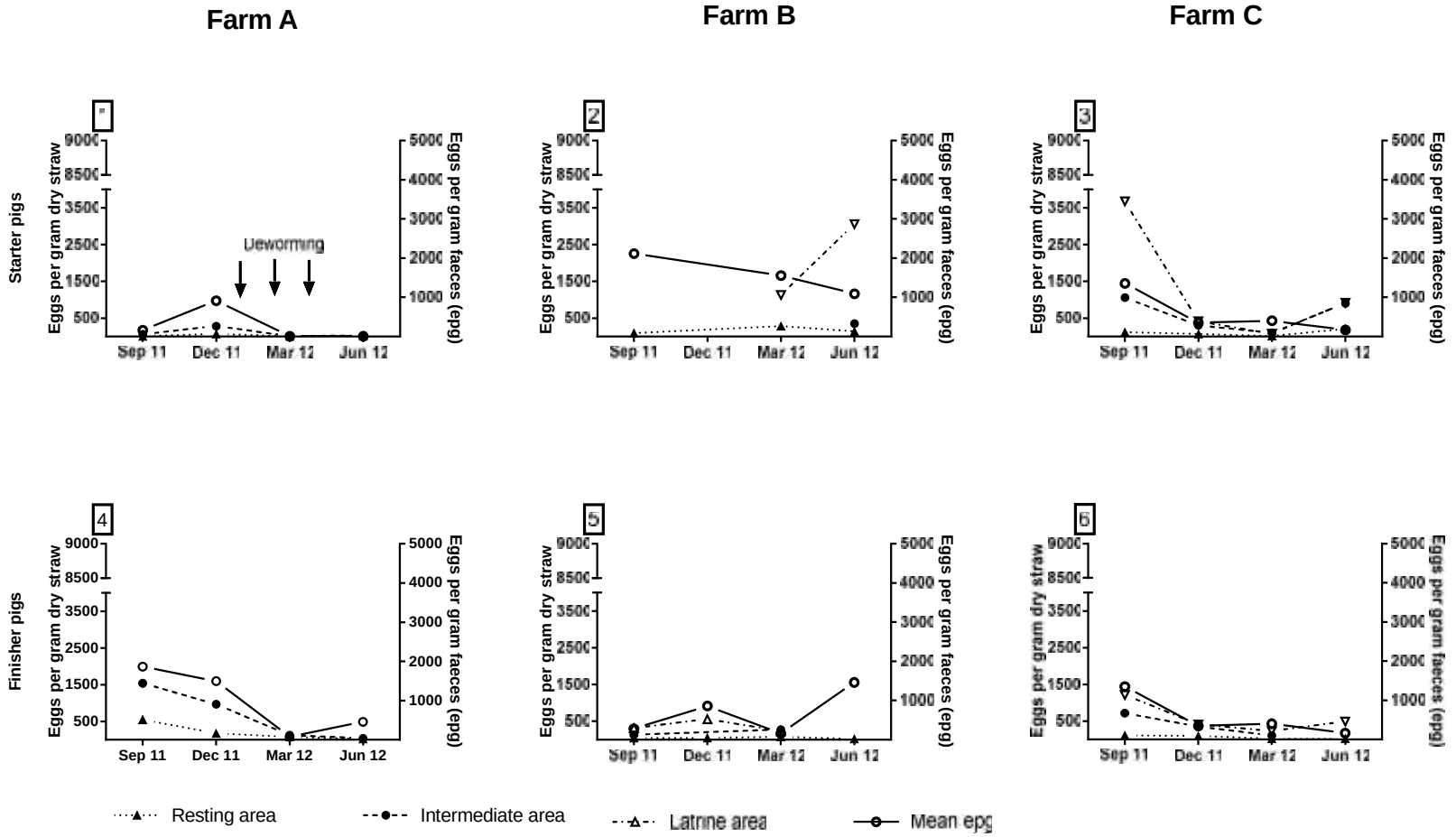

Fig. 2.Total number of *Ascaris suum* eggs/g dry straw (left Y-axis) in resting, intermediate and latrine areas of pens and mean epg of pigs (right Y-axis) for starter pigs (1, 2 and 3) and finisher pigs (4,5 and 6) on shallow litter farms (A, B and C) during different seasons. On farm A, starters were dewormed regularly since December 2011 after second sampling and there were no latrine areas. On farm B there were no intermediate and latrine areas in September 2011 and no pigs in December 2011. There were no intermediate areas in December 2011 in finisher pens on farm B.

