Development of a sandwich ELISA for quantification of immunoglobulin G in mink blood

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Preface

Dear Reader,

Scientifur Volume No. 40 (3/4) contains the Proceedings of the IFASA 2016 congress held in Helsinki, Finland of 23-26 August and hosted by the Finnish Fur Breeders' Association. As editors we are pleased to have 71 oral and poster presentations for the IFASA 2016 congress. We were happy to find out that most scientists wanted to give an oral presentation of their work to their colleagues. This was a challenge to the group of editors resulting in a difficult task of disappointing some. Therefore we have done our best to provide good location and sufficient amount of time for poster presentations. The Proceedings contain the papers on the topics: Health and Disease, Breeding, Genetics and Reproduction, Nutrition, Feeding and Management, Behaviour and Welfare and a theme Aleutian Disease.

We would like to thank all those who have made the XIth International Scientific Congress in Fur Animal Production possible in Helsinki, Finland. We hope you will enjoy the congress as well as the Proceedings!

The Editors

Asko Mäki-Tanila
Jarmo Valaja
Jaakko Mononen
Tarja Siren
Olli Vapalahti

Part 1. Health & Disease

Fecal microbiota of healthy and diarrheic farmed arctic foxes *(Vulpes lagopus)* and American mink *(Neovison vison)* – a case-control study

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Abstract

Fecal samples from 21 healthy and 14 diarrheic foxes as well as six healthy and 11 diarrheic mink from ten farms were collected in September 2015. The fecal microbiota was studied using a next generation sequencing (NSG) method. For the comparison of bacterial flora, the V3 and V4 regions of 16S RNA gene were sequenced. For the fecal virome analysis a random amplification followed by NGS was conducted.

The metagenomic analysis suggested that foxes have more diverse bacterial microbiota than the mink (mean Shannon species diversity index 2.7 vs. 2.0; $P < 0.001$). There were no differences in the bacterial species diversity between healthy and diarrheic animals. Diarrheic arctic foxes had more bacteria of the phylum *Firmicutes* than the healthy controls ($P < 0.001$), whereas the healthy foxes had more members of phylum *Bacteroidetes* than diarrheic foxes ($P < 0.01$). In the genus level, the healthy foxes had more members of *Bacteroides* ($P < 0.01$) and *Prevotella* ($P < 0.05$) genera than diarrheic foxes, whereas diarrheic foxes had more genus *Streptococcus* members ($P < 0.01$) than the healthy controls. There were no clear differences in the fecal microbiota of healthy and diarrheic mink. However, there were farm-specific differences in the bacterial microbiota of mink.

Keywords: 16S, metagenomics, gut, bacteria

Introduction

The influence of the gut microbe community on host health has recently become an expanding area of biomedical research. Enteric diseases in fur animals influence animal welfare and induce economic losses for the farmers. Further, some of the suspected enteric pathogens of fur animals, such as *Clostridium perfringens* and the members of genus *Campylobacter* are zoonotic, therefore posing a health risk for the farm workers. However, there is a significant lack of knowledge on the microbial communities of fur animals, as well as on the evidence of causality between distinct potentially pathogenic microbes and enteric diseases. Likewise, the role of bacterial and viral co-infections in enteric diseases of fur animals is ill-known. Importantly, the limited knowledge on the causality between microbes and enteric diseases, as well as the limited availability of diagnostic methods may lead to unnecessary use of antibiotics, which is globally a significant risk for both human and animal health.

The objective of this study was to clarify the role of bacterial microbiota in diarrhea of two species of fur animals: arctic foxes and mink. We conducted culture-independent 16S sequence analysis on bacterial diversity of fecal samples collected from both healthy and diarrheic animals.

Material and methods

The fecal samples of a total of 42 foxes (27 healthy and 15 diarrheic) and 30 mink (18 healthy and 12 diarrheic) were collected from 12 farms in September 2015. Fecal samples from foxes were available from 9 farms. Five of these had both diarrheic and healthy foxes and four had only healthy foxes. Fecal samples from mink were studied from six farms. Four of these had both diarrheic and healthy mink, whereas two had only healthy mink. Three farms had both foxes and mink. Two of these had both healthy and diarrheic animals, whereas one had healthy animals only. From each farm and each species, fecal samples from three healthy and three diarrheic animals (one sample per animal) were collected. The study material is summarized in Table 1.

Table 1. *The number of farms from which the samples were collected. The number of animals is given in the brackets.*

	Species			
	Fox	Mink	Both	**Total**
Healthy only	3 (9)	1 (3)	1 (6)	**5 (18)**
Diarrheic and Healthy	3 (18)	2 (12)	2 (24)	**7 (54)**
Total	**6 (27)**	**3 (15)**	**3 (30)**	**12 (72)**

For bacterial metagenomics analysis DNA was extracted from the fecal samples using PowerFecal DNA isolation kit (Mo Bio Laboratories Inc. Carlsbad CA USA). Sequencing library targeting V3 and V4 regions of prokaryotic 16S ribosomal RNA gene (16S rRNA) was prepared using Illumina 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA) according to manufacturer´s instructions. NEBNext Library Quant Kit for Illumina (New England Biolabs, Ipswich, MA, USA) was used for library quantitation. The sequencing was conducted using the Illumina MiSeq system and MiSeq Reagent Kit v3 with 300-bp paired-end reads. Reads were demultiplexed, adapter sequences were removed, and FASTQ files were produced with the MiSeq reporter. The sequence reads were analyzed using MiSeq reporter 16S metagenomics workflow. Bacterial taxons with >50 reads were included in the subsequent analyses.

Results

The most common bacterial phyla in all studied samples were *Bacteroidetes, Firmicutes Proteobacteria* and *Fusobacteria* (Fig 1). These four phyla accounted for over 99% of all sequence reads with both foxes and mink. The sole exception for this were healthy mink, where the phylum *Tenericutes* (2.8% of the reads) was more common than the phylum Bacteroidetes (1.3% of the reads).

However, there were large differences between the bacterial communities of foxes compared to those of mink. With foxes, a significant proportion of fecal microbiota comprised of the phylum *Bacteroidetes* (45.1% and 29.5%, with healthy and diarrheic foxes, respectively), whereas with mink, only approximately 1% of the reads (both with healthy and diarrheic animals) were classified into this phylum. Accordingly, the phyla *Firmicutes* and *Proteobacteria* predominated the fecal microbiota of mink, since over 90% of the sequence reads from both healthy and diarrheic mink were classified into these two

phyla. Furthermore, at species level, foxes had more diverse bacterial species composition than mink (mean Shannon species diversity index 2.7 vs. 2.0; $P < 0.001$).

The comparison between healthy and diarrheic animals suggested that, diarrheic arctic foxes had more phylum *Firmicutes* bacteria than the healthy controls ($P < 0.001$), whereas the healthy foxes had more members of phylum Bacteroidetes than diarrheic foxes ($P < 0.01$). Accordingly, at the genus level, the healthy foxes had more members of *Bacteroides* ($P < 0.01$) and *Prevotella* ($P < 0.05$) genera than diarrheic foxes, whereas diarrheic foxes had more genus *Streptococcus* members ($P < 0.01$) than the healthy controls. There were no differences in the bacterial species diversity between healthy and diarrheic animals. Notably, on average only 61% (range 38-74%) of the sequence reads could be classified at the species level, whereas on the genus level 90% (82-94%) of the sequence reads were classified.

There were no clear differences in the fecal microbiota of healthy and diarrheic mink. However, there were farm-specific differences in the bacterial microbiota of mink.

Discussion

The intestinal microbiota has a critical role in maintaining gut homeostasis and host health. Driving factors for bacterial diversity in the gut include age (Yatsunenko *et al.*, 2012), diverse lifestyles (Schnorr *et al.*, 2014), diet (Muegge *et al.*, 2011, Vital *et al.*, 2015), host phylogeny and body-size (Godon *et al.*, 2016).

So far, in the family *Canidae* the gut microbiome of domestic dogs has been characterized most thoroughly (Ericsson *et al.*, 2016,Middelbos *et al.*, 2010,Swanson *et al.*, 2011). In agreement with these studies, the fecal samples of arctic foxes were dominated my four bacterial phyla *Bacteroidetes, Firmicutes, Proteobacteria* and *Fusobacteria*. However, the relative abundances of *Proteobacteria* and *Fusobacteria* appear to be different among arctic foxes and domestic dogs, since *Fusobacteria* has been reported to comprise approximately 20% of the bacterial composition of healthy domestic dogs, whereas only 6.8% of the microbes detected from healthy arctic foxes were classified into this phylum. Previously, the fecal bacterial communities have been described from two captive arctic foxes from a zoo (Vital *et al.*, 2015). Overall, the fecal bacterial communities of the fur-breed arctic foxes described in the present study were similar to those described from captive arctic foxes from a zoo. However, the relative abundance of the phylum *Bacteroidetes* was higher (>70%) in the healthy captive arctic foxes kept in the zoo compared to healthy fur breed animals described in our study (45%). This is most likely due to differences in the diets of these animals. It has been shown previously, that carbohydrate digestibility is higher in farm-raised compared to wild arctic foxes (Ahlstrom *et al.*, 2003). Improved digestive capacity may be associated with changes in gut microbiota.

Currently, only limited data is available about the gut microbiome of mustelids. In agreement with our results from mink, the fecal microbiome of ferret *(Mustela putorius furo)* is dominated by two bacterial phyla: *Firmicutes* (approx. 50%) and *Proteobacteria* (approx. 50%), whereas with river otter *(Lontra canadensis)* the fecal microbiome is composed of three phyla *Bacteroidetes, Firmicutes* and *Proteobacteria* with approximately equal abundances (Vital *et al.*, 2015).

Althogether, the comparison between the different species of canids and mustelids suggests both host phylogeny (canid vs. mustelid) driven differences and host diet driven differences. The host phylogeny associated differences may be related to distinct gut physiology. The more diverse fecal microbiota in arctic foxes is complementary to previous findings of higher fermentation rates and activities of bacterial enzymes in colon of silver fox *(Vulpes vulpes)* compared to mink (Gugolek *et al.*, 2015). Mink

and ferrets that show similarities in gut microbiome are both obligate carnivores and largely unable to digest plant matter, whereas arctic foxes are known to feed also on berries and seaweed. Accordingly, mink and ferret lack a cecum and have a short digestive tract with very limited bacterial activity in the colon whereas in foxes some bacterial fermentation takes place in the cecum and colon (Ahlstrom & Skrede., 1998). These physiological and ecological differences are most likely reflected in the species diversity of fecal microbiota.

The major objective of our study was to compare the bacterial communities in diarrheic vs. healthy of fur animals. With arctic foxes there were clear differences between fecal bacterial communities of healthy and diarrheic animals suggesting perturbation of intestinal microbiota during diarrhea. It should be noted however, that these differences do not confirm causal relationship between distinct bacterial taxons and diarrhea, since changes in bacterial communities may also reflect secondary consequences of diarrhea. The causality of bacterial microbiome in diarrhea should be studied further with prospective studies. Further, the role of potential co-infections with viruses should be addressed.

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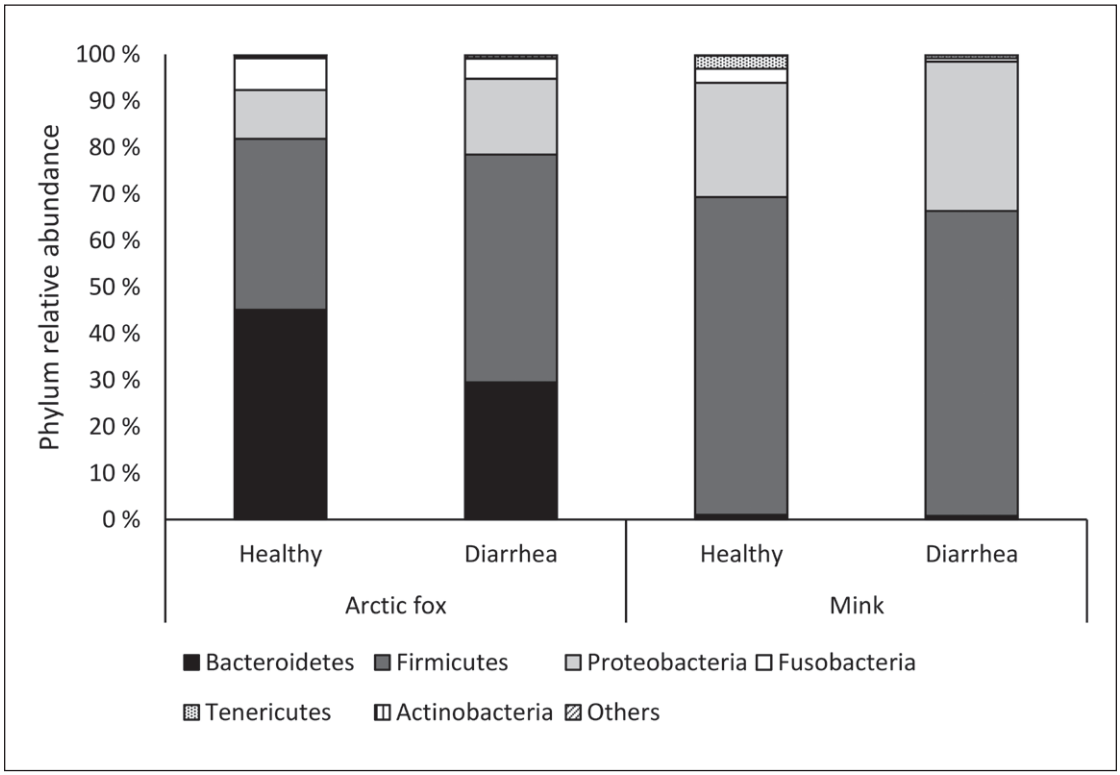
Figure 1. *Phylum level composition of fecal microbiota. Bar chart shows the relative abundances of bacterial phyla in healthy and diarrheic animals. The mean abundances in each group is shown.*



Epidemiologic study on Fur Animal Epidemic Pyoderma in Finland

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Abstract

Background: Fur Animal Epidemic Necrotic Pyoderma (FENP) is a severe skin disease affecting all fur animals farmed in Finland; mink, foxes and Finnraccoons. It manifests differently in all species, but pyoderma and necrosis is the common feature. Signs are severe with even lethal outcome and the disease causes both important animal welfare problems and financial loss. In 2011, an epidemiologic study was conducted concerning FENP in Finnish fur farms during 2009-2011.

Results: The results described the geographical distribution of the disease in Finland, spread of the disease between farms, and the clinical signs in more detail. The study suggested also some possible sources of infection, as well as revealed potential risk factors which may be involved in contributing to the disease.

Conclusions: FENP is an emerging disease among Finnish fur animals. It is a multifactorial disease, in which the bacterium *Arcanobacterium phocae* as well as other infectious factors, for instance a novel *Streptococcus* sp. are involved. However, immunological and environmental factors seem to contribute to the disease as well. By improving the biosecurity level of the farm, including adequate quarantine procedures, farms may reduce the risk of contagious diseases such as FENP.

Keywords: skin disease, mink, fox, Finnraccoon, epidemiologic survey

Introduction

New kind of signs were detected in 2007 in Finnish fur animals. In mink *(Neovison vison)* necrotic pyoderma of the facial skin as well as feet was detected. Blue foxes *(Vulpes lagopus)* experienced severe conjunctivitis, which easily spread to the eyelids and to the entire facial skin. Simultaneously Finnraccoons (*Nyctereutes procyonoides*, a raccoon dog bred for fur), which generally are in very good health, developed painful abscesses in paws between the toes. The disease caused pain and mortality creating a notable welfare problem. It also caused financial loss to the farmers due to the increased mortality as well as due to the diminished pelt quality.

Similar lesions in mink were documented in the USA in 1970 and in Canada in 1996 (1). Afterwards also other countries producing fur animal pelts have encountered the disease. Nordgren *et al.* described signs, gross- and histopathological changes in diseased animals and introduced the name Fur Animal Epidemic Necrotic Pyoderma, FENP, due to the common features detected in all affected species; necrosis and pyoderma. The Finnish study also introduced the bacterium *Arcanobacterium phocae* as a

potential causative organism to FENP (2). The finding of this bacteria was extremely interesting as *A. phocae* was previously isolated in the skin inflammations of the seals (3), and in North-America the signs in mink were simultaneous with the initiation of feeding mink with seal meat (1). In the samples of the diseased animals a novel Streptococcus sp., with probable marine origin too, was detected. However, it was not detected as frequently as *A. phocae*, and it was seldom detected in the samples of the foxes (2).

The disease continued to spread between the farms and on the farms affecting an increasing number of Finnish fur animals and farms. To find out the number of the farms affected, the geographical distribution and spreading of FENP, as well as to describe signs in more detail and to find out possible risk factors or sources to the disease, an epidemiologic survey was performed in 2011 as a joint project between Finnish Fur Breeders' Association (FFBA), Finnish Food Safety Authorities (Evira) and University of Helsinki (UH).

Materials and methods

The study was performed as an epidemiologic questionnaire mail survey. The questionnaire was sent to all farmers belonging to the FFBA (N=958) at the time of the study. The questionnaire concerned the years 2009-2010 and the first six months of the year 2011. The questionnaire consisted of multiple choice questions, yes/no questions and questions where numerical or written data was required.

The questions concerning the signs in different species as well as the characteristics of the diseased animals were asked. The procedures performed to the diseased animals (medication, culling), the medicines used as well as the effect of the medication evaluated by the farmer were inquired. The farm and farmer characteristics were registered as well. The introduction of imported and purchased animals to the study farms were listed out. The health status (other diseases detected on the farm, preventive health care), management procedures (feed and feeding, water and water supply, bedding materials, manure handling etc.) and biosecurity level (quarantine, wash and disinfection procedures etc.) were asked.

The study was analyzed as case-control study. Logistic regression was used to count odds rations and their 95% confidence intervals for potential risk factors.

Results

Questionnaires were returned by 25 % of the farmers belonging to the FFBA. The study farms and farmers were compared to all Finnish fur farms and farmers and they represented the study population well. The preliminary results of the study indicate that the study could reveal some evident risk factors for the spreading of the FENP. The study also clarified the signs in different species detected by the farmers and it yielded information about the geographical distribution of FENP in Finland and the occurrence of FENP on Finnish fur farms. The results also gave new information about the general circumstances on Finnish fur farms.

Discussion

FENP is an emerging fur animal disease in Finland. The etiology of FENP is likely multifactorial. Although there is an association between FENP and *A. phocae* and novel *Streptococcus* sp., also other factors acting as predisposing or contributing risk factors, are involved in the pathogenesis of FENP.

Acknowledgements

We thank all the farmers participating to the study, Maija Mäkinen for her valuable work on handling the returned questionnaires. DVM Heidi Rosengren for participating in the study and questionnaire design, DVM Anna-Maria Moisander-Jylhä for participating in the questionnaire design and FFBA for funding and sending the questionnaires to the farmers.

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Pooling of faecal samples for quantitative virus diagnostics by real-time PCR

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Keywords: astrovirus, diarrhea, mink

Abstract

Diarrhoea is a multifactorial disease with a large impact on mink health and substantial economic losses to mink farming. Several viruses have been implicated as contributing causes of diarrhoea, although few have been identified as unambiguous etiological agents of mink diarrhoea, because the virus is often found in both symptomatic and asymptomatic animals. A quantitative PCR diagnostic approach has been undertaken for diagnosing mink enteritis virus (MEV), astrovirus, rota- and coronaviruses with a PCR diagnostic package on faecal samples from mink *(Neovison vison)*. Each assay was validated on faecal samples from mink submitted to the National Veterinary Institute either for routine diagnostic purposes, as base-line submissions or as case-control samples from mink farms with diarrhoea. Here we present data to evaluate the use of pooled samples in the diagnostic package. The faecal samples were pooled submission wise and according to case or control category. Two different pooling methods were tested. Pools and single samples were tested by quantitative RT-PCR for astrovirus and the results were compared. Both pooling methods showed a good agreement with the average of the individually tested samples. Testing of pooled samples reduces the costs of testing and thus promotes the use of the diagnostic PCR package. This will improve the proper treatment of mink with viral diarrhoea and potentially reduce the use of antibiotics.

Introduction

Gastrointestinal (GI) disorders are the main cause for submitting mink *(Neovison vison)* carcasses for post-mortem examination at the National Veterinary Institute in Denmark, and have been described as the predominant cause of disease and mortality in the Danish mink production (Rattenborg et al., 1999). Diarrhoea in mink can be associated with infectious agents (virus, bacteria and parasites) and feed-related/multifactorial conditions (Jensen et al., 2016) . Known enteric viral infections are caused by mink enteritis virus (MEV) and mink astrovirus. Coronaviruses and caliciviruses have also been implicated as potential causes or contributors to diarrhoea in mink. Rotavirus is poorly described in mink, but has previously been demonstrated by electron microscopy in faeces from mink pups with and without clinical signs (Jorgensen et al., 1996). The pathogenicity of these viruses could be related to viral load, virulence and the age of the mink. Therefore, there is a need for a quantitative diagnostic approach. We have developed new and/or adapted previously published real-time PCR/RT-PCR assays for MEV, astrovirus, rota- and coronavirus for quantitative diagnostics. Here we present the evaluation for the use of pooled samples based on astrovirus testing data.

Materials and methods

Sample material

Mink faeces was collected from June to November 2015. The sample material was collected by farmers or veterinarians and submitted to the National Veterinary Institute at the Technical University of Denmark (DTU Vet) or was taken during diagnostic necropsy of mink submitted to DTU Vet.
Three sample types were collected:

1) Samples from mink submitted for diagnosis at DTU Vet;
2) Faeces from five young healthy animals submitted by twelve farmers every second week – these served as the baseline;
3) Samples from five healthy and five diarrhoeic mink submitted by veterinarians whenever they visited a farm where diarrhoea was present – these constitute the case-control samples.

Pooling of samples

The faecal samples submitted by veterinarians and farmers were pooled submission-wise and according to case or control category, so that one pool consisted of five samples. Two different pooling methods were used for every five samples. By the first method (pool1), equal measures of faeces by weight from five samples were mixed and diluted 10-fold in PBS. By the second method, equal measures of supernatant from 10 % faeces in PBS were mixed (pool2).

Nucleic acid extraction

Viral RNA and DNA was extracted from 10 % faeces in PBS with QIAsymphony DSP Virus/Pathogen Mini Kit (QIAGEN, Denmark) on the QIAsymphony (QIAGEN, Denmark) extraction robot. Nucleic acid eluates were stored at -80°C until analysis.

Quantitative real-time RT-PCR testing for mink astrovirus

Individual faecal samples and faecal pools were tested by quantitative real-time RT-PCR using an assay adapted from (Ullman et al., 2004) on a Rotor-Gene Q machine (QIAGEN, Denmark). All runs included negative water controls and a positive plasmid control of a spectrophotometrically predetermined concentration. The plasmid control, which included the target sequence of the assay with primer and probe-binding sites, was tested in a series of eleven ten-fold dilutions to generate a standard-curve. This standard-curve was used to calculate the astrovirus concentration in the faecal samples.

Presence of other viruses

Selected samples were also tested for the presence of rotaviruses, MEV, calici and coronaviruses by in-house designed and/or adapted real-time PCR assays, using a quantitative approach with plasmid controls for MEV, calici and coronavirus (Escutenaire et al., 2007; Jiang et al., 1999; Pang et al., 2004).

Statistical analyses

Analysis of pooling methods was done on the basis of the quantitative real-time RT-PCR testing for astrovirus for the sample types 2 and 3. Calculated concentration data was sorted in data sets (5 individual samples and two pools) and grouped according to the number of positive samples out of five e.g. all data sets, where two out of five individual samples were positive in the quantitative real-time RT-PCR. The ideal virus concentration in a pool (pool-ideal) was calculated as the arithmetic mean of all five individual sample concentrations. The results from pool1 and pool2 were compared to the pool-ideal for each data set and the numerical differences were plotted as a function of log10 to the virus concentration of the pool-ideal. This comparison was done for groups of zero out of five positive individual samples, one out of five positive samples, two out of five positive samples and so forth and for all data sets in total.

Results

Samples

A total of 207 samples of type 1, 610 samples of type 2 and 250 samples of type 3 were collected and 232 pools were constructed.

Presence of virus in samples

Astrovirus, coronavirus and calicivirus were found in a large proportion of the samples. MEV and rotavirus were found in only few samples.

Analysis of pooling methods The differences between the results of the two pooling methods and the calculated pool-ideal showed the same tendency in all the six different groupings of number of positive samples per data set. Both pools were generally within 1 log-value of difference to the pool-ideal, with pool2 performing slightly better.

Discussion

The knowledge on viral infections causing diarrhoea in mink is very limited, however a quantitative correlation to disease to some degree might be expected, as discussed in a review of viral gastroenteritis pathogen quantification in children (Corcoran et al., 2014). DTU Vet can now offer a quantitative diagnostic package of real-time PCR tests for viral mink diarrhoea agents. The use of pooled samples will presumably encourage and lead to increasing number of submissions of multiple samples. Our study on pooling methods on the basis of quantitative astrovirus real-time RT-PCR data showed a good correlation to the average viral load of the individual samples independently of the number of positive samples per data set. Empirically, this is expected also to be true for other quantitative real-time PCR assays included in the diarrhoea package and it enables us to diagnose viral causes of diarrhoea on pools of five samples per farm, reducing the costs for testing and encouraging the testing of multiple samples. In the long run this will hopefully lead to a more prudent use of antibiotics on the farms.

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Epidemiology of the Fur animal epidemic necrotic pyoderma associated bacteria at the farm level

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Keywords: *Arcanobacterium phocae, Streptococcus sp.*, transmission, disease containment

Abstract

The fur animal epidemic necrotic pyoderma continues to spread from farm to farm. We have been able to show a strong correlation between *Arcanobacterium phocae*, and to a slightly lesser degree, a novel *Streptococcus* sp. and the disease. This has made it possible to better target diagnostics and also ascertain the pathogens were susceptible to antibiotics. This knowledge has however failed to stem the progress of the disease. More comprehensive information was needed on how the bacteria spread from farm to farm and from animal to animal.

We studied the routes that *A. phocae* and the novel *Streptococcus* sp. take to transfer from animal to animal. The goal of our study was to identify the spreading mechanisms of the pathogen(s) and to use this information to design effective means to stop the disease once it has entered a farm.

We collected samples from farms acutely affected by the disease, farms that no longer have active cases or only a few, sporadic flare-ups and farms that have never had the disease. We sampled the animals, cages of both diseased animals and those next to them, machinery, feed, other animals that move around the farm and caretakers. The sampling was done over a two year period targeting the colder seasons when most cases of FENP can be found.

The samples were taken by sterile swabs. The fur animals, rodents, cats and birds were also necropsied. The swabs were cultured on blood agar plates and secondary identification and probing of the tissue samples was done by PCR. Preliminary results show possible routes of transmission.

Introduction

Despite advances made in the recognition and diagnosis of animals suffering from the fur animal epidemic necrotic pyoderma (FENP) [Nordgren et al. 2014], the disease continues to spread to new farms and revisit farms which have regained disease-free status. Field experience of veterinarians suggests that the disease ebbs and vanes possibly due to the animal population turnover resulting in new susceptible animals with no protecting antibodies.

The two bacteria connected to this disease are not well characterized yet but knowledge of related

species [Aly *et al.* 1977 and Hernández-Castroa *et al.* 2005], and studies on microbial epidemics at the farm level, lets us hypothesize on the mechanisms of the infection and the modes the pathogens use for spreading in and between animal populations. [de Bruin *et al.* 2013, Prieto *et al.* 2014, Truong *et al.* 2013] Studies have shown that nearly any vector, inanimate or animate, on a fur farm can harbor pathogens and thus facilitate the transmission of bacteria or viruses. Especially the equipment and handlers coming into contact with the animals and that potentially move between the farms, pose the greatest risk. [Prieto *et al.* 2014]

As no working prophylaxis exists against this disease and the response to antibiotic treatment remains poor [Nordgren *et al.* 2014], there is a clear need for better understanding on how the bacteria spreads in a farm environment. The aim of this study was to define the transmission routes of the bacteria and then define the biosafety measures that could inhibit the spread of the pathogens to new farms and aid in the recuperation of already infected farms.

Materials and methods

Samples

Samples were collected from farms selected based on their current and previous disease status. Samples were collected from two farms with beginning primary epidemic, five farms which had had the disease for years, two farms that had suffered an epidemic but currently had only isolated, mild cases and two farms with no known cases of FENP.

All three fur animal species were included in the sampling. Samples were also obtained from other animals inhabiting the farm environment where possible. These included rodents, cats, dogs and birds. The rodents were trapped using standard kill traps and the cats and birds were sampled from euthanized or culled animals during basic maintenance of the animal populations. Only non-invasive swabs were taken from the dogs.

Swabs for bacterial cultures and PCR were taken from the animals' skin and mucosa. Serum was collected from animals treated by veterinarians either for FENP, other disease or dietary defects and also from euthanized animals. Dead animals were necropsied and samples were collected from mucosal membranes, eyes, nose, skin, lungs, spleen, liver and intestine.

Swabs were also taken from the environment, anything coming into contact with the animals and animal caretakers. The sampling was concentrated on the cages of the sick animals and those closest to them, the feeding trucks, forceps used to handle the animals, clothing of the caretakers and the hands of the caretakers. 400 swabs were taken over two separate collections in November 2014 and 2015.

Bacterial Cultures and PCR

The 200 swabs collected in 2015 were plated onto blood agar plates with 4% horse blood and incubated over night at +37 °C. The resulting cultures were read and further sampled for PCR for species confirmation (Nordgren et al. 2014). DNA was extracted from the original swabs of 2015 and those from 2014 as well as from all the tissue samples and then analyzed by PCR specific for *Arcanobacterium phocae* and the novel *Streptococcus* sp. The resulting PCR products will be sequenced to confirm the bacterial species. PCR based on the 16s ribosomal RNA gene will be done on selected interesting bacterial isolates to determine their species.

Results and Discussion

The bacterial cultures showed interesting results with several possible sources of bacteria identified. The bacteria must still be characterized further to ascertain species. The methods used to collect the samples were successful and yielded sufficient live bacteria as well as high quality DNA.

To ascertain air-borne infectious particles, a collection membrane could be used inside a shed with sick animals. Further study subjects may arise from other incidental bacterial findings not connected to FENP. The normal microbiome of the skin and mucosa of the fur animal species is largely unstudied and would be of great interest.

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Risk factors for use of antimicrobials in mink *(Neovison vison)*

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Abstract

The patterns and trends in antimicrobial use in mink were described, and a multi-variable variance analysis was carried out with the objective of identifying risk factors for antimicrobial use on herd level. The study was based on register data from 2007–2012. Information on antimicrobial use was obtained from the national database VetStat, monitoring all medicinal products used for animals on prescription level. Data on microbiological feed quality was obtained from the Voluntary Feed Control under the Mink producers Organization. Data on herd size, and the relation between farm and feed producer was obtained from the registers at Kopenhagen Fur, based on yearly reporting from the mink producers. In conclusion, antimicrobial use on herd level was significantly associated with the microbiological feed quality, the feed producer, and the veterinarian. The prescription patterns varied significantly between veterinarians, and some veterinarians were associated with both larger and more frequent prescriptions of antimicrobials herd level. Herd size was associated with different prescription patterns. Finally, infections with *P. aeruginosa*, astrovirus, influenza virus and *Salmonella* spp. diagnosed at the Danish National Veterinary Institute were associated with an increase in antimicrobial use.

Keywords: Medicine, mink, consumption

Introduction

Use of medicines, including antimicrobials, is of outmost importance in order to ensure welfare of the animals in the livestock productions in order to cure infectious diseases. In livestock, fluctuations in antimicrobial use are often due to emergence of disease (eg. PMWS in the pig production, DANMAP 2005) or improvement of the control of disease, but may also reflect changes in the legislation regarding the prescription and use of veterinary medicines (DANMAP 2010; eg effect in the pig production, Jensen et al., 2014). However, the total amount of antimicrobials prescribed for use in the Danish mink production has been steadily increasing (Chriél *et al.*, 2012) without any obvious reason. The purpose of this study is to identify risk factors associated with the usage of antimicrobials in the Danish mink production.

Material and methods

The study was a cross-sectional study across the period 2007-2012. A farm is defined by an identity code (CHR-ID) within the Central Husbandry Registers (CHR). The data on the relation between feed producer and CHR-ID, and the herd size was obtained from the registers at Kopenhagen Fur. The average weight of a dam and the progeny for a given month was estimated from growth curves (Anon., 2013), time of birth and pelting, and weight. The amount of antimicrobial needed to treat an average

animal on the farms fluctuates strongly over the year due to the intense growth of the litters; this had to be taken into account in order to estimate the proportion of animals treated. For each calendar month, the average biomass related to each dam was estimated. The total live biomass on a given farm was estimated based on the recorded number of dams on the farm in a given month and year. Data on all prescriptions of antimicrobial medicines and vaccines for mink within the study period were extracted from the Danish Veterinary Medicine Statistics, VetStat (Stege et al., 2003). All records on sales of antimicrobials for local gastrointestinal (GI) or systemic treatment were extracted. Medicines for topical application were not included in the analyses because the dosage cannot be generalized, and because the amounts used topically are negligible. From the laboratory database at the National Veterinary Laboratory (NVI), data on CHR-ID and date of sample submission was obtained for confirmed laboratory diagnoses of astrovirus, Aleutian disease virus (ADV), influenza virus, mink enteritis virus (MEV), canine distemper virus (CDV), *Pseudomonas aeruginosa*, and *Salmonella spp*. For each feed producer, a voluntary feed control is carried out at least once a month through test of a daily batch of the moist feed (Christensen *et al.*, 2013). The estimated monthly treatment proportion was calculated as number of antimicrobial daily doses, DADD, divided by the estimated biomass*days for a given farm in a given month and year.

Two generalized linear models were developed using the GENMOD procedure in SAS® version 9.4. Model A, where the response variable was reduced to a binary outcome (prescription/no-prescription) on monthly level. The data was fitted to a binomial distribution, describing the number of month with prescription out of 12 trials (months) per farm per year (farm-year). Model B, where all zero treatment proportion (TP) responses were omitted and the positive TP's were log-transformed to meet the assumption of normal distribution. Thus all (81,272) observations with TP=zero were omitted resulting in a data set with 13,480 observations.

Results

A total of 1320 farms were active throughout the study period, and the final dataset comprised 1316 farms. After applying the inclusion criteria, the recorded prescriptions in the final dataset corresponded to 87.5 % of the antimicrobials prescribed for mink during the study period. The descriptive analysis of antimicrobial use in mink on the national level showed a clear seasonal variation in antimicrobial use: the crude antimicrobial use peaked in May and in autumn; also, the level was high between the peaks compared to the winter months. The peak in May–July was very prominent, when the mink kits were weaning and thus, the biomass low. Compared to 2007, the annual treatment proportion had more than doubled (102% increase) by 2011. Half of all mink farms received antimicrobial in 2007, increasing to 82% in 2012. From 2007 to 2012, oral prescription increased from 23 to 45 DADD/1000 biomass*days and parenteral prescription increased from 0.45 to 0.87 DADD/1000 biomass*days.

The generalized linear models showed significant effects of feed producer, feed quality, and the outbreak of *P. Aeruginosa*, astrovirus, influenza virus and *Salmonella spp*. Smaller herd size was associated with more infrequent prescription and larger monthly prescription in the months with prescription probably an effect of package size, as prescriptions in one month may cover the need for treatment in several months in small herds. Some of the veterinarians were associated both with frequent antimicrobial use an high antimicrobial use per animal in individual months indicating a significantly positive effect on overall antimicrobial use, whereas the opposite was observed for other veterinarians.

Discussion

Many factors affect the decision for medical treatment, e.g. economic break-even between price for the medicine and the animal of concern, and work load associated with the administration of the medicine (Chriel and Dietz, 2003). In the mink production, the new generation of feed trucks with automated dosing equipment for medication has eased the use of e.g. antimicrobials. Based on submissions to the National Veterinary Institute the antimicrobial use is high in the early summer, most likely due to diarrhoea in the kits. In autumn, the crude antimicrobial consumption is high, most likely due to outbreaks of respiratory disease. The risk factors investigated in this study explained much of the variation in antimicrobial use, but not the gradual increase over time. However, the observed patterns suggest that, the public debate and implementation of regular veterinary visits is associated in time with an increase in parental use as an focus on the individual animal, but a much larger increase in oral treatment may be a side effect the mandatory veterinary visits.

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Experimental infection with Arcanobacterium phocae and Streptococcus halichoeri in farm mink (Neovison vison)

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Keywords; Mink, necrotizing pyoderma, *Neovison vison*, disease, wounds

Abstract

Understanding the pathology resulting from *Arcanobacterium phocae* and *Streptococcus halichoeri* polymicrobial wound infections is of great importance for mink production due to the apparently increasing prevalence of severe wounds infected with these bacteria on some farms and the resistance of these wound infections to antimicrobial treatment. Severe illness was recorded after three days in mink inoculated with high dose of polymicrobial infections with *Arcanobacterium phocae* and *Streptococcus halichoeri*. Necropsy of the euthanized animals revealed severe widespread necrosis. Wound contraction was significantly delayed by mono-species infection with high dose of *Arcanobacterium phocae*, and wound size increased during the observation period, which is similar to what has been reported for mink with necrotizing pyoderma associated with this bacteria. These preliminary results indicate a possible synergetic effect between *Arcanobacterium phocae* and *Streptococcus halichoeri*, which may contribute to the severity of the wound infection.

Background

Outbreaks of necrotizing pyoderma characterized by the occurrence of unusual wounds in the face and paws of mink were diagnosed on five Danish farms in 2014-2015 (Hammer et al. 2015). Similar disease previously referred to as "pododermatitis",*" face and footrot"* or – "fur animal epidemic necrotic pyoderma" has been reported from several fur producing countries including Canada in 2000 (Brøjer 2000, Chalmers et al. 2015) and Finland in 2007 (Nordgren et al. 2014). The outbreaks of necrotizing pyoderma in Danish mink farms were associated with occurrence of unusual wounds in the face and paw region, and with increased mortality. Researchers at the University of Copenhagen have diagnosed the disease based on traditional microbiological culture, molecular biological methods and pathology. Findings were similar to reports from other fur producing countries. Copenhagen University have analyzed data and material collected on 6 Danish farms. The cause of disease has not been identified and it has not been possible to identify a common risk factor. Since the disease occurs simultaneously in many genetic types on the farms it does not appear to be genetically inherited. FENP is a disease of concern for mink breeders worldwide, compromising animal welfare and causing considerable economic losses to farmers. The purpose of this study was to assess the pathogenicity of two bacterial agents suspected to be causal factors of FENP.