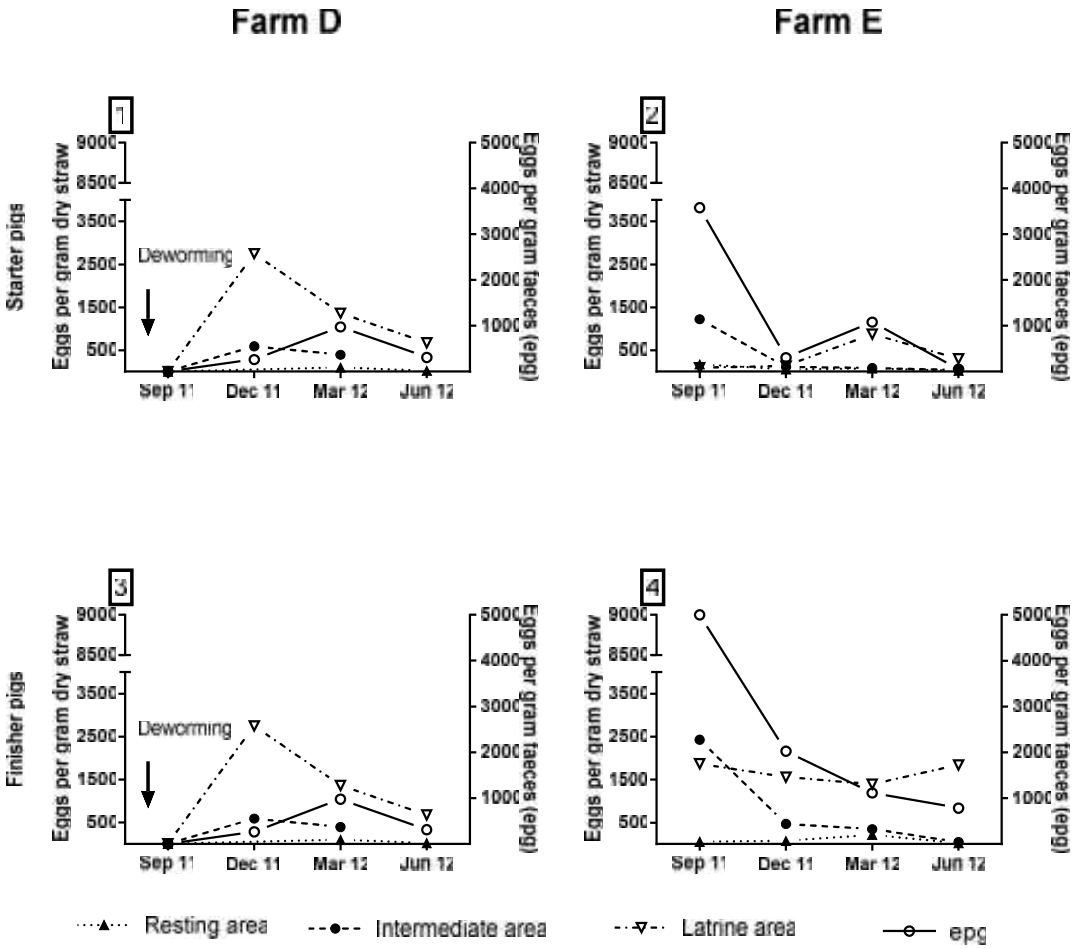

Fig. 3. Total number of *Ascaris suum* eggs/g dry straw (left Y-axis) in resting, intermediate and latrine areas of pens and mean epg (right Y-axis) for starter pigs (1 and 2) and finisher pigs (3 and 4) on deep litter farms (D and E) during different seasons. On farm D, the pigs were dewormed two weeks prior to sampling and there was no intermediate area in finisher pens in June.