Methods

The study was designed as a parallel group experiment where a total of 10 healthy male white colortype mink, 9 months of age, were divided into groups randomly assigned to 5 experimental regimens (A-E). All groups were subjected to wound surgery. Group A were inoculated with a combination of *Arcanobacterium phocae* and *Streptococcus halichoeri* in high dosage. The isolates were cultured from Danish

farm mink diagnosed with FENP and species confirmed by Maldi TOF and 16S sequencing. Group B were inoculated with *Arcanobacterium phocae* in high dosage. Group C were inoculated with *Streptococcus halichoeri* in high dosage. Group D received a combination of Arcanobacterium phocae and Streptococcus halichoeri in reduced dosage. In the control group, Group E, the experimental wounds were not inoculated with bacteria. The surgical procedures were carried out l*ege artis* at the experimental facility. Mink were weighed and anaesthetized with a combination of ketamine and medetomidine intramuscularly after premedication with an intramuscular injection of buprenorphine. The mink were surgically prepared by removing the fur at the surgical site with electrical clippers and washing with chlorhexidine-cetrimide followed by 70% ethanol. All mink were subjected to wound surgery. A small one centimetre long incision wound were created on the front leg in the shoulder region using sterile scalpels. The wound were placed on the leg, because FENP wounds are often found on the legs and feet of the mink. Using sterile scalpels and scissors and a two centimetre deep pocket were created in subcutis – in which the inoculation of bacteria took place by use of a Eppendorf micropippette. After the procedure, anaesthesia was reversed by intramuscular injection of atipamezole and the mink were returned to their cages, receiving meloxicam as painkiller for a minimum of 24 hours post-surgery. The wounds were left for secondary intention healing without intervention, but with daily monitoring of the condition and feed intake of the mink. The mink were housed singly in standard mink cages according to Danish legislation and were allowed a five days acclimatization period prior to surgery at the experimental facility at the University of Copenhagen. They were fed with a commercial mink diet and had free access to tap water. Experienced animal caretakers were responsible for the routine care of the animals. The handling of the animals and the experimental procedures were approved by the Danish Animal Experimental Act.

Results and discussion

Clinical evaluation of the mink showed quick recovery and good general state of health in all mink the day after anaesthesia. The animals were eating well and seemed unaffected by the skin wound. The mink in group A were euthanized on day 3 because of acutely appearing severe clinical symptoms including severe lameness (not supporting weight on the leg), swelling, depression and anorexia. Necropsy of these mink subjected to infection with a high dose of polymicrobial infection with *Arcanobacterium phocae* and *Streptococcus halichoeri* revealed severe widespread necrosis in the area near the inoculation site and spreading to the distal leg and surrounding shoulder region. Haemorrhage and serous exudation from the wound were recorded.

The mink in group B and one of the mink in group C were euthanized on day 8. The mink in group B were euthanized because the wound increased in size and moderate lameness (rarely supporting weight on the leg) not responsive to pain killers was seen. One mink in group C were euthanized on day 8 because of/due to mild, but persistent lameness (sometimes relieving weight from the leg) unresponsive to painkillers.

In group B wound contraction was significantly delayed by mono-species infection with high dose of *Arcanobacterium phocae.* Haemorrhage and serous exudation from the wound were recorded. The wounds in this group were significantly increased in size on day 8 when the animals were euthanized. In group C and D wound contraction seemed comparable to the control group E. Animals in group D and E seemed unaffected by the wound all through the study period, while one of the animals in group C developed lameness. The general state of health remained good for all animals in group C-E and haemorrhage and serous exudation from the wound were not recorded at any time during the observation period. The wounds were dry and without exudation and swelling. The remaining mink in group

C and the mink in group D and E were euthanized on day 14. The preliminary results of this infection trial results indicate a dose dependent pathogenicity of *Arcanobacterium phocae* and *Streptococcus halichoeri* in mink. The results also indicate an interaction of bacterial species within mixed-species wound infections and possible a synergy effect in mixed infections with Arcanobacterium phocae and Streptococcus halichoeri, resulting in greatly increased severity of clinical symptom development in animals receiving a high dose of *Arcanobacterium phocae* and *Streptococcus halichoeri* in combination. Results of histological, microbiological and molecular biological methods and serological analyses are not included in this preliminary report.

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Blue fox parvovirus in semen

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Abstract

Blue fox parvovirus (BFPV) affects blue fox *(Vulpes lagopus)* at any stage of life. Adult animals are asymptomatic, but young cubs might develop diarrhoea. BFPV was isolated for the first time in 1983 from Finnish farm-bred blue fox (1). It has been established that BFPV causes significant reproductive failures due to early embryonic death. Antibodies to BFPV disappear rapidly and thus animals are susceptible to infection during pregnancy each year.

Blue Fox parvovirus is a pathogenic virus which affects pregnant vixens in early stage of pregnancy and causes reproduction failures in farms or in individuals. It has been thought earlier that after the onset of the disease in a farm, the significance of BFPV is marginal and preventive measures are not necessary. Now it seems that parvovirus infection during the pregnancy might be a bigger issue than expected. To our knowledge there have been no studies about possible infection via fresh semen used in artificial insemination (AI) at farms.

Semen samples were collected in spring 2016 and are being analysed for the presence of BFPV. The results are inconclusive at this time and more analyses are needed to conclude anything about the possibility of BFPV infection via artificial insemination.

Keywords: reproduction failure, viral disease, sperm, BFPV

Introduction

Parvoviruses are widely spread and infect a vast variety of hosts. Many parvovirus cause diarrhea and for example Porcine parvovirus is known to kill the fetuses in early stage of pregnancy. To prevent reproductive failures, diarrhea and other symptoms that parvoviruses may cause, at least sows, cats, dogs and mink are vaccinated against species specific parvovirus being PPV, FPV, CPV and MEV respectively. Also raccoon dogs *(Nyctereutes procynoides)* are often vaccinated with MEV vaccine to prevent diarrhea in young cubs.

It has been established that parvovirus infection in early stages of pregnancy is detrimental to whelping result in blue foxes (2). It has been shown that antibodies acquired by vaccination have short lifespan, and antibody titers decline to low levels in five to six months (2). Thus, it is possible that blue fox vixen are infected by BFPV during pregnancy each year resulting in poor reproductive yield.

Infection routes are not very well known, but it is clear that at least animal to animal and via fomites (personnel, handling equipment, cages etc.) are possible. We have taken several serum samples from different farms with reproduction problems and high parvovirus antibody titers are often found. Al-

most all blue fox vixens are artificially inseminated 2-3 times either with fresh semen collected from same male or with different males. Vixens are inseminated every other day twice or three times, in some cases there might be four or even more inseminations. There is a report (3) of parvovirus infection in mice gametes and embryos. The possibility exists that fresh semen is an infection source for the inseminated vixens, and remains to be studied.

Materials and methods

The semen samples were collected from six different fox farms. One farm sampled all the breeding males during the season, some of the males were sampled several times. Also at the end of the season, swabs were taken from the rectum and the preputial sack. Another farm was sampled in one day during which semen and rectal and preputial samples were taken. Four farms collected semen samples from 5-12 blue fox males during 7-10 days and samples were then shipped to the laboratory. Altogether 150 semen samples were obtained and an equal amount of rectal and preputial swabs were taken. All samples were cooled before shipping to the laboratory. All semen samples were diluted with commercial AI-fluids most often used with boar semen. Sampled males have never been vaccinated against parvovirus. At the time this manuscript is written, whelping is still ongoing and there is no data about pregnancy rate or puppy result because blue fox pregnancies are not monitored.

In laboratory polymerase chain reaction and virus culture were used to determine the presence of the blue fox parvovirus in fresh diluted blue fox semen. The laboratory analyses are ongoing.

Discussion

The significance of blue fox parvovirus infection to animal reproduction is well established in previous studies (2). The infection route and long-term significance to reproduction, and the role in causing diarrhea is to be determined. Field samples indicate that there is some significance to health of the cubs and also to reproduction even though BFPV is present in all fox farms in Finland. Antibody titers vary a lot and therefor it is possible to acquire the infection every year by at least some part of the breeding animals. Economical aspect is also important because blue foxes have only one breeding season per year, and if this is affected somehow, the economic losses to farmer are significant. More research is needed before blue fox parvovirus is prevented and the economic and animal welfare issues are solved.

Acknowledgements

We thank the farmers who provided the samples for our study: Jarmo Jylhä, Jukka Tofferi, Hannu Sillanpää, Harri Myllymäki, Kari Kattilakoski ja Peter Järnström.

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Pediculosis (Stachiella larseni) in farmed mink in Ontario, Canada

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Abstract

In early January 2015, two dead unpelted sapphire mink with rough hair coats bearing large numbers of small ectoparasites (Figure 1), alopecic skin abrasions (Figure 2), and chewed tail tips (Figure 3) were submitted by an Ontario mink producer to the Animal Health Laboratory, University of Guelph, Ontario, Canada for identification of the ectoparasites. Less than two years previously, this same producer had submitted mink to the lab for the same complaint.

The ectoparasites were identified based on the following taxonomic features:

1) Lice: Order *Phthiraptera*
2) Biting lice:
1. Head wider than thorax.
3) Suborder: *Ischnocera:*
1. Mouthparts without palps.
4) Genus: *Stachiella:*
1. Host species (mink)
2. Three segmented antennae without a club
3. Characteristic body lengths: females 1.1 mm, males 0.8 mm (Figure 4)
5) Species: *S. larseni.*
1. Abdominal tergal plates without narrow heavily sclerotized bands
2. Two pairs of distinctive anterior dorsal abdominal spine-like setae and sparse fine setae on the rest of the abdomen *(Figure 5)*

These two features distinguish *S. larseni* from *S. ermineae*, which are found primarily on weasels, and from *S. retusus*, which are found primarily found on weasels and martens. Both of these species can also be found less frequently on mink. Similarly, *S. larseni* occurs on mink typically, but has also been reported on weasels.

The literature contains very little information describing lice from mink (Emerson, 1962) but it is recognized that lice are typically very host-specific and are often found on only one host species or closely related species, so these lice are not considered to have originated from dogs. If lice are present on farmed animals, populations may be at undetectable levels for most of the year, typically peaking in the winter when animals are kept indoors and close together. The winter of 2014/2015 in Ontario, Canada was exceptionally cold for prolonged periods of time and although the mink were housed in pens inside sheds, this extended cold could have provided sufficient additional stress, especially for colour mutation mink, during a time when the mink were also being prepared for the upcoming breeding season.

The quality of the mink pelt dictates the value of the pelt, therefore if farm animals are already infested with lice, keeping the ectoparasite burden under control is imperative. Since this farm experienced clinical pediculosis in the recent past, since lice populations can fluctuate widely, and since the farm has been consistently populated with mink for years; it is likely that a low-level louse population has been present on this farm for a few years.

When considering treatment and control recommendations, the life cycle of the parasite needs to be considered given the very short cycle with eggs hatching in approximately one week. The eggs are not affected by insecticides, and, since the lice are mostly associated with the animal and less often in the environment, treatment of the animal must be done on a weekly basis. Treatment with permethrin, pyrethrin, or malathion powders in the nest box on a multi-weekly basis should be sufficient. If nest box bedding is replaced, it will need to be retreated.

Ectoparasites are not often identified on mink farms, but monitoring should still be part of herd management. Considering that quality pelts are the primary product of mink production, external examination during handling times will help identify underlying populations before they impact the health, wellbeing, and pelt quality of the mink.

Keywords: farmed mink, lice, pediculosis, *Stachiella larseni*

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Figure Legends

Figure 1:
Large numbers of pinpoint tan lice are on the tips of the fur over the head, neck, and body of the mink.

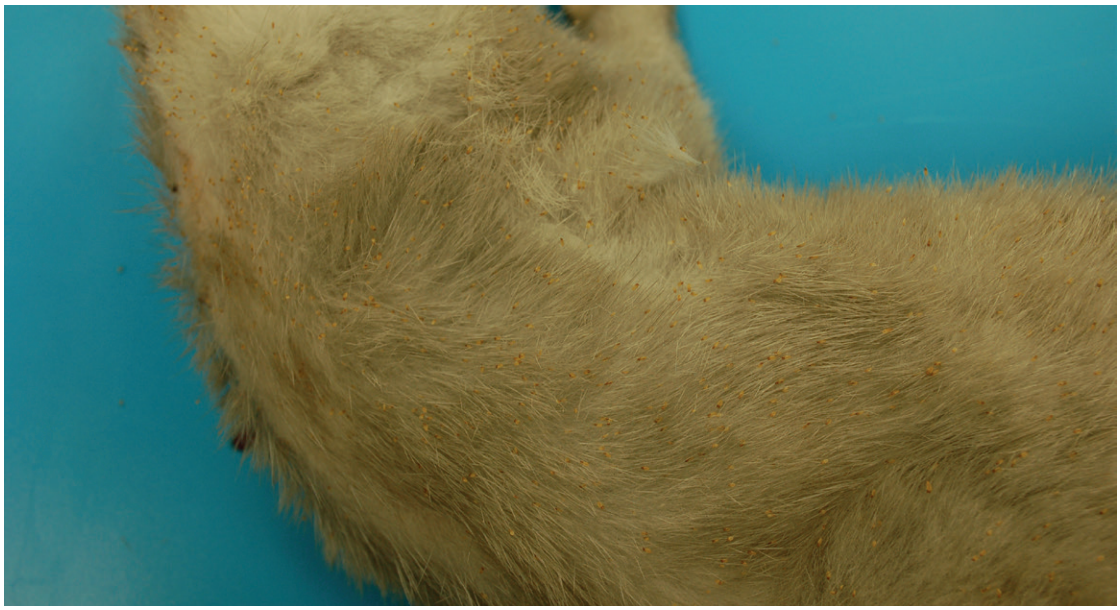


Figure 2:
Alopecic skin abrasions and whirls of damp, disheveled fur are over the lateral and ventral abdomen.

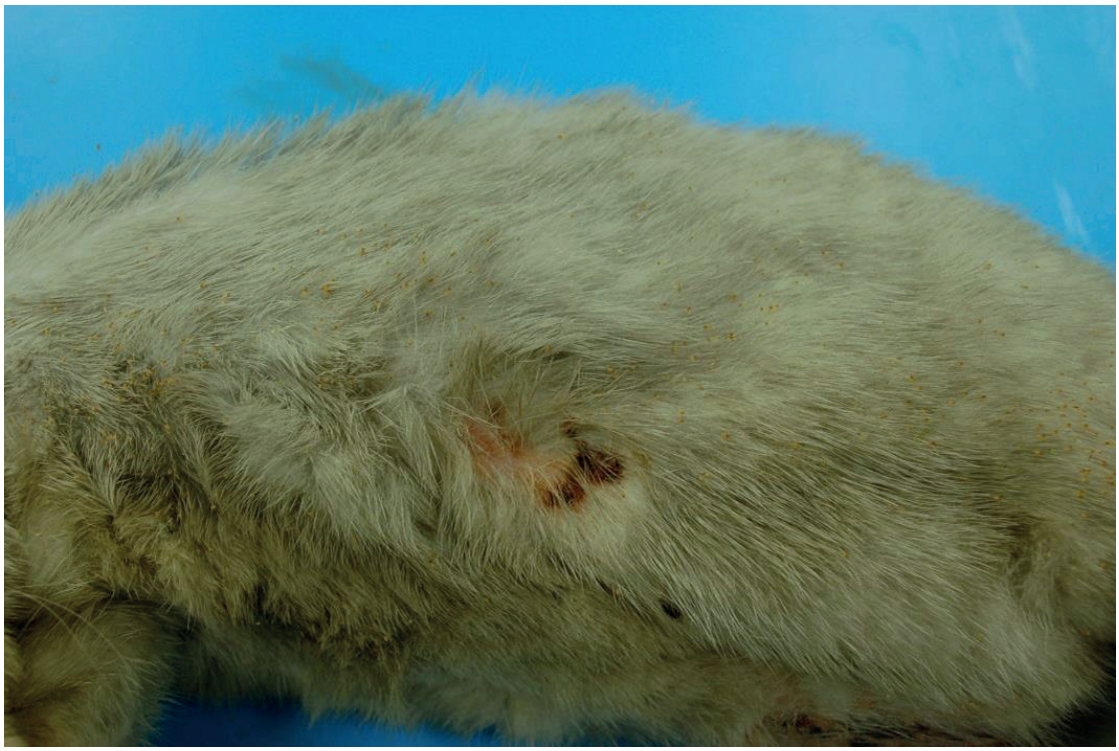
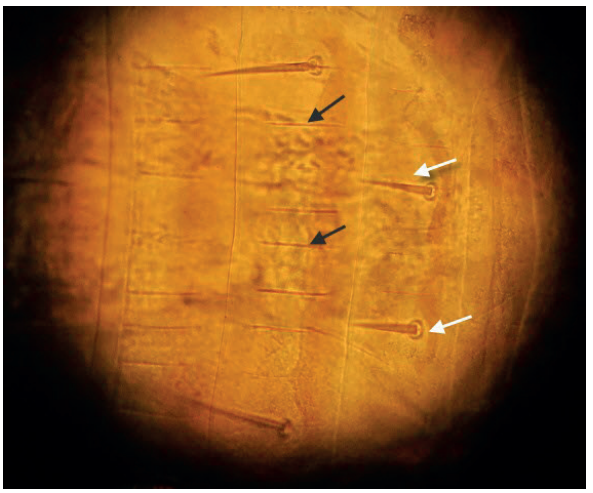


Figure 3:
Chewed tail tip. A few lice are present on the fur.

Figure 4:
Stachiella larseni male louse, 0.8 mm long.

Figure 5:
Stachiella larseni male louse. Note the 2 pairs of distinctive anterior dorsal abdominal spine-like setae (white arrows) and sparse fine setae (black arrows) on the rest of the abdomen. Head of the louse is to the right.



Eimeria vison on Dutch mink farms

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Abstract

From each of six Dutch mink farms (A-F) 20 random fecal samples were collected in the first week of June 2015. Samples were collected from cages housing female minks and their offspring, all without signs of disease. Farm A and C each submitted six additional fecal samples from pups, of which three at farm C originated from animals in the sick bay. The number of oocysts per gram (OPG) of fecal material was determined by flotation using the McMaster method with zinc sulfate (ZnSO4). Coccidia were found in 61 percent of the random samples (40%-90% of samples per farm), with an average OPG per farm ranging from 107 to 2,005. Most (11/12) pup samples contained oocysts with an average OPG of 18,343 (farm A) and 4,785 (farm C). The average OPG from the three samples of sick pups was 1,540. For each farm a pool of oocysts was sporulated. Morphology, length, width and length/width ratio of sporulated oocysts was consistent with Eimeria vison, a known intestinal pathogen of minks. The high prevalence on farms and the high OPG in pup feces indicate coccidiosis is a possible health problem for Dutch mink pups in June and a potential complicating factor in other intestinal diseases.

Keywords: coccidiosis, June blues, pups, protozoa, parasites

Introduction

Coccidiosis is the clinical disease caused by coccidia. Transmission is feco-oral and tends to be self-limiting. Since severity of infection is dose-dependent, clinical disease is associated with extensive contact with contaminated feces. This might explain why coccidiosis is rarely, if ever, reported in Dutch mink farms using wire-cages. Unexpectedly, a recent monitoring program identified large amounts of coccidia in minks with diarrhea although the farmer did not note 'abnormal amounts of diarrhea' on a herd level, raising the question if low-grade infections are underdiagnosed. In other species low-grade non-lethal infections with coccidia are known to have a large impact on production (Daugschies and Najdrowski, 2005; Gauly *et al.*, 2004; Voeten *et al.*, 1988), and in minks these infections have previously been implicated as a cause or contributing factor to overall poor performance and intestinal problems in June and July, called 'June blues'. To determine if coccidia are common in Dutch minks, and which species are involved, a small screening of six farms was organized.

Material and methods

Samples

In the first week of June 2015, farmers of six mink farms (A-F) each collected 20 fecal samples. Samples were collected from group housed mink, and each sample likely represented feces from multiple animals. Samples contained either diarrhea (34/120) or normal feces (86/120). Additional samples were collected at the end of June from pups at farm A (6 healthy pups) and C (3 healthy pups and 3 pups in sick bay). Samples were stored at 4˚C.

Measurements

For each sample the number of oocysts per gram of (OPG) feces was determined using a modified McMaster method utilizing solutions of zinc sulfate (ZnSO4). Additionally a pooled sample was created for each farm. Oocysts from each pool were sporulated according to a modified version of the method from Ryley *et al* (Ryley *et al*., 1976). Length and width of sporulated oocysts were measured using Cellsense software (Olympus, Japan) in accordance with Long's method (Long et al., 1976).

Results

Oocyst detection

In the 120 samples collected in the first week of June, 61 percent contained oocysts, with a minimum 40 percent and a maximum of 90 percent per farm (Table 1). Incidence of coccidia in diarrheic samples was not significantly different from normal samples.

Table 1. *Average number of oocysts (Av. OPG) as well as oocyst incidence from the first 120 samples, listed by farm and subdivided into diarrhea and normal samples*

Farm	Diarrheic samples		Normal samples		Total	
	Av. OPG	Positive/total	Av. OPG	Positive/total	Av. OPG	Positive/total
A	396	4/5 (80%)	660	10/15 (67%)	594	14/20 (70%)
B	165	6/8 (75%)	220	7/12 (58%)	198	13/20 (65%)
C	693	8/10 (80%)	561	10/10 (100 %)	627	18/20 (90%)
D	-	0/0	2,005[1]	12/20 (60%)	2,005[1]	12/20 (60%)
E	66	1/5 (20%)	154	7/15 (47%)	132	8/20 (40%)
F	55	2/6 (33%)	130	6/14 (43%)	107	8/20 (40%)

[1]The average OPG at farm D is strongly affected by a single high measurement. Without this outlier the average OPG at farm D is 165.

All but one of the additional pup samples from farms A and C contained oocysts (Table 2). The average OPG (18,343 and 4,785) was significantly higher than the OPGs in the first 120 samples (Table 1). The three samples from the sick bay (farm C) had a lower average OPG (1540) than the three pup samples from the healthy population at farm C (8030).

Table 2. *Average number of oocysts (Av. OPG) as well as oocyst incidence in 12 pup feces samples from farms A and C*

Farm	Sick bay		Healthy population		Total	
	Av. OPG	Positive/total	Av. OPG	Positive/total	Av. OPG	Positive/total
A	-	0/0	18,343	6/6 (100%)	18,343	6/6 (100%)
C	1,540	2/3 (67%)	8,030	3/3 (100%)	4,785	5/6 (83%)

Oocyst identification

Intact sporulated oocysts were only found in pooled samples from farms A, C and D. Length, width and length/width-ratio was determined for 37 of these oocysts. Average length was 23.6 µm and average width was 15.3 µm, which is comparable to values reported by Hindsbo and McTaggart for Eimeria vison (Hindsbo, 1995; McTaggart, 1960) (Figure 1 and 2). Additional morphologic characteristics of these oocysts were also consistent with descriptions of *E. vison* (Levine, 1948; McTaggart, 1960). No other Eimeria or *Isospora* species were recognized.

Figure 1.
Frequency of length of sporulated oocysts (µm), compared with data from Hindsbo and McTaggart

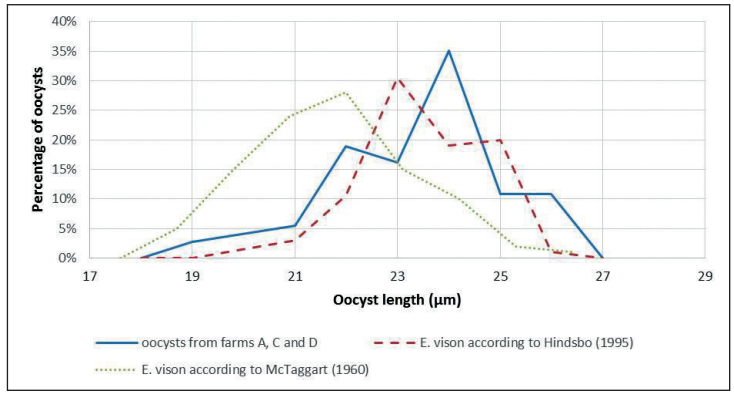
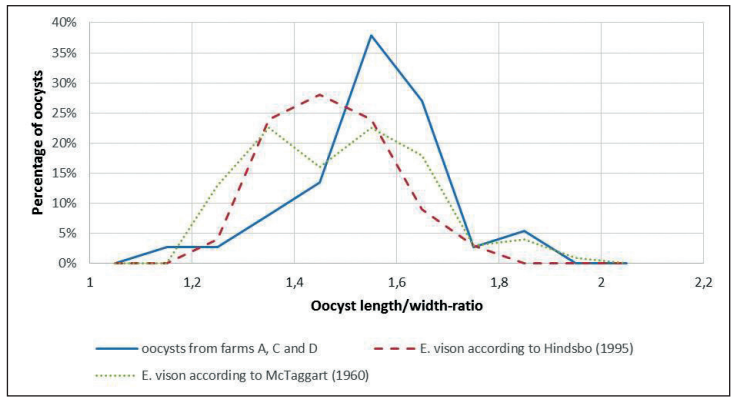


Figure 2.
Frequency of length/width ratio of sporulated oocysts compared with data from Hindsbo and McTaggart



Discussion

Coccidia were found in samples from each farm at a rather high incidence (40% to 90% of samples per farm), but generally at low levels (≤ 2,005 oocysts per gram feces). In contrast, the fecal samples from pups where almost all positive and often contained well above 10,000 oocysts per gram, which has been suggested to be the level above which infections are of clinical importance (Henriksen, 1986). These oocyst numbers exceed those in other recent reports of Eimeria vison infections in minks, and we suggest that this might be due to the fact that the current screening was done in June, while previous reports focus on infections in later months. In June the pups lose the protection by maternal antibodies and will generally not yet have built up their own antibody levels against the parasites, potentially leading to increased susceptibility to infection with higher replication and shedding. In this context it seems paradoxal that OPG in animals with diarrhea is not significantly different from OPG in animals with normal feces and that the pups in the sick bay actually had a lower OPG than those in the general population. Sampling sick animals could lead to underestimation of coccidial burdens in field situations. Concluding, our results support the suggestion by Gorham (Gorham, 2005) that

coccidia could play an important role in the increased incidence of intestinal problems in June and July, sometimes referred to as 'June blues'.

Acknowledgements

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Treatment of wounds in mink kits in June and effect on skin quality

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Abstract

The objective of this study was to investigate the treatment effect of skin wounds in mink *(Neovison vison)* in June, and to evaluate how skin wounds in mink kits affect the skin tensile strength and quality. The treatments included a spray containing antimicrobials (tetracycline based), and two sprays not including antimicrobials: a Cu/Zn based spray and a physiological saline spray. The collected data showed no significant difference in the treatment effect of the three topical products used in June. The evaluation at pelting revealed a significantly higher rate of torn pelts in the group treated for skin wounds compared to the control group. Furthermore, scaring on the leather side of the pelts were highly prevalent in the animals treated for skin wounds (80% of the treated pelts had scaring). The scar tissue was assessed to affect the quality of the skin.

Keywords: mink, *Neovison vison*, disease

Background

Cutaneous wounds in farmed mink are considered an indicator of reduced welfare in mink production as stated by the EU Scientific Committee on Animal Health and Animal Welfare (2001) and studies have shown that wounds may be a significant cause of mortality in mink kits (Hansen et al. 2007, Clausen 2016). The increased focus on the development of antibiotic resistance is also an important motivation to increase knowledge concerning prevention and treatment of skin wounds in mink kits. The fur industry in Denmark has established guidelines for the management of injured mink (Kopenhagen Fur 2016), though decisions regarding choice of therapy or euthanasia in individual cases - as well as the effect of various therapeutic protocol - are not evidence-based. Identifying alternative treatment protocols not including antibiotics may reduce development of antimicrobial resistance. The objective of this master's thesis was to investigate the treatment effect of skin wounds in mink (Neovison vison). The treatment effect with and without antibiotics was evaluated as well as the impact of skin wounds in June on the quality of the fur.

Materials and methods

The study was performed as a field study in 2014. The aim was to investigate the treatment effect of different topical products. June and October were selected as trial periods based on previous knowledge of the occurrence of skin wounds in farmed mink. June included three topical treatment groups.

The treatments included a spray containing antimicrobials (Cyclospray), and two sprays not including antimicrobials: a Cu/Zn based spray (Repiderma) and a physiological saline spray (Apotekets Sårskyl Steril). Macroscopic evaluation of the wounds was completed during the treatment period. The

wounds were measured and photographed on day 0 and day 14. The treatment effect was calculated as the reduction in the wound area from day 0 to day 14. At pelting macroscopic evaluation of the fur was performed. Tissue samples were collected at pelting for histopathological evaluation.

Results and discussion

The collected data did not show a significant difference in the treatment effect of the three topical products used in June. This study included relatively few animals and further studies are necessary in order to provide evidence based recommendations for fur veterinarians and farmers in this field. The results from June showed that there was a significant reduction in wound area depending on infection status of the wounds. The evaluation at pelting revealed a significantly higher rate of torn pelts in the group treated for skin wounds compared to the control group. Furthermore it was found that there was a considerable amount of scaring on the leather side of the pelts in the animals treated for skin wounds. 80% of the pelts from mink treated with wounds had macroscopically visible scaring. Histological examination of dark areas of scarred skin revealed delayed maturation of hair compatible with descriptions of skin lesions in mink with experimental bite lesions (Hansen et al 2014). The presence of scar tissue was assessed to affect the quality of the fur.

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Data recording by use of treatment cards on farms with outbreaks of diarrhea during the lactation period

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Abstract

In 2015 a case-control study was conducted on mink farms with outbreak of diarrhea in the pre-weaning period with control farms that had a low or no occurrence of diarrhea in the same period. A treatment card was designed and applied for systematic registration of data concerning litter treatment, and including date of birth, coat color and litter size. Data was included for 2,440 litters on eight farms (four case farms and four control farms). The majority (73.6%) of the treated litters were initially big litters (defined as a litter size of seven or more kits when litter size was initially recorded) and the majority (83.5%) were born before 3rd of May. The cards were found to be a useful tool for registration of litter data and treatment data during field studies of diarrhea in the pre-weaning period.

Keywords: mink, *Neovison vison*, disease, health

Introduction

Diarrhea in the pre-weaning period (also known as sticky kits) is a major cause of reduced growth and increased mortality in mink kits (Clausen 2010), representing both a production-economic problem as well as a welfare problem. The disease is not monitored, so there is no clear overview of incidence and economic impact. It is generally difficult to gather systematic data from mink farms with disease outbreaks during the lactation period and literature on the prevention and treatment of diarrhea in mink in all age groups is sparse and mainly based on individual observations and experiences (Henriksen 1987, Hyldgaard-Jensen 1989). Recent studies indicate that the symptom complex is a general physiological response to diarrhea in mink kits, rather than a response to a specific etiological cause, and that there are etiologically distinct types of diarrhea in the pre-weaning period (Hammer et al. 2007, Hansen et al. 2014). In other words - the clinical signs of diarrhea in the pre-weaning period are approximately the same, but may have various causes. Etiological types should probably be separated in order to identify risk factors and tools to prevent outbreaks. The aim of this pilot study was to assess the applicability of treatment cards for systematic data recording from litters subjected to treatment, including date of birth, color type, litter size and treatment data. This report presents the analysis of treatment card data from four control farms and four case farms.

Materials and methods

This pilot study is part of a case-control study conducted in May 2015 on 30 Danish mink farms located in Jutland and on Funen. The case and control farms were selected on the basis of medical history in which case farms have had major problems with diarrhea during lactation in recent years, and control farms had low or no occurrence of diarrhea during lactation.

In order to collect data on litters treated for diarrhea, we distributed treatment cards to the farms. On the cards data were systematically recorded including date of birth, color type, litter size and treatment. Data was entered into Excel for further analysis. For each farm information was also collected on farm size, number of litters born and average litter size at first count.

Results and Discussion

According to information obtained from the farmers the overall percentage of litters with diarrhea at four case farms was: 14.9% - 46.3% and four control farms: 3.4% - 5.9%. On the eight farms card data was recorded for a total of 2,440 litters treated for diarrhea with antibiotics. Additional 18,651 litters on the eight farms were not registered on treatment cards, because diseases were not observed. In case farms, 4.2% to 24.3% treated litters were recorded, and in control farms from 3.4% to 4.4% treated litters. The majority (83.5%) of the treated litters were born to and including 2nd of May, suggesting that premature litter are more likely to develop diarrhea. However, a previous study documented that diarrhea mainly occurred in litters born later on the month (Chriél 1997).

The treatments were conducted form the 3rd of May to 11th of June 2015. The majority (73.6%) of the treated litters were large litters (defined as litters containing seven kits or more). This distribution could indicate that large litters are more likely to have diarrhea than small litters. However, it should be kept in mind that 89 cards lacked information about litter size, giving the possibility of changing the above mentioned findings.

Overall, 96.5% of litters were treated the first time at an age of eight to 31 days. The most treatments were conducted when the pups were 28 days old and decreased very fast in the days thereafter. The reason for this may be that the kits developed age-related resistance around this age.

The case and control farms in this study showed a very similar pattern in treatments. It is an important achievement in relation to assess if the data from these farms are comparable. In other words, the results suggest, that the disease incidence on case- and control farms represents different degrees of the same problem, and that it would be appropriate to proceed with further studies of factors.

The cards were found to be a useful tool for registration of litter data and treatment data during field studies of diarrhea in the pre-weaning period. The cards may also be applied for data registration in clinical trials evaluating the effect of measures taken for disease prevention or treatment.

Acknowledgements

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Cutaneous diphtheria-like disease with Corynebacterium ulcerans in mink and its presence on nasal mucosa of healthy mink

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Abstract

Three dead mink were submitted from a farm suffering of increased mortality after vaccination. They had severe necropurulent inflammation at the injection site. Large numbers of *Corynebacterium ulcerans* were cultured from two of the wounds, while *Clostridium perfringens* was cultured from one of these two, as well as from the wound of the third mink. To determine whether *C. ulcerans* is commonly present in mink, nasal swabs were collected from clinically healthy mink at three farms during the pelting period. *C. ulcerans* was found in 28/62, 29/56 and 6/60 swabs. Five isolates per farm as well as the isolates from the wounds were investigated for presence of the tox gene by PCR and production of toxin by ELEK test. Their identification was confirmed by MALDI-TOF MS, 16S partial sequence analysis and biochemical profiling. The tox gene was found in the isolates from the wounds and in 0/5, 4/5 and 5/5 isolates from the farms. Toxin production was only detected in the isolates from the wounds. This is the first report of cutaneous diphtheria-like disease with toxigenic C. ulcerans in mink. Screening results suggest C. ulcerans is a common inhabitant of the nasal mucosa of healthy mink.

Keywords: wounds, diphteroid, post-vaccination, skin

Introduction

In February 2015 increased mortality was reported shortly after vaccination on a Polish mink farm. We describe the necropsy results from this case as well as results from a screening on six Dutch farms. The screening was based on nasal swabs collected from healthy mink to determine whether the potentially zoonotic bacteria cultured from the dead Polish mink are commonly present in healthy mink.

Materials and Methods

Field case

Necropsy was performed on three dead mink in good body condition, which had died in the days following intramuscular vaccination in the hind leg. The farmer reported increased mortality post-vaccination, associated with large firm swellings at the injection site.

At the injection site all three mink had a subcutaneous and partly intramuscular nodule of approximately 2.5x2x2 cm, with a necropurulent to necrohemorrhagic aspect on cut surface. Animal three also had splenomegaly, hepatomegaly with yellow discoloration (hepatic lipidosis) and congested lungs. No other gross abnormalities were noted.

Histological examination of the injection sites showed extensive necrosis affecting both the muscle and the hypodermis. In mink one and two the necrosis was accompanied by massive infiltration of neutro-

phils, while in animal three infiltration with neutrophils was only mild to moderate. In the necrotic areas of the first two animals there were many bacterial colonies consisting of numerous pleiomorphic bacilli, 1-2 µm in diameter, which colored uniformly gram-positive using the modified Brown and Brenn gram-stain.

After superficial disinfection by flame, each injection site was sampled and plated on two different blood agars. These were incubated overnight at 37°C; one under normal atmospheric conditions and the other in a sealed container under anaerobic conditions. Typing of colonies was done using biochemistry as well as MALDITOF-MS. Results were: abundant *Corynebacterium ulcerans* (mink one), abundant *C. ulcerans* and mild growth of *Clostridium perfringens* (mink two) and mild growth of *Cl. perfringens* mixed with *Cl. sordellii* (mink three). The *C. ulcerans* isolates were tested for *tox* gene carriage by PCR and for toxin production by the Elek test at the National Institute of Public Health and the Environment (RIVM). All isolates carried the *tox* gene and all isolates produced toxin.

Screening

Sampling

The isolation of potentially zoonotic toxigenic C. ulcerans in the described field case raised concerns about the possibility that mink are a reservoir host for this bacterium. It was therefore decided to screen mink on a selected number of farms for nasal carriage of corynebacteria. In 2015 approximately 60 nasal swabs were collected from three Dutch mink farms during pelting time (62 from farm A, 56 from farm B, 60 from farm C) within one hour after euthanasia by CO inhalation. None of the sampled animals showed signs of disease or trauma. Directly after sampling the swabs were transported at below 8°C to the GD laboratory for immediate processing.

Culture and identification

Swabs were plated on blood agar and incubated overnight at 37°C. Each distinct colony was typed using MALDITOF-MS. Based on the high yield of *Corynebacterium* colonies using this method on samples from the first farm (Farm A) it was decided not to use additional selective Corynebacterium-culture media for the remainder of the screening.

Toxin production

Five random *C. ulcerans* isolates per farm were additionally typed at RIVM using 16S partial sequence analysis and extensive biochemical profiling and subsequently tested for presence of the tox gene using PCR. The tox gene is necessary for toxin production. All isolates that tested positive were also tested for toxin production using the Elek-test.

Results

The results of *C. ulceran*s isolation, PCR and Elek testing, are listed in Table 1. Other *Corynebacterium* spp. isolated from the nasal swabs included *C. amycolatum* (34), *C. freneyi* (5), *C. pseudotuberculosis* (3), *C. xerosis* (3), *C. ammoniagensis* (3), *C. confusum* (2) and *C. casei* (1).

Table 1. *Isolation of Corynebacterium ulcerans from nasal swabs, detection of the tox gene using PCR in five isolates per farm and results of the Elek test for toxin production in the tox gene PCR positive isolates*

Farm	C. ulcerans (positive/tested)	Tox-gene PCR (positive/tested)	Toxin production (positive/tested)
A	28/62	0/5	-
B	29/56	4/5	0/4
C	6/60	5/5	0/5

Discussion

In human medicine *C. ulcerans* strains that harbour the toxin-encoding *tox* gene are mostly known for their ability to cause exudative pharyngitis indistinguishable from diphtheria, occasionally with severe sequela. Cases of diphtheria-like disease caused by toxigenic *C. ulcerans* actually outnumber classical diphtheria cases caused by *C. diphteriae* (Wagner et al., 2010; Zakikhany and Efstratiou, 2012). Toxigenic *C. ulcerans* can also cause cutaneous lesions indistinguishable from classical cutaneous diphtheria (Dewinter *et al.*, 2005; Mattos-Guaraldi *et al.*, 2008; Werchniak *et al.*, 2007). Recently one such case was associated with transmission of *C. ulcerans* from a domestic cat with lesions on paw and mouth mucosa to its owner (Corti *et al.*, 2012). In veterinary medicine *C. ulcerans* is primarily known as a cause of mastitis in cattle. Raw dairy products as well as well as close contact with cattle are historically important sources of zoonotic transmission. However, *C. ulcerans* is increasingly found in domestic dogs and cats (De Zoysa *et al.*, 2005; Hogg *et al.*, 2009; Lartigue *et al.*, 2005) as well as in pigs (Boschert *et al.*, 2014; Schuhegger *et al.*, 2009), posing a zoonotic risk for their owners (Berger *et al.*, 2013). The current report describes isolation of toxigenic *C. ulcerans* from lesions in mink and non-toxigenic *C. ulcerans* in nasal swabs from many healthy mink. Although isolates from healthy mink did not produce toxin, they often carried the *tox*-gene (non-toxigenic *tox* gene-bearing strains). Only three farms were screened, but these results could indicate that *C. ulcerans* is a common inhabitant of the nasal mucosa in mink. Since our report is the only known case of disease in mink associated with the bacterium, the pathogenic potential of *C. ulcerans* in mink is thought to be low. However, the zoonotic potential should be considered, especially in periods when people have close contact with the animals, such as during pelting time.

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Development of a sandwich ELISA for quantification of immunoglobulin G in mink blood

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Abstract

A major concern amongst the Danish mink farmers is the incidence of the syndrome pre-weaning diarrhea. The syndrome causes major management issues and decreases the welfare of the mink and increases mortality in the pre-weaning period. The etiology of the syndrome is considered multifactorial as a specific cause is not fully established or understood. Adding to an increased risk of developing pre-weaning diarrhea is the fact that the mink kits are born with very low levels of circulating immunoglobulins. Rapid achievement of high levels of immunoglobulins in the bloodstream is essential for the kits early immunity and thus their resistance against pathogenic agents found in the environment. This study describes a sandwich ELISA for quantification of the concentration of total immunoglobulin G in mink blood. The ELISA was validated with serum samples from females (n=8) and their kits (litters of 4-12). Preliminary results show that the IgG concentration among kits from the same litter was similar, while litter to litter variation was high.

Keywords: mink serum IgG, ELISA, validation

Introduction

The role of maternal immunity with regard to pre-weaning diarrhea has not been fully elucidated. One approach to getting closer to an understanding of the importance of the female mink immune system and its effect on the immune system of the mink kits is to take a closer look at the concentrations of total IgG in the kit serum. The mink kits are born with very low levels of IgG (Coe and Race, 1978). In this study we designed and validated an immunoglobulin class specific ELISA for mink IgG in order to quantify the levels in mink blood. As there are no commercially available mink ELISA kits or antibodies towards mink IgG we developed and validated a sandwich ELISA based on a commercially available goat anti-ferret IgG antibody, which cross-reacts with Ig from mink (Martel and Aasted, 2009).

Material and methods

Animals:

Eight female minks *(Neovision vision)* and their litters (n=4-12) were obtained from two commercial mink farms in Zeeland, Denmark. They were housed in separate cages and fed a standard mink diet.

Sample collection:

Peripheral blood samples were taken from the mink dams and kits. Clotted blood was centrifuged at 4000 G for 15 min at 4 °C. Serum was collected and stored at -20 °C prior to ELISA.

IgG purification

10 ml of the mink serum pool were passed through a column packed with 4 ml of Protein G Sepharose High Performance (GE Healthcare, Bio-Sciences, Uppsala, Sweden). The column was washed extensively with washing buffer (0.2 M NaOH, pH 8.8) and eluted with 0.1 M glycine/HCl, pH 2.8. Absorbance at 280 nm was determined on a Nanodrop spectrophotometer and used to estimate the protein concentration of the eluted fractions, which were pooled and dialyzed against PBS overnight at 4°C. The resulting IgG pool was analyzed by SDS-PAGE (12 % Bis-Tris NuPAGE, Invitrogen, Carlsbad, California, United States) and stored at -20 °C prior to ELISA.

ELISA:

The optimal dilutions of the catching antibody and detection antibody were determined by checkerboard titration. The catching antibody was a commercially available goat anti-ferret IgG (Sigma-Aldrich), which cross-reacts with mink IgG (Martel and Aasted, 2009). This was diluted in 0.05 M carbonate buffer (pH 9.6) and then coated overnight at 4°C on a Maxisorp plate (Nunc, Roskilde, Denmark) at a concentration of 0.5 µg/ml. Wells were then emptied and washed 4 times and blocked with 1% Bovine Serum Albumin (BSA, Sigma-Aldrich, St. Louis, Missouri, United States) in PBST (PBS with 0.05% Tween 20) for 1 hour at room temperature with shaking. After four washes the serum samples and 2-fold dilutions of the mink IgG pool (used as standard and prepared as described above, 0.5 µg/ml) were diluted in acetate buffer (0.05 M, pH 5.5). The diluted samples and standard were added to the wells and incubated with shaking at room temperature for 1 hour prior to 4 washes. Horseradish peroxidase (HRP) conjugated goat anti-ferret IgG (Sigma-Aldrich, St. Louis, Missouri, United States) diluted in 1% BSA + PBST to the concentration of 1.25 µg/ml was added to the wells and incubated for 1 hour at room temperature with shaking and then washed 4 times. Then substrate TMB (Kem-En-Tec, Taastrup, Denmark) was added to the wells and when a suitable color development was observed the reaction was stopped using 0.5 M sulfuric acid. The optical density (OD) of wells was read at 450 nm, and unspecific coloration was subtracted at 650 nm using an automatic plate reader (Thermo Multiskan Ex spectrophotometer, Thermo Scientific, Waltham, MA, USA). All samples including standard were analyzed in duplicates. Sample values were calculated from the curve fitted to the readings of the standard (using Ascent software v. 2.6, Thermo Scientific, Waltham, MA, USA).

SDS-PAGE

The eluted IgG fractions from the protein G purification was analyzed using SDS-PAGE on NuPAGE 12% Bis-Tris gels (Invitrogen, Carlsbad, California, United States) and the samples/bands were visualized using silver staining.

Results

Protein G Sepharose affinity chromatography was used to purify serum IgG (see materials and methods). The purity of this mink IgG preparation was demonstrated by SDS-PAGE comparing it to an existing purified mink IgG preparation (a kind gift from Bent Aasted, University of Copenhagen). We confirmed the molecular weight of mink IgG heavy chain and light chain to be 54 kDa and 25 kDa, respectively. The ELISA was thoroughly validated and had a lower limit of quantification at 0.008 µg/mL, a good reproducibility with low intra- and inter-assay variability, and was linear for serum samples within a relevant dynamic range. Our results using this sandwich ELISA indicated a within litter effect on the serum concentrations of IgG in mink kits.

Discussion

There are no commercially available mink IgG ELISA kits and no commercially available reagents with defined specificities against mink immunoglobulins. This study describes the development, validation and optimization of a sandwich ELISA for the quantification of the concentrations of total IgG in mink serum. Quantification of total IgG in serum will be very useful for estimating the immunological status of the mink dam and kits with respect to availability and transfer of maternal antibodies during the suckling period. The preliminary finding of within litter clustering of IgG concentrations indicates that within a litter all kits obtain the same IgG serum concentrations, which may suggest that the maternal supply of IgG may be the determining factor in the efficiency of transfer of IgG from the mink female to her kits. Future uses of the ELISA include its use as a tool for predicting which female will be able to let their kits attain optimal serum IgG concentration within an optimal time window.

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Diarrhea in the pre-weaning period in farmed mink (Neovison vison) – preliminary results from interviews from a case control study

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Abstract

Diarrhea in the pre-weaning period of mink kits also known as "sticky kits", "greasy kits" or "wet kit syndrome" affects mink kits of 2-5 weeks of age. The clinical picture is characterized by diarrhea and the appearance of a sticky/greasy exudation starting in the neck region and on nails. Fulminant diseased kits get a red swollen anus, exhibit a distressed vocalizing behavior and may become dehydrated with the risk of dying. The disease complex/syndrome is known in all mink producing countries in the northern hemisphere and is an economic problem for the breeders as well as a welfare problem for the animals. Several pathogens have been incriminated as triggering factors for the syndrome, but it still seems that the cause(s) shall be found in an interaction between etiologic agents and environmental conditions. The present study was a part of a case control study involving 30 farms in 2015. The results from two interviews with the mink farmers concerning different aspects of management in the pre-weaning period are presented.

Keywords: "sticky kits", "wet kits", wet kit syndrome, "greasy kits", risk factors, farm level

Introduction

Several bacteria including staphylococci, streptococci and *E.coli* have been isolated from diseased mink kits with diarrhea in the pre-weaning period. However, the findings are inconsistent and the same microorganisms are often also found in healthy mink kits (Vulfson *et al.* 2003, Guardabassi *et al.* 2012, Hammer *et al.* 2014, Jørgensen *et al.* 1996, Sledge *et al.* 2010).

In 2001, *Astrovirus* was suggested to be an important cause of pre-weaning diarrhea (Englund *et al.* 2001). In later studies, *astrovirus* has been confirmed to be a significant risk factor (Englund *et al* 2002, Ullman *et al.* 2004, Ullman *et al.* 2006, Hammer et al. 2006, Hansen *et al.* 2013). Calicivirus has also been found to be a significant risk factor for pre-weaning diarrhea (Englund *et al.* 2002, Ullman et al. 2006).

When an outbreak of pre-weaning diarrhea occurs, the mortality may be as high as 30% on a farm (Clausen and Dietz, 2004). Often one or two kits per litter die. Even in cases of low mortality, treatment of the animals is very time consuming and surviving animals can be unthrifty with affected pelt quality. The syndrome is still regarded as a multifactorial disease complex and with certain farms having a history of repeating outbreaks each year, emphasis has been on identification of possible environmental risk factors.

Methods

As a part of a case control study carried out in 2015, 30 farms were visited from the 11th of May to the 2nd of June. The mink farmers were interviewed and follow-up interviews were performed after weaning. 15 farms with a former history of outbreaks were selected as expected case farms and 15 farms without a history of pre-weaning diarrhea were randomly selected among farms receiving feed from the same feed kitchens as the case farms. The case-farmer called our team for a visit when an outbreak started, and the control farms were visited concurrently. At the end of the period, the percentage of affected litters was recorded and the case control status was finally determined.

The questions included aspects on housing, production efficiency (empty females, kits per litter) composition of color types, gender and age of the animals, management, biosecurity and hygiene initiatives and concurrent frequency of mastitis on the farm. The data were entered into MS Excel and transferred to SAS (SAS Institute Inc.) and evaluated in univariate and multivariate analysis using Student's t-test, Chi-square/Fishers exact test and logistic analysis with the estimation of mean differences and odds ratios between case and control farms. A 95% significance level was chosen.

Results

Case farms had between 13 and 77% affected litters in the period, whereas control farms had between 0 and 8% affected litters. One predefined control farm changed status to be a case farm, and two predefined case farms appeared to have so limited problems that they were re-categorized from case to control farms. The rest of the farms (27) kept their expected status. Univariate analytical results showed statistically associations. The expected farm status was significantly associated with the percentage of affected litters ($P<0.05$) and case farms had am higher accumulated percentage of affected litters at the time of visit compared to control farms (7.9% versus 1.1%). There was an increased risk of being a case farm if farm size was above median (OR=5.5, $P<0.05$) and case farms had a significantly higher proportion of 1-year old females compared to control farms (63.3% and 53.1% respectively). Case farmers more often bred females which had diseased litters in the previous season compared to control farms ($P<0.05$). Having dogs on the farms gave an OR of 9.3 ($P<0.05$) of being a case farm compared to farms where dogs were not allowed to enter. Having a proportion of mastitis greater than 10% among the females was also found to be significantly more common in case farms compared to control farms ($P< 0.05$). The case farms had a reduced number of weaned kits compared to the control farms (5,6 and 6,1 kits per parturient female, respectively) but it was not statistically significant. There were no effects of housing or material in the nest boxes and no differences in hygiene initiatives between case and control farms. There was no effect of feed additives to the feed, number of feedings per day or in the way the farmers used litter equalizations. As an effect of diarrhea staff on the case farms spent more time nursing and treating the animals than staff on the control farms did (5.8 hours/1000 females and 3,7 hours/1000 females, respectively).

Discussion

In this study we found an association between farm size and being a case farm with outbreak of pre-weaning diarrhea, and this have also been demonstrated in other studies (Olesen and Clausen, 1990; Boersting, 1997). This may be due the larger number of animals, increased animal density on the farm and infection pressure, but also the total staff employed on the farm increases, possibly affecting the variability of the management procedures, traffic in and out of the farm etc. The results showed that case farms had a greater proportion of 1-year old females compared to control farms which

corresponds very well to the knowledge, that young females are at greater risk of having litters with pre-weaning diarrhea than older females (Olesen and Clausen, 1990). This is probably because 1-year old females are the least immune-competent animals, which may be reflected in the passive transport of immunoglobulins to their offspring. From other production animals it is also well known that the farm immunity depends on the composition of the dams according to their age and parity (Zimmerman et al., 2012, Evans et al.,2008) This interview based study did not find significant associations on hygiene initiatives and of biosecurity measures, only that dog access to the farms was associated with pre-weaning diarrhea. Biosecurity initiatives turned out to be very important in a recent study about canine distemper on Danish mink farms (Gregers-Jensen et al., 2015), but maybe the agents incriminated in pre-weaning diarrhea, e.g. astrovirus is so widespread on commercial mink farms that biosecurity measures do not affect the occurrence of diarrhea in the pre-weaning period. It is not known whether dogs can act as a reservoir for e.g. mink astrovirus, but a fecal oral transmission route is not obvious, since dogs normally do not have access to the wire mesh where the feed is delivered. Maybe allowing dog access to the farms is correlated with a somehow more incautious behavior of the farm staff, increasing the risk of pre-weaning diarrhea. Breeding of females from diseased litters in the previous season and the increased need for manpower can be regarded as effects of having outbreaks of high morbidity on the case farms. However, it might also prevent the mink farmer from accomplishing a strict selection of the most immuno-competent or resistant animals. A high prevalence (>10%) of mastitis on the farm seemed to be a risk factor for being a case farm. A study has shown that mastitis is not a necessary cause for pre-weaning diarrhea/"greasy kits" (Clausen and Dietz, 2000). However since the chronic forms of mastitis can be difficult to identify clinically, the true prevalence of mastitis might be underestimated by the farmers in both case and control groups. The results from this study suggest that mink breeders having repeating outbreaks of pre-weaning diarrhea should pay attention to several management procedures including the parity profile of 1-year old females and older females when selecting dams for the next breeding season.

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Health & Disease

Oral presentations

Fecal microbiota of healthy and diarrheic farmed arctic foxes *(Vulpes lagopus)* and American minks *(Neovison vison)* – a case-control study

Epidemiologic study on Fur Animal Epidemic Pyoderma in Finland

Pooling of faecal samples for quantitative virus diagnostics by real-time PCR

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Development of a sandwich ELISA for quantification of immunoglobulin G in mink blood

Diarrhea in the pre-weaning period in farmed mink *(Neovison vison)* – preliminary results from interviews from a case control study

Part 2. Aleutian Disease

Study of distribution of AMDV load in infected mink farms environment

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Abstract

In order to study the distribution of the AMDV load in infected farms, 85 environmental samples from seven mink farms were analyzed by qPCR between the years 2013-2015. All farms included in the study were infected between 2012 and 2014, and had developed a high seroprevalence (CIEP/ELISA) in a time period shorter than 12 months. Samples were classified into eight categories, based on the type of sample and degree of proximity to the source of infection (animals). 73 of 85 samples tested positive to qPCR. The results of this study indicate a high contamination of the environment on these farms. In addition, quantification of positive samples shows a decrease in the virus concentration from the areas close to the animal barns to the periphery of the farm. Also, our results demonstrate the contamination of elements with direct contact with animals, street clothes, vehicles of farm workers and personal protective equipment used for sampling. Statistical analysis reveals significant differences between diverse categories. Finally, possible implications related to biosecurity measures against AMDV are discussed.

Keywords: Aleutian disease, environmental distribution, qPCR, biosecurity

Introduction

Aleutian Mink Disease Virus (AMDV), recently reclassified as *Carnivore amdoparvovirus 1*, is the responsible agent of Aleutian Disease (AD), also known as Mink Plasmacytosis. AD affects both wild and farmed mink, where it causes severe economic losses, and is present in most of fur-producing countries (reviewed by Canuti *et al.*, 2015). The virus may produce a progressive wasting syndrome in adults, characterized by weight loss and anorexia associated with generalized systemic disease and also reproductive disorders as reduced litter size, decreased pregnancy rate, embryonic death and abortion (Eklund *et al.*, 1968; Broll and Alexandersen, 1996), while in kits the virus may cause an interstitial pneumonia with typically high mortality (Alexandersen and Bloom, 1987).

In farmed mink, eradication strategies include serological screening by counter-inmunoelectrophoresis (CIEP) or enzyme-linked inmunosorbent assay (ELISA) and, depending on the results, it is decided between culling of infected animals or stamping-out procedure (Cho and Greenfield, 1978; Gunnarsson, 2001). AMDV is extremely persistent in the environment (Hahn *et al.*, 1977); thus, the success of the eradication action is closely related to cleaning and disinfection procedures. From the point of view of fur-producing sector, it is critical to prevent the AMDV transmission from infected farms to free farms. This transmission can occur through introduction of infected animals, employment of contam-

inated farm materials, shared personnel between farms, visitors, or even through the contamination of farmers or workers who visit contaminated premises (e.g. infected farms, pelting centers).

Biosecurity and cleaning and disinfection protocols have been supported on assumptions based on experience and epidemiological evidences. However, the frequent failure of eradication strategies suggests that there are some unknown issues related to virus reservoirs. The first AMDV detection in environmental samples has been published recently (Prieto *et al.*, 2014). The mentioned study represents a step forward to the understanding of AMDV distribution and concentration under certain conditions.

Thus, the aim of this study was to evaluate the AMDV distribution and load in the environment of farms with active/recent infection, by means of environmental samples analyzed by qPCR, including not only farm facilities but also workers, visitors and vehicles that can act as reservoirs and/or carryover vectors.

Material and methods

Seven AMDV-positive farms were selected for this study. Six of them are situated in northwest Spain (Galicia) and the remaining farm (nº 82) is placed in the south of France. Table 1 shows the evolution of seroprevalence for all included farms. All of them were infected between 2012 and 2014, and presented the common characteristic of developing a high seroprevalence (CIEP/ELISA) in a time period shorter than 12 months. The serological analysis were performed at three different laboratories: CFE Laboratorium (Nederasselt, the Netherlands), LASAPAGA (Lugo, Spain) and CIALI (Vigo, Spain).

Table 1. *Evolution of seroprevalence from negative status in the seven farms for the study period.*

Farm ID[a]	Date	Method	% Seroprevalence	Sampling date
61	18/01/2012	CIEP	0%	24/07/2013
	22/10/2012	CIEP	24.65%	
10	04/01/2012	CIEP	0%	22/10/2015
	29/10/2012	CIEP	4.17%	
	29/10/2014	ELISA	36.63%	
19	18/01/2012	CIEP	0%	30/10/2013
	11/06/2013	CIEP	2.78%	
	04/10/2013	CIEP	32.92%	
12	0·/01/2013	CIEP	0%	19/09/2014
	26/05/2014	CIEP	16.56%	
	05/01/2015	CIEP	68.18%	
109	06/06/2014	CIEP	0%	19/09/2014
	14/10/2014	CIEP	16.85%	
	25/11/2014	CIEP	26.02%	
22	20/01/2014	CIEP	0%	16/12/2014
	08/01/2015	CIEP	42.97%	
82	25/05/2011	CIEP	0%	14/11/2014
	17/10/2013	CIEP	47,19%	
	16/07/2014	CIEP	68,82%	

Sample categorization and sampling procedure

Sampling was done between 2013 and 2015. Prior to visit the farms, eight categories of samples were established based on the type of sample and degree of proximity to the animals. Table 2 shows the number and type of samples included in each category. All samples (n = 85) were taken by using a dry sterile cotton swab of 11mm in diameter, swabbing the area of sampling for 20-30 seconds. Next, swab heads were place into sterile 12ml screw-cap tubes by breaking the wooden stick of the swab, and once in the laboratory the tubes were frozen at -20°C until processed. Prior to DNA isolation, 5 ml of PBS-T (Phosfate Buffer Saline with 0.05% of Tween 20) were added directly to the tube and vortexed for 1 minute. All used reagents were supplied by Sigma-Aldrich (Missouri, United States). After 15 minutes of settling, 1 ml from each tube was taken and placed in a sterile Eppendorf tube, and then kept at -20°C until DNA isolation.

Table 2. *Categorization of samples, with number and type of samples included in each category.*

Category nº	Category name	n	Description
1	Cages	13	Cages and nests walls
2	Soil/manure	10	Barns soil, manure under cages
3	Gloves	6	Working gloves (for animal immobilization)
4	Facilities	17	Warehouses, changing room, dining room, feed kitchen, freezer, offices/dwelling place
5	Street	9	Street clothes and footwear, farmers´ vehicles
6	Visitors	10	Coveralls and boot covers
7	Effluents	7	Water outputs from the farms
8	Periphery	13	Vehicle ruts, parking area, farm walls, silos, soil from farm entrance

DNA isolation and qPCR performance

A commercial kit was used for DNA isolation (Nucleospin® Soil, Macherey-Nagel GmbH & Co KG, Düren, Germany), by using SL1 reagent supplied with the kit in the first step and then following the manufacturer´s instructions. DNA was collected using a 100 µl volume of elution buffer. For qPCR, a commercial kit that detects NS1 (a single copy gene) was employed (Genesig Advanced Real-Time PCR Detection Kit for Aleutian Disease Virus, PrimerDesignTM Ltd., Southampton, UK). Quantification of NS1 copies was performed by means of a standard curve built with six ten-fold serial dilutions of a synthetic positive control supplied by the manufacturer.

Statistical analysis

Kruskal-Wallis test and Dunn *post-hoc* analysis for pairwise multiple comparisons adjusted with Bonferroni correction were used for studying statistical differences among categories.

Results

73 of 85 samples tested positive to qPCR. Table 3 resumes the obtained results for the AMDV quantification for each sample category. Statistical analysis showed significant differences (Bonferroni´s adjusted p-value < 0.05%) in the two first categories (“Cages” and “Soil/manure”) regarding the two last categories (“Effluents” and “Periphery”).

Table 3. *Number of qPCR positive samples, range of AMDV copies, mean of AMDV copies and standard error for each category.*

Category nº	Category	n	Nº positive samples (%)	Range	Mean AMDV copies	Standard error
1	Cages	13	13 (100%)	1.87x104 - 1.27x108	4.20x107	1.35x107
2	Soil/manure	10	10 (100%)	1.53x105 - 3.31x107	8.64x106	3.77x106
3	Gloves	6	6 (100%)	1.83x105 - 3.73x107	7.64x106	6.03x106
4	Facilities	17	13 (76.5%)	1.63x104 - 6.06x106	1.35x106	5.19x105
5	Street	9	8 (88.9%)	4.11x104 - 4.73x106	9.63x105	5.51x105
6	Visitors	10	10 (100%)	4.87x104 - 7.60x105	3.54x105	8.94x104
7	Effluents	7	5 (71.4%)	7.22x103 - 2.79x104	1.48x104	3.78x103
8	Periphery	13	8 (61.5%)	6.94x103 - 2.00x106	3.16x105	2.44x105

Discussion

The delay in the successful completion of the AMDV control programs can be caused by the infection persistence and the introduction of new infections on farms, and also by an incomplete understanding about recontamination sources (Themudo *et al.,* 2011). In this study, the authors´ intention was to apply a previous knowledge about environmental sampling for AMDV detection (Prieto *et al.*, 2014) in order to establish how AMDV is distributed in the environment of an infected mink farm.

To begin with, two main aspects related to the study performance must be considered. Firstly, the included farms presented a high seroprevalence; therefore, environmental virus load could fluctuate under other conditions. Secondly, the detection of AMDV DNA does not imply infectivity since PCR is unable to discriminate between viable and unviable virus. However, the obtained results can be used for compare the distribution of AMDV contamination among different places or facilities in the farms.

The eight described categories represent a progressive distancing from the animals, the main source of contamination. In fact, average virus load and number of positive samples decrease in the same direction (table 3), with the exception of category 7 "Effluents" (discussed later).

Categories 5 and 6 ("Street" and "Visitors") represent mobile elements which move among the remaining categories and out from the farm, being able to act as carryover vectors to other places, directly or indirectly. The average contamination level of these 2 categories is similar to the obtained value for "Periphery" (in the case of "Visitors") or for "Facilities" (in the case of "Street" category). It is deduced that workers have a high potential to carry the virus to other farm dependencies ("Facilities") as well as to their street clothes and vehicles ("Street"). For this reason, "Street" category has a huge significance as virus reservoir during cleaning and disinfection procedures, as well as about biosecurity due to their mobility. In the case of "Visitors", they are contaminated when they move among the animal barns, and this contamination depends on the tasks carried out and also on the time spent in the farm (in this study, 30 minutes walking around inside the farms). This fact also makes them a significant risk for biosecurity, as they can visit several farms in the same day. Thus, the use of personal protective equipments (coveralls and boot covers) is necessary.

Among the regular elements of a farm, the areas with the highest contamination are the cages, manure

and gloves for animal immobilization. AMDV contamination decreases in the annexed facilities, and the lowest values are detected in the periphery elements and effluents. These results are especially relevant in regards to planning the cleanup and disinfection procedures in farms which are performing a stamping-out program, and for monitoring these processes. Besides, this is significant for biosecurity issues, as it seems logical to infer that the risk of contamination is lower in the "periphery" of the farm than in the "core" of the same. Nevertheless, biosecurity measures have to be taken anyway when annexed facilities or the periphery of a positive farm are visited.

"Effluents" category must be considered separately, because this category includes the water that comes from the most contaminated areas in the farm, and however it presents the lowest detected AMDV load. This fact is probably related to the water action that favours the virus lavage and leaching; therefore, it can be more appropriated to practice, previously to DNA isolation, a concentration protocol over a higher amount of sample (Ahmed *et al.*, 2015).

To conclude, the detection of AMDV on environmental samples by qPCR has allowed to objectively assess the AMDV distribution and virus load in positive farms and the results has been the basement of a review of risks and biosecurity protocols. It has also had a deep influence on the awareness of farmers and other agents of the industry about specific risks.

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Evaluation by qPCR of personal protective equipments for visitors against AMDV

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Abstract

In 2015, a study for the Galician Association of Mink Breeders (AGAVI) was conducted in order to evaluate the effectiveness of different Personal Protective Equipments (PPEs) used for routine visits to mink farms. Sampling was carried out during a visit to an infected farm. Sampled elements included two types of protective coveralls, two types of boot covers and other elements (unprotected hair, beard and paper forms) using a sterile swab which was subsequently analyzed by qPCR. In order to assess the viral load present in the farm environment, samples inside the barns where animals are housed were also taken. The results show that only the coveralls with certification for protection against suspended solid particles were effective in preventing the pass-through of viral particles, while the simple coveralls allowed the passage of the virus. Footwear contamination was also observed despite having used double boot covers. Other unprotected elements, as the hair or the paper form used for data collection, were also contaminated during the visit to the farm. The results of this study served as the basis for reviewing the recommendations on the PPEs and biosecurity protocols for farm visitors.

Keywords: Aleutian disease, qPCR, biosecurity, environmental surveillance

Introduction

Aleutian Mink Disease Virus (AMDV) is a parvovirus responsible of Mink plasmacytosis or Aleutian Disease (AD). There is evidence that AMDV is environmentally persistent (Hahn *et al.*, 1977) but the sources of repeated reappearance of AD on cleaned ranches are not clearly understood. One possibility is the transmission via shared materials or personnel among free and infected farms, as well as visitors who move from farm to farm. Also, detection of AMDV in environmental samples has recently demonstrated (Prieto *et al.*, 2014). This study was conducted in order to evaluate the effectiveness of different Personal Protective Equipments (PPEs) used for routine visits to mink farms by means of qPCR detection.

Materials and methods

The selected farm presented a AMDV seroprevalence of 36.63% in the last enzyme-linked immunosorbent assay (ELISA). Prior to entering into the farm, street clothes and working shoes were sampled, and then three persons were dressed with a disposable coverall (CC: certificated coverall; Biztex® Microporous 6/5 Coverall, Portwest Ltd., Westport, Ireland) with certification against airborne solid particulates and liquid chemicals (UNE-EN ISO 13982-1:2005/A1:2011 and UNE-EN

13034:2005+A1:2009). Additionally, two of them were also dressed with a second coverall without any certification (SC: simple coverall). One of these assays was performed in a different day with the same protocol. The three persons were fitted with two types of boot covers (external and internal boot covers). Hair and beard were unprotected in one person, and after the visit samples were taken from these locations.

A walk (over 30 minutes) was performed within farm barns. During this visit, samples from different farm elements *(Table 1)* were taken in order to evaluate the environmental AMDV load. Once out of the farm, coveralls (1 external CC, 2 CC under the SC and 2 external SC), street clothes under CC, external and internal boot covers, working shoes, paper form, hair and beard were sampled *(Table 2)*.

Sterile cotton-tipped swabs of 11 mm in diameter were used. DNA isolation, amplification and detection was basically the same described by Prieto et al., (2014). In this case, two commercial DNA-isolation kits (Nucleospin® Soil, Macherey-Nagel GmbH & Co KG, Düren, Germany, and High Pure PCR Template Preparation Kit, Roche Diagnostics GmbH, Mannheim, Germany) were used for the DNA extraction following the manufacturer´s instructions. In order to detect AMDV, a commercial kit was used (Genesig Advanced Real-Time PCR Detection Kit for Aleutian Disease Virus, PrimerDesignTM Ltd., Southampton, UK). Once all samples were analyzed with the two extraction protocols, the highest AMDV load was registered in positive samples by both methods.

Results

Results from samples taken in the farm during the visit are showed in table 1.

Table 1. *Virus load in the tested farm elements.*

	CT	DNA copies/swab
Cages	23.40	3,999,014
Shed ground	23.94	2,789,762
Shed Truss	24.08	2,541,106
Shed roof	25.02	1,357,675
Parking ground	28.61	123,914

Samples taken from street clothes and working shoes before visit were negative. Table 2 shows the results from samples taken after the visit.

Table 2. *AMDV load from samples taken after the visit.*

	CT/ result	DNA copies/swab
External boot covers	28.42	140,652
Internal boot covers	23.55	3,618,365
Working shoes	28.04	181,215
External certificated coverall	26.55	489,444
Street clothes under certificated coverall	Negative	
Simple coverall- 1	29.18	84,732
Certificated coverall under simple coverall- 1	34.63	2,237
Simple coverall- 2	25.90	754,996
Certificated coverall under simple coverall- 2	29.63	62,766
Paper form filled in farm	30.01	48,716
Hair	34.40	2,608
Beard	40.86	35

Discussion

Our results show the existence of AMDV contamination in the PPE´s used for a farm visit under the described conditions. CC was effective protecting street clothes from contamination. However, SC let virus to pass through it and virus was detected over the CC layer under it. Besides, the use of double boot covers did not prevent the detection of virus DNA in the working shoes.

Both hair and beard samples were also positive. These elements are not always covered during the visits to farms. Other elements as the paper form filled in the farm and the parking area also obtain a positive result.

These results can be used for evaluating and comparing the effectiveness of different biosecurity PPE´s and farm visit protocols:

- Certificated coveralls (UNE-EN ISO 13982-1:2005/A1:2011 and UNE-EN 13034:2005+A1:2009) should be used for routine farm visits.
- The coverall hood should be used too, at least for visits to positive farms.
- The working shoes, even with double boot covers, should be disinfected after visit the farm, before they can contaminate the car or other elements.
- Alternative ways for carrying papers should be implemented (e.g. Whatsapp pictures).
- Car should be parked away from the farm area.
- After visiting positive farms, a shower and change of clothes is advisable.

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Outbreaks of Aleutian mink disease in farmed mink *(Neovison vison)* in Denmark: molecular characterization by partial NS1 gene sequencing

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Keywords: plasmacytosis, phylogenetic analysis, AMDV

Abstract

Aleutian Mink Disease (AMD) is a devastating infectious disease of mink and a major cause of economic losses in global mink farming. The disease is caused by Aleutian mink disease virus (AMDV) that belongs to the *Amdovirus* genus within the *Parvoviridae* family. Several strains have been described with varying pathogenicity and the severity of infection may also depend on the host's genotype and immune status. Clinical signs include respiratory distress in kits and unthriftiness and poor pelt in adults. The infection can also be subclinical.

In 1976 systematic eradication of AMD from Danish mink farms was initiated. Until recently, the disease was largely restricted to the very Northern part of the country, with only occasional outbreaks outside this region. However, during 2015 several outbreaks of AMD have occurred at Danish mink farms throughout the country. Phylogenetic analyses of partial NS1 gene sequences from viruses originating in these farms have revealed new clusters of viruses that do not include previously known strains. To study the origin of the viruses, a global approach has been undertaken.

Introduction

Aleutian mink disease virus (AMDV) is the cause of serious disease with several different symptoms in minks. The pups usually develop pneumonia leading to a high mortality, whereas the adults often develop chronic disease. This chronic disease is caused by the immune system's reaction to the virus, which leads to increased mortality and reduced fertility, thereby affecting the mink production significantly (Alexandersen 1994; Porter 1969).

A test and stamping out policy of AMDV positive mink was initiated in Denmark in 1976, and legislation was issued in 1999 in order to force all Danish mink farmer to test the breeders. The prevalence of AMDV in Danish farmed mink has been only 5 % since 2001 and AMDV positive farms were until recently restricted to the northern part of Jutland and one food-borne outbreak in the southern part of Jutland in 2003. However, in the autumn of 2015 outbreaks of AMDV occurred, now affecting farms all over Denmark. To this date the outbreak is still ongoing.

To trace the source of the current outbreak in Denmark an analysis of the partial NS1 gene was carried out. Previous studies have suggested the ability to distinguish different viral strains of AMDVs using partial NS1 sequencing (Christensen *et al.* 2011; Knuuttila *et al.* 2009; Leimann *et al.* 2015; Olofsson 1999; Persson *et al.* 2015). To investigate origin of the source(s), mink samples from European countries, America and Canada were collected and compared to sequences from Danish farmed and wild mink.

Material and methods

Samples

The Danish mink samples were collected by Kopenhagen Fur (Kopenhagen Fur, Glostrup, Denmark), who sampled the spleen and mesenteric lymph node from euthanized animals, and submitted them to The National Veterinary Institute of Denmark at Technical University of Denmark for further processing. Several foreign veterinarians working in mink farms contributed to the project. Ideally 10 farms from each country were sampled, and five animals from each farm were submitted. The individual animals assumed to be infected with AMDV were selected by the veterinarian or the farmer. All samples were obtained with the farmers consent, and donated to the project for research. Mink samples from abroad were submitted directly to The National Veterinary Institute of Denmark, either as carcasses, blood or spleen samples. From each carcass a blood sample from the heart was taken along with samples of the spleen, the mesenteric lymph node, the liver, the kidneys and the lungs. In total, more than 400 animals were sampled and analysed. So far 12 foreign countries contributed with samples: Canada, Finland, Greece, Holland, Iceland, Italy, Latvia, Lithuania, Poland, Sweden, Ukraine and USA.

DNA extraction, PCR and sequencing

DNA extraction and PCR was performed essentially as previously described (Jensen et al. 2011), but with PCR enzyme AmpliTaq Gold (Thermo Fisher Scientific, Copenhagen, Denmark). PCR products were sequenced with the PCR primers at LGC Genomics, Berlin, Germany.

Phylogenetic analysis

The nucleotide sequences were analysed in the program CLC main workbench version 7.5 (www.clcbio.dk , QIAGEN, Aarhus, Denmark). For each sample, sequence chromatogram files resulting from the forward and reverse primers were contiged, and manually proof-read. The primer binding regions were trimmed off manually to generate 328 bp consensus sequences from each sample. Consensus sequences were aligned at the nucleotide level with existing partial NS1 sequences in GenBank NCBI at February 2016, using the "MUSCLE" alignment algorithm. Neighbor-Joining trees were created with 1000 bootstrap repetitions. Tree visualization was performed in FigTree version 1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

The Danish AMDV sequences from farmed mink grouped in three clusters. One cluster was closely related to the Sæby strain that has been circulating in Northern Jutland for at least last two decades. The other two clusters did not closely resemble sequences of known AMDV strains available from Genbank. Clustering was correlated to the geographic site of sampling. One strain was isolated only in Jutland and Funen, while the other was only isolated at Zealand. None of the foreign sample sequences were identical to the strains of the Danish outbreaks in Jutland and Funen, but 2 Swedish sequences were closely related to the outbreak in Zeeland. The strains within the three Danish clusters have a sequence identity of approximately 97-100 %.

Discussion

The phylogenetic analysis shows that the partial NS1 gene sequences can be used to distinguish between major clusters. It was clearly seen that the geographical location of outbreaks correlated to the cluster of virus detected. Within cluster analysis, however, would require more sequence information. The source of the major clusters of Danish viruses was not determined.

Acknowledgements

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Emerging infections at the interface of wildlife and livestock

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Emerging infections are one the biggest health threats of today. Determining the major steps in the emergence and spread of infectious diseases will help to identify which prevention strategies to implement. This study focuses on understanding pathogen spread in ecosystems, in particular at the boundary of wildlife and livestock. The most significant infectious disease of captive carnivores is aleutian disease, caused by aleutian mink disease virus (AMDV). It can cause an immune complex disease in American mink. AMDV has been detected also in several other mustelid species with potential negative impact on their health and population. We have reported the presence of anti-AMDV antibodies and/or AMDV DNA in 54% of feral American mink, 27% of badgers, and 7% of polecats in Finland. This study showed that AMDV is prevalent in free-ranging mustelids and widely distributed across the country. Here, we continued the project by analysing AMDV-positive samples from mink farms in 2015 and 2016, and report the simultaneous circulation of highly variable strains, of which some are also found in free-ranging mustelids, but some only on farms.

Keywords: Aleutian disease, mustelid, infectious disease

Introduction

Aleutian mink disease virus (AMDV) causes an immune complex disease, Aleutian disease (AD), in farmed and free-ranging American mink *(Neovison vison)*. The disease is also known as plasmacytosis. Natural infection has been detected in other members of the Mustelidae family – ferrets, polecats, European mink, stoats, martens, and otters – in Europe, Japan, and North America (Farid, 2013; Fournier-Chambrillon *et al.*, 2004; Mañas *et al.*, 2001; Murakami *et al.*, 2001). In addition to Mustelidae, evidence of AMDV infection has been found in raccoons, striped skunks, bobcats, common genets, and foxes (Farid, 2013; Fournier-Chambrillon *et al.*, 2004; Ingram & Cho, 1974; Oie *et al.*, 1996).

Finnish farmed mink carry at least three types of AMDV strains, suggesting at least three separate introductions (Knuuttila *et al.*, 2009a). In other wild mustelid species, the viruses causing the infection and antibody response have not been clearly characterised and the details of their evolutionary relationships are not known. Although the presence of clinical disease in wild mustelids other than feral mink is largely unreported, the feral mink may pose a threat to populations of indigenous species by transmitting AMDV to them. Furthermore, if the virus is present in the wild, it has to be taken in to account in the biosecurity measures at mink farms. For these reasons, we designed this project with the aim to describe the AMDV strains circulating in Finland.

Materials and Methods

Blood and spleen samples were collected from free-ranging mustelids in Finland during the years 2006–2014. Samples were provided by Finnish Game and Fisheries Research Institute (RKTL), Finn-

ish Food Safety Authority (Evira), and small game trappers through Finnish Forest Research Institute (Metla), Finnish Hunters' Association, and the Finnish Wildlife Agency.

The antibody response to AMDV infection was measured from all filter paper samples with AMDV-VP2 ELISA (Knuuttila et al., 2009b). For mink samples, the diagnostic sensitivity of this test is 99.0% and specificity 97.0% (Knuuttila *et al.*, 2009b).

DNA was extracted from blood samples of all initially seropositive samples, and equal amount of seronegative samples. DNA was isolated with Qthe iagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. If necessary, DNA was isolated also from 10 mg of spleen. A 640-nucleotide fragment of the AMDV NS1 gene was amplified from the isolated DNA with qPCR (Knuuttila *et al.*, 2015).

Blood and spleen samples from farmed mink were collected in collaboration with Finnish Fur Breeders association. Blood samples were first screened by Fin FurLab for the AMDV antibodies, and spleen samples were subjected to AMDV PCR as described above.

Selected PCR-positive samples were sequenced for the phylogenetic analysis using Sanger sequencing (Haartman Institute sequencing unit, Helsinki, Finland). Phylogenetic trees were constructed using maximum likelihood (ML) method with MEGA5 using 1000 bootstrap replicates and Bayesian method with BEAST 2. General time reversible model was used in both methods.

Results and Discussion

308 samples of wild mustelids were analysed with ELISA of which 92 also with qPCR. Anti-AMDV antibodies were detected in 35 and viral DNA in 34 mustelid samples. All ELISA and/or PCR positive animals (n=39) were regarded as infected (Knuuttila *et al.*, 2015).

Partial AMDV sequences were obtained from three species: feral mink, two badgers, and one polecat. Additional sequences were obtained from Estonian wild mustelids, and farmed mink in Finland, and combined with a dataset retrieved from the GenBank. On phylogenetic analysis, no clear clustering according to species, location, or year could be identified. The most distinctive finding was divergent badger sequences that formed a novel sister group to all other AMDV.

We have confirmed a high AMDV prevalence in feral mink, identified a new host species, European badger, and found a markedly divergent AMDV strain, possibly representing a novel amdoparvovirus (Knuuttila et al., 2015). The infection was widely distributed geographically, excluding only few regions. Most of the strains in free-ranging mustelids were similar to farmed mink strains and free-ranging mustelids, especially feral mink, seem to have an important role in transmitting the virus.

The study on free-ranging mustelids (already published in Knuuttila et al., 2015) has been complemented by analysing the AMDV strains currently circulating on mink farms in Finland. The preliminary analyses have shown the continuous presence of highly variable strains, of which some seem to originate from wildlife. Certain strains, however, seem to be more restricted to circulation on farms.

The prevalence of AMDV in feral mink (54%) and badgers (27%) was striking compared to that of farmed mink (13% in 2012). As feral animals obviously can act as reservoirs and may transmit the virus to AMDV-free farms, this has to be considered in the biosecurity measures at mink farms. Our

study clearly shows that closely related strains occur in both free-ranging mustelids and farmed mink. If contacts between wild and farmed animals cannot be prevented, eradication of AMDV from farms will be challenging. On the other hand, the escape and release of infected farmed mink may be a threat to wild mustelids.

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Dot immunoenzyme assay for the diagnosis of Aleutian mink disease

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Abstract

In 1992, we reported a method to detect antibodies against Aleutian mink disease (AD) virus in blood samples spotted onto nitrocellulose membranes. We called it dot immunoenzyme assay (DIA). Since then, the initial DIA procedure has been modified several times. In the current version, viral antigen produced in the baculovirus expression system and alkaline phosphatase as an enzyme label are used. Because of minute amounts (0.5 microliter) of blood required for testing, the method obviates the need for capillary tubes. Instead, standard disposable lancets are used for blood sampling and application onto membranes. This approach dramatically saves sampling time, excludes mink bleeding and reduces sampling errors. Large sets of membranes are processed together to test tens of thousands of samples simultaneously thereby providing the highest throughput among the AD diagnostic methods. DIA has proved to be efficient in AD eradication programs. Since licensing in 2012, it has replaced counterimmunoelectrophoresis in 70% of tests for AD in Russia.

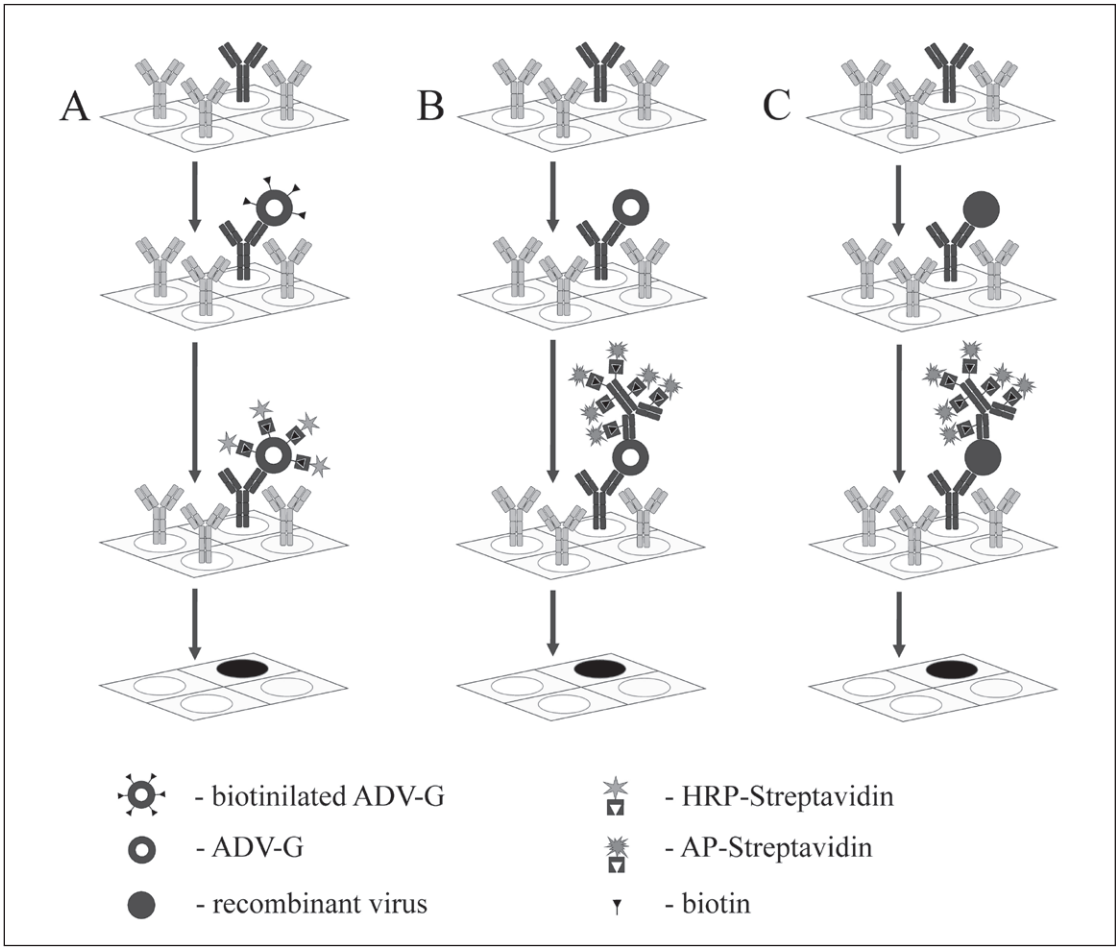
Aleutian mink disease (AD) is a persistent viral infection induced by the AD parvovirus (AMDV) (Bloom et al. 1994). The disease manifestations in adult mink are hypergammaglobulinemia, glomerulonephritis, arteritis, and reduced reproductive performance. In newborns, AD may cause acute fatal pneumonia. Reduced fertility and increased mortality of infected minks can result in serious economic losses to the mink farmers. So far, no effective treatment for the disease exists, and the only available method for AD control is removal of infected minks. The infection may be diagnosed by the presence of either viral DNA or virus-specific antibodies in mink blood. A plethora of serodiagnostic tests have been reported for AD (Aasted and Bloom, 1983; Andersson and Wallgren, 2013; Chen *et al.*, 2016; Cho and Greenfield, 1978; Dam-Tuxen *et al.*, 2014; Knuuttila *et al.*, 2014; Wright and Wilkie, 1982). However, there is still a need for a method combining diagnostic accuracy with high throughput and low costs.

At the IFASA meeting held in Oslo in 1992, we described a method for detection of AMDV-specific antibodies in mink sera spotted onto nitrocellulose membrane (Miroshnichenko *et al.*, 1992). We called this method dot immunoenzyme assay (DIA), as the presence of antibodies in the tested samples appeared as a colored dot on the membrane. The idea was that simultaneous processing of multiple membranes should make mass diagnostic studies substantially less time- and labor-consuming.

The initial version of DIA used mink blood sera as samples (Fig 1A). The AMDV-specific antibodies immobilized on the nitrocellulose membrane were detected by a complex of biotinylated antigen and horseradish peroxidase (HRP)-labeled streptavidin. Nonpathogenic ADV-G strain of AMDV propagated in CRFK cells was used as the antigen (Miroshnichenko *et al.*, 1992). During the past years, the DIA procedure was modified twice. The second version used crude lysate of the ADV-G-infected cells as the source of antigen. Virus binding was detected using secondary biotinylated AMDV-specific

antibodies. In addition, alkaline phosphatase was used as an enzyme label instead of HRP (Taranin *et al.*, 1996). The procedure was longer since it included an additional step (Fig 1B). However, it was advantageous in that it reduced antigen consumption. In the third and currently used version that was developed in 2001, ADV-G has been replaced by the recombinant viral antigen (VP1-VP2) produced in the baculovirus expression system (Fig 1C) (unpublished).

Fig 1. *Schematic representation of the DIA evolution. A - version 1; B – version 2; C – version 3*



During 1992-2005, a number of studies comparing the diagnostic performance of DIA and counter-immunoelectrophoresis (CIEP) have been performed. Side-by-side comparisons of these studies are not straightforward because of the use of different versions of DIA and the antigens of different origin for both DIA and CIEP. When using the same antigen (VP1-VP2 produced in the baculovirus expression system), CIEP and DIA demonstrated comparable performance. Table 1 shows an example of comparative testing in a farm with 5% prevalence. In this particular population, DIA shows a specificity 99.95% (95% CI = 99.80-99.99%) and sensitivity 98.17% (95% CI = 95.37-99.50%) under assumption that CIEP is a "gold standard" method (Gardner and Altman,1989). However, the DIA specificity was >99.99% when calculated on the basis of testing of mink farms considered AD-free. This is similar to the performance shown by recently reported ELISA tests (Chen et al., 2016; Dam-Tuxen et al., 2014; Knuuttila et al., 2014)

Table 1. *Cross-classified results of DIA and CIEP tests in a farm with low AD prevalence*

		DIA		
		positive	**negative**	**total**
CIEP	**positive**	214	4	218
	negative	2	3700	3702
	total	216	3704	3920

The most important feature of the current version of DIA is the capacity to detect AMDV-specific antibodies in the whole blood samples. As only 0.5 microliter of whole blood is required for testing, the method obviates the need for capillary tubes. Instead, usual lancets are used for both blood sampling and sample spotting onto the membrane *(Fig. 2A and 2B).* This approach has a number of advantages: i) the time of the sampling is reduced 2-3-fold compared to that with the use of capillary tubes; ii) there is no need to cut toenails; iii) minks do not bleed after sampling; iii) the risk of sampling errors is minimized.

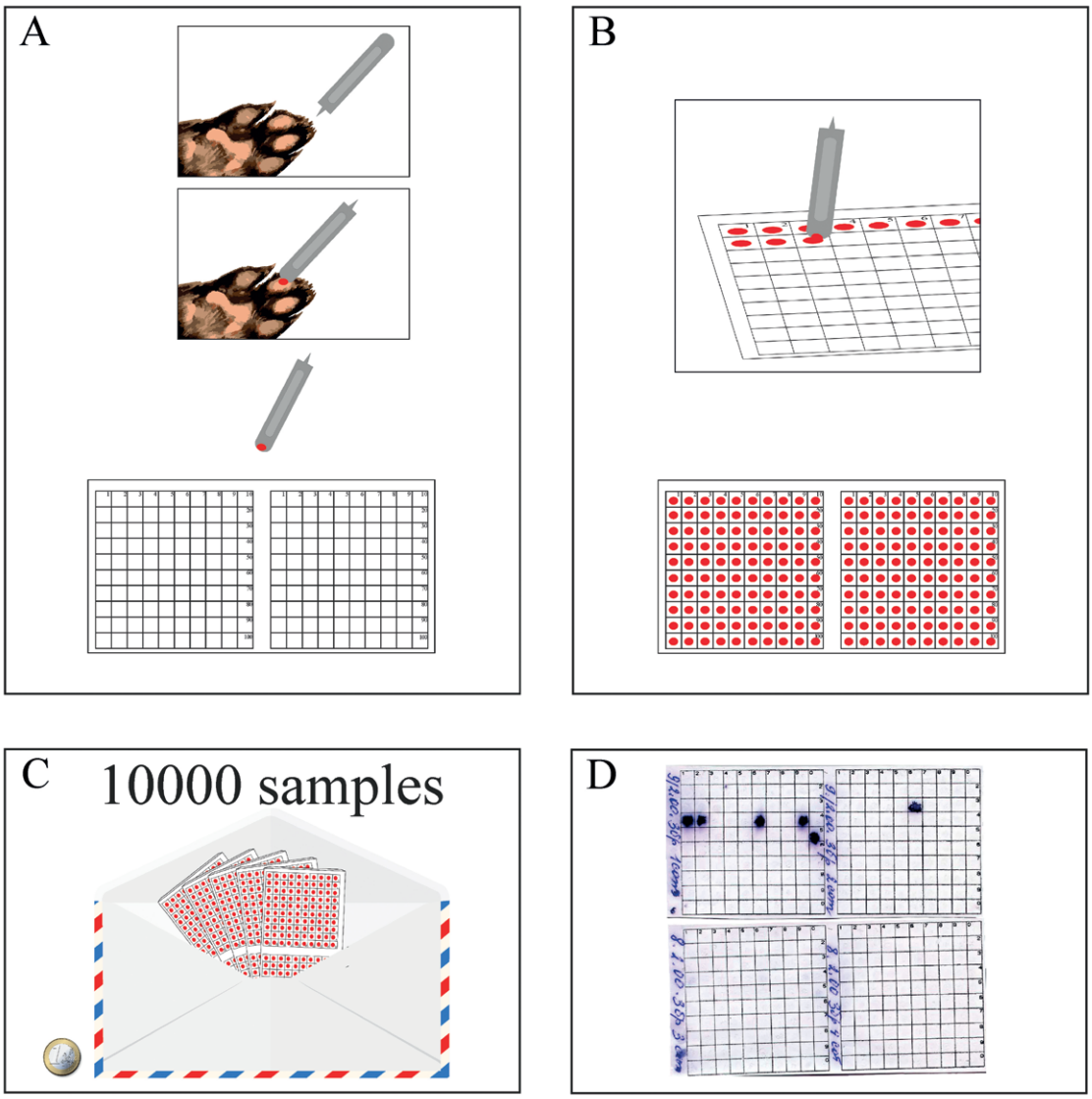
The latter advantage, in our opinion, is of particular importance for successful AD eradication. It is clear that erroneous attribution of positive and negative samples to the tested animals may cause uncontrolled spread of the disease. The CIEP protocol is highly prone to errors of that kind (mixed up or broken capillaries, swapping samples while loading into gel wells, errors in reading the results and data recording). The recently introduced automated ELISA (Dam-Tuxen *et al.*, 2014; Knuuttila *et al.*, 2014) reduces the possibility of sampling errors. However, like CIEP, ELISA requires transmission of samples from one carrier (filter paper combs or cards) to another (microwell plates). Furthermore, the automatic ELISA station must be instructed which comb/card corresponds to which group of animals. Both of these steps are the possible source of errors. In this respect, DIA provides the least risk of sampling errors because the reaction is developed directly on the membrane used for sampling.

The studies for AD using DIA are organized as follows. The lab located in Novosibirsk distributes membranes among the farmers wishing to use this method. Each membrane is designed for application of 200 blood samples. After sampling, the farmers return the membranes to the lab. All the markings on the membranes (date and place of sampling) are made with a usual pen. As 50 membranes (10,000 samples) weight just 35 g, they may be sent in a standard envelope by regular or express mail *(Fig 2C).* The procedure tolerates up to three week interval between blood sampling and processing (Taranin *et al.*, 1996). The membranes are developed in bulk in the lab. The only piece of equipment required for this procedure is a thermostat shaker. A single operator can process up to 200 membranes with 40,000 samples per day. To our knowledge, this is the highest throughput among the known diagnostic methods. Positive results (if any) are communicated to the farmers by e-mail in a form of a datasheet and/or a set of scanned images *(Fig 2D).*

During 1992-2005, DIA was used on a regular basis by just three farms housing from 15,000 to 20,000 breeding females. In two of them, the AD prevalence was reduced from 7-11% seropositive minks to zero during three years (data not shown). The third farm has been failing to eradicate the disease for many years. While no serious AD outbreaks happened in this farm during this period, the prevalence fluctuated in the range of 0.05 to 2.6% (data not shown). It was only after changing the sanitary measures and the testing strategy by a new owner that the farm became AD-free.

Table 2 shows three most recent examples of the DIA performance in AD eradication. Farm 1 (20,000

Fig 2. *Organization of AD diagnostic studies using DIA. A – mink blood sampling using lancets; B – blood application onto membranes; C – mailing the membranes to the lab; D – presentation of DIA results*



females) started to use DIA in the situation of high (>85%) prevalence of AD (not shown). After herd renewal, the number of positive animals decreased to an average of 10%. It took four years to reduce this number to zero. During the past four years the farm remains AD-free.

Two other cases show that DIA can help successfully control AD outbreaks. Farm 2 (35,000 females) and Farm 3 (1,000 females) were AD-free for many years and acquired the infection after purchasing new mink stocks (Table 2). After two exhaustive (on the whole herd) DIA studies and culling out all positive minks, no reactors have been revealed in two subsequent studies. In 2012, DIA was licensed on demand of the Russian Fur Breeder's Association. By October 2015, the method had replaced CIEP in 18 of 36 Russian mink farms (~800,000 or >70% of all tests for AD in Russia in 2015). At the moment 14 farms using DIA on a regular basis are regarded AD-free (no positive reactors). The four

remaining farms had 0.25 to 1.9% seropositive minks, as shown by the last study (Feb 2016).
In summary, our long-term experience supports the applicability of DIA for AD eradication programs. While demonstrating diagnostic efficiency comparable with that of CIEP and ELISA, DIA outperforms the competitors in many ways. Its design provides the fastest blood sampling procedure with minimal risks of sampling errors. Mailing of the samples has never been so easy and convenient. The DIA procedure shows the highest throughput, thereby providing quick release of the test results and attractive prices.

Table 2. *Summary of DIA tests for Aleutian mink disease in three mink farms*

Date of bleeding	Farm 1 (18,000)[1]		Farm2 (35,000)[1]		Farm 3 (1,000)[1]	
	# of tests	# of positive	# of tests	# of positive	# of tests	# of positive
2008 Oct	14,200	1,400	6,400	0	600	0
2009 Feb	18,200	480	8,800	0	100	0
Jun	10,000	17	1800	0	0	0
2010 Feb	15,600	320	1800	0	100	0
2011 Feb	18,000	56	0	0	100	0
Oct	16,000	51	7,600	0	200	0
2012 Feb	20,400	0	6,000	0	200	0
Oct	13,600	2	11,600	0	200	0
2013 Feb	27,400	0	21,400	0	200	0
Oct	17,600	0	8,600	0	200	0
2014 Feb	25,300	0	26,400	0	0	0
Aug	0	0	14,000	33	0	0
Oct	19,100	0	120,000	650	0	0
2015 Feb	23,400	0	45,000	38	200	0
Sep	0	0	0	0	200	2
Oct	21200	0	133,000	0	1700	35
Nov	0	0	0	0	1200	1
2016 Feb	25,400	0	48,800	0	1100	0

[1] # of mating females

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Detection of Raccoon Dog and Fox Amdoparvovirus Infection and Viral Genetic Characteristics

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Extended abstract

The study was to examine raccoon dog *(Nyctereutes procyonoides)* and fox amdoparvovirus (RFAV) infection and characterize RFAV genetic traits. Three methods including PCR, AMDV-specific counter immunoelectrophoresis (CIEP) and serum iodine agglutination test (IAT), were used to test samples of the suspected animals infected by RFAV. Furthermore, the purified RFAV DNA amplicons were sequenced directly by Sanger method using the conserved primers within the genus Amdoparvovirus. Then, RFAV sequences obtained were used to perform RFAV bioinformatics analysis. During 2012 to 2015, RFAV PCR positive animals accounted for 83.8% of 161 suspected sick raccoon dogs from 12 farms in three provinces in China. One hundred and five racoon dogs were both RFAV PCR and AMDV-G CIEP positive. Moreover, there was a high serum IAT positive rate of 77.8% (93/129) in RFAV PCR positive blood. Meanwhile, the results of PCR, CIEP and IAT were negative for the samples of healthy raccoon dogs. Six representative strains of RFAV share a similarity of less than 76.7% and 92.1% in NS1 and VP2 amino acid sequences, respectively, with AMDV. The NS3 sequences of RFAV are shorter than AMDV and GFAV within genus *Amdoparvovirus*. RFAV has the S420 (417DTLS/A421) of VP2 protein and the 226EE/T228 of NS1 protein at the caspase cleavage sites, which differ from the corresponding residues of the AMDV and GFAV. A neighbor-joining tree of the VP2 hypervariable region indicated that RFAV isolates cluster into a unique clade between AMDV and GFAV species. The testing results indicate CIEP test can detect RFAV infection using AMDV-G antigen, and IAT can identify the high γ-globulin content caused by RFAV infection in raccoon dog. The RFAV genetic features reveal that RFAV is a new amdoparvovirus species distinct from AMDV, and naturally infects farmed raccoon dogs and arctic foxes *(Alopex lagopus)*, suggesting that effective measures should be taken to prevent RFAV infection of raccoon dog and fox.

Keywords: raccoon dog and fox amdoparvovirus (RFAV), infection, detection, genetic characteristics

Introduction

Amdoparvoviruses, members of the autonomous parvoviruses, belong to the genus Amdoparvovirus in the subfamily *Parvovirinae* of the family *Parvoviridae* (Cotmore, *et al.* 2014). To date, *three* species have been reported: *Carnivore amdoparvovirus 1*, which comprises only Aleutian mink disease virus (AMDV) and *Carnivore amdoparvovirus 2*, which comprises only gray fox amdoparvovirus (GFAV) (Li, *et al.* 2011). *Carnivore amdoparvovirus 3*, which comprises raccoon dog and fox amdoparvovirus (RFAV) (Shao, *et al.* 2014). RFAV was discovered from farmed raccoon dogs and arctic foxes in China. And our study confirmed that RFAV is most likely the etiologic agent of the sick raccoon dogs causing

Aleutian mink disease-like symptoms. It is believed that additional new species will likely be discovered in their susceptible hosts (Bodewes, *et al.* 2014; Canuti, *et al.* 2015).

Natural Infection of AMDV mainly occurs in the Mustelidae family. AMDV has a single-stranded (ss) DNA genome of 4.7 kb, which comprises two large open reading frames (ORFs), encoding nonstructural (NS1, NS2, and NS3) and structural proteins (VP1 and VP2), respectively (Qiu, *et al.* 2006; Huang, *et al.* 2014). Among various isolates of AMDV, including isolates from farmed minks in China (Sang *et al.* 2012; Wang, *et al.* 2014), the NS1 and VP2 genes present high genetic diversity (Oie et al. 1996; Olofsson, et al. 1999), from which AMDV genotypes have been identified (Christensen *et al.* 2001; Knuuttila, *et al.* 2009). The AMDV-G strain is one of the rare culturable strains (Porter, et al. 1977), which is prepared for antigen to detect antibodies to AMDV. In mink infected with AMDV, Counter Immunoelectrophoresis (CIEP) was used to detect antibodies to AMDV (Cho and Greenfield,1978), and Iodine Agglutination Test (IAT) to determine high γ-globulin contents in serum (Henson, *et al.* 1962). In this study, we extended the survey of RFAV infection in raccoon dog. CIEP, IAT assays for AMDV infection were used to examine the sera from diseased raccoon dog with growth retardation, to verify the feasibility of the two existing tests for RFAV infection. Additonal RFAV isolates were colleted and sequenced, the analyses of increasing RFAV sequences revealed its genetic characteristics within the genus *Amdoparvovirus.*

Materials and methods

Animals and sampling

From July to December of 2012 and 2015, 161 sick raccoon dogs and 10 arctic foxes, which were farmed for fur products on twelve farms in Jilin, Liaoning and Hebei provinces in China, were received for quarantine inspection at the Fur Animal Disease Laboratory, Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences. Clinical signs of the diseased raccoon dogs were similar including anorexia, emaciation, growth retardation, thirst, chronic diarrhea and unkempt fur; necropsy often revealed cyanosed splenomegaly, enlargement of mesenteric lymph nodes, renal cortex congestion, and brittleness. Some sick raccoon dogs presented reversible albino on the nasolabial plate due to having iron-deficiency anemia. Data of the animals used in this study are summarized in Table 1 (Shao, *et al.* 2014; Shao, *et al.* 2015).

Nucleic acid extraction, PCR detection and genome sequencing

Animal tissues (spleen, kidney, and mucosal tissue) were collected and subjected to three cycles of freeze-thaw-homogenization in phosphate buffer solution (PBS), pH 7.4. Homogenized samples were centrifuged at 10, 000 x g for 5 min, and the supernatant was collected for DNA extraction using a MiniBest Viral RNA/DNA Extraction Kit (TaKaRa, Dalian, China).

Nucleic acid samples were used for direct PCR, using AV7 primers (AV7-F, 5'-ccaacaagtaatgacaccttg-gt-3'; AV7-R, 5'- cctgctggtattatccattcagga-3', ~786bp amplicon). We designed AV7 primers, which were based on the conserved region of published AMDV and GFAV genomes, for PCR detection of potential amdoparvoviruses in the samples. AMDV-G virus was used as a positive control. PCR was performed as follows, using *EX-Taq* enzyme (TaKaRa): 94°C for 3 min, 35 cycles of 94°C for 40 s, annealing temperature for 40 s, 72°C for 50 s, and final extension at 72°C for 5 min. Amplified DNA was analyzed on 2% agarose gel electrophoresis, and confirmed by direct sequencing.

The PCR products of the expected size were extracted and subjected to direct sequencing on a Genetic

Analyzer 3100 (Applied Biosystem®, USA) using the amplification primers and the Big Dye Terminator v.3.1 Cycle Sequencing Kit.

Detection of RFAV infection

Serum samples from healthy and sick raccoon dogs and foxes were assessed for AMDV-specific antibodies by counter-immunoelectrophoresis (CIEP) using AMDV-G antigen, which was purchased from the Danish Fur Breeders' Laboratory. The γ-globulin content in serum was determined by iodine agglutination test (IAT).

Sequences analysis of RFAV

RFAV sequences obtained in this study were compared to the *Amdoparvovirus* genus's sequences by NCBI BLASTn searching GenBank. Sequence similarity of the new amdoparvoviruses was analyzed by Lasergene 7.10. Furthermore, RFAV and other published amdoparvovirus sequences were aligned using the MUSCLE program in MEGA6.0 (Tamura, *et al.* 2013). The neighbor-joining tree was reconstructed for nucleotide sequences of VP2 hypervariable regions in the *Amdoparvovirus* genus using the Kimura 2-parameter model. Other tree-building method, maximum likelihood, was used to confirm the topology of the neighbor-joining tree.

Results

Detection of RFAV in sick animals

During the survey of Aleutian mink disease, Raccoon dog cases were observed with Aleutian mink disease-like symptoms. The similarity of symptoms enlightened the findings. We first used AMDV-specific CIEP to test serum samples from the sick raccoon dogs. Next, we designed conserved amdoparvovirus primers AV7 for PCR detection of amdoparvovirus in blood or tissues of the sick animals. Results of sample testing by PCR, CIEP and IAT are summarized in **Table 1**. The overall positive rates of CIEP antibody and IAT were 72.6% (105/135) and 77.8% (93/129), respectively, in RFAV PCR positive raccoon dogs. Four raccoon dogs had IAT-positive sera, but their samples were PCR and CIEP negative.

Genetic characterization and phylogenetic analysis of RFAV

AV7 primers-amplified PCR products from the tissues of the diseased animals were purified and directly sequenced (partly deposited GenBank accession no. KJ396347- KJ396358). Notably, RFAV sequences were uniformly presented in sick animals. By BLASTn search, Six representative strains of nearly complete genome sequences, four of them deposited in GenBank as accession no.: KJ396347-KJ396350, were found to share a similarity of 82% and 90% with AMDV NS1- and VP2-coding sequences, respectively. The four RFAV strains share a similarity of 92.1% and 76.7% similarities with AMDV VP2 and NS1 amino acid sequences, respectively. Among the six RFAV strains, NS1 and VP2 protein sequences showed a divergence of 5.1 % and 5.2%, respectively. The four RFAV strains have VP1 of 676 - 688 amino acids (aa), VP2 of 633 - 645 aa, NS1 of 641 aa, NS2 of 114 aa, and NS3 of 66 aa. Of note, sequencing analysis of the VP2 hypervariable region of RFAV DNA amplified from samples of sick raccoon dogs and foxes from Farm A revealed an identity of over 99%, suggesting that only one RFAV strain circulates on this farm.

The neighbor-joining tree of amdoparvoviruses shows that RFAV strains cluster into a unique clade between AMDV and GFAV species (Figure 1). The NS3 sequence of RFAV was shorter than that of two known species in genus *Amdoparvovirus* (Huang, *et al.* 2014). The two key pathogenic residues on VP2 of AMDV-Utah, V352 and D534(Fox, *et al.* 1999; Stevenson, *et al.* 2001), are reserved on RFAV VP2.

However, the S420 (417DTLS/A421) of RFAV VP2 differs from the D420 of the AMDV and GFAV VP2 (417DLLD/G421 and 417DTID/S421, respectively), which is a caspase cleavage site of AMDV VP2 (Cheng, *et al.* 2009). The level of AMDV VP2 protein is limited by the presence of the D420, which is responsible for the caspase-mediated cleavage (Cheng, *et al.* 2009). The S420 of RFAV VP2 may decrease the cleavage of VP2 and in turn, increase the stability of the capsid. Additionally, the 226EE/T228 of RFAV NS1 differs from the corresponding residues of AMDV or GFAV NS1, which is a caspase cleavage site of NS1 (Best, *et al.* 2003). The cleavage of NS1 at this site is a key to AMDV DNA replication (Best, *et al.* 2003). The key residues of RFAV were shown in *Figure 2*.

Discussion

CIEP and IAT tests for RFAV infection

Spleens and kidneys of sick raccoon dogs contained AMDV-G-related antigen, which were RFAV PCR positive. The evidences suggest that RFAV shares similar antigenic compositions with AMDV-G (Shao, *et al.* 2014). Positive rates of RFAV DNA and antibodies in sick raccoon-dog samples were 83.8% and 66.5% respectively, and the positive rate of CIEP antibody was72.6% (105/135) in RFAV PCR positive raccoon dogs. The antibodies raised against RFAV and AMDV-G antigen showed positive CIEP test. That is, CIEP using AMDV-G antigen can detect RFAV infection. Additionally, Although IAT test is unspecific to RFAV infection. IAT still can be used to determine high γ-globulin in sera due to chronic RFAV infection.

Molecular evidence for a new amdopavovirus species

We report herein a group of amdoparvoviruses from farmed raccoon dogs and foxes in eight counties in Jilin, Liaoning and Hebei provinces, China, belonging to the *Amdoparvovirus* genus, and forming a species phylogenetically positioned between *Carnivore amdoparvovirus 1* (AMDV) and *Carnivore amdoparvovirus 2* (GFAV). Although sharing similar antigenic compositions with AMDV-G, RFAV has a short NS3 protein, a serine at aa 420 of the VP2 and glutamic acid at aa 227 of the NS1 protein, which are genetically distinct from AMDV. A less than 85% aa identity in the NS1 protein has been proposed to demarcate a different species within the family *Parvoviridae* (Cotmore, *et al.* 2014). The six representative RFAV strains share a similarity of only 76.7% in the NS1 protein sequence with AMDV or GFAV, and therefore, RFAV must belong to a new species, an addition to the *Amdoparvovirus* genus. Based on the similarities of the NS1 and VP2 sequences, RFAV has a closer relationship with AMDV than with GFAV.

RFAV naturally infects raccoon dogs and foxes

RFAV DNA or specific antibodies persisted in the sick raccoon-dogs and arctic foxes. High viral DNA levels were found in both blood and spleen samples from the sick raccoon-dogs, suggesting a high level of RFAV replication in sick raccoon-dogs. These data collectively indicate that raccoon-dogs are naturally susceptible to RFAV infection (Shao, *et al.* 2014).

RFAV sequences from the sick raccoon dogs or arctic foxes of different farms showed a high homology. Only RFAV was detected from famed raccoon dogs and arctic foxes, but RFAV was not detected from the mink in the same farm, indicating RFAV naturally infects raccoon dogs and arctic foxes. However, arctic foxe's cases were a few with RFAV.

Raccoon-dogs and foxes are widely distributed and farmed in Eurasia. RFAV was detected in the blood, urine and intestinal mucosa of sick animals, suggesting that RFAV can be transmitted through body fluids and excreta. We found that, on Farm A, foxes were infected with RFAV followed by infec-

tion of raccoon-dogs at the same period. RFAV sequences, obtained from the samples from both sick raccoon-dogs and foxes, revealed a highly homologous VP2 hypervariable sequence, indicating that RFAV was transmitted among foxes and raccoon-dogs, especially under the conditions of high-density farming and wildlife predation. Whether RFAV infects other canids and beyond requires further investigation.

In the previous study (Shao, *et al.* 2014), the sick raccoon dogs manifest similar disease symptoms as AMDV-infected minks. Notably, the positive or strong serum IAT results were associated with a chronic infection like Aleutian mink disease (Gorham, *et al.* 1965). Sick raccoon dogs manifested chronic viral infection long-term weight loss, growth retardation, cyanosed splenomegaly and nephritis. RFAV is the etiological agent responsible for the disease manifestations of the sick raccoon dogs.

Notably, after the sick raccoon dogs were removed from farms, raccoon dogs with similar symptoms were found on the same farm the next year, and spleens from four asymptomatic raccoon dogs showed obvious spleen lesions with blood samples positive for PCR and CIEP assays. Together with the observation that a raccoon-dog