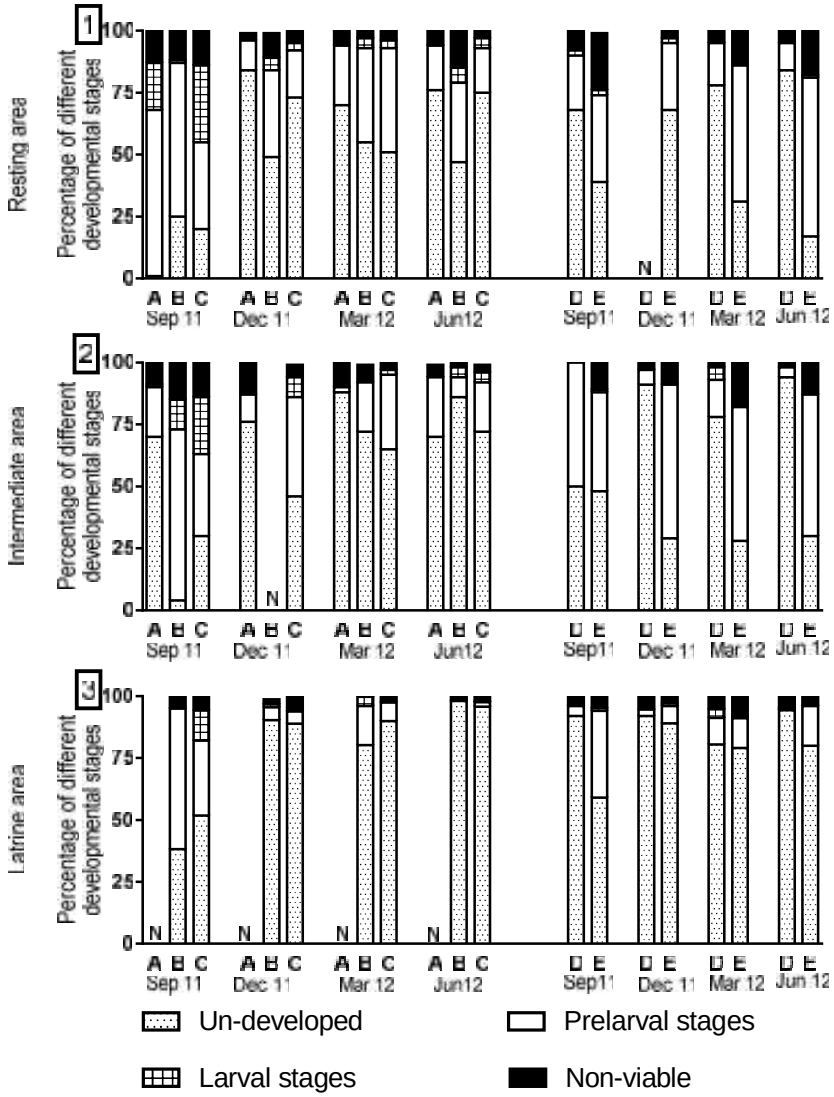

Fig. 4. Percentage of different developmental stages of *Ascaris suum* eggs in bedding material (top 10 cm)from three different areas (resting (1), intermediate (2) and latrine (3) areas) of pens on three shallow litter farms (A, B and C) and two deep litter farms (D and E). N denotes the absence of a given area in that particular farm. Farm E: only finishers were included as the starter pigs were kept on shallow straw.

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PhD student (name and student no.)	Kiran Kumar Katakam
Department	Department of Veterinary Disease Biology
Principal supervisor	Stig Milan Thamsborg

2. **This co-author's declaration applies to the following article or maniscript**
Viability of *Ascaris suum* eggs in stored and separated liquid slurry

The extent of the PhD student's contribution to the article is assessed on the following scale

A. has contributed to the work (0-33%)
B. has made a substantial contribution (34-66%)
C. did the majority of the work independently (67-100%).

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1. Formulation in the concept phase of the basic scientific problem on the basis of theoretical questions which require clarification, including a summary of the general questions which it is assumed will be answerable via analyses or concrete experiments/investigations.	B
2. Planning of experiments/analyses and formulation of investigative methodology in such a way that the questions asked under (1) can reasonably be expected to be answered, including choice of method and independent methodological development.	B

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4. Presentation, interpretation and discussion of the results obtained in article form.	C

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Date	Name	Title	Signature
27 06 2013	Allan Roepstorff	Assocaite Professor	
01.07.2013	Olga Popovic	Post doc	
27/6/13	Niels C. Kyvsgaard	Associate Professor	
27/6/13	Stig Milan Thamsborg	Professor	
20/2/14	Anders Dalsgaard	Professor	

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1. **General information**	
PhD student (name and student no.)	Kiran Kumar Katakam LC3088
Department	Department of Veterinary Disease Biology
Principal supervisor	Stig Milan Thamsborg

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4. Signatures of Co-authors

Date	Name	Title	Signature
26.02.2014	Helena Mejer	Assocaite Professor	
20/2-2014	Anders Dalsgaard	Professor	
26/2/14	Niels C. Kyvsgaard		
24/2/14	Stig Milan Thamsborg	Professor	

5. PhD student's signature
K. Ikuta

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PhD student (name and student no.)	Kiran Kumar Katakam LC3088
Department	Department of Veterinary Disease Biology
Principal supervisor	Stig Milan Thamsborg

2. **This co-author's declaration applies to the following article or maniscript**
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26/2/14	Niels C. Kyvsgaard		
24/2/14	Stig Milan Thamsborg	Professor	

5. PhD student's signature
K. Ikuon Lu

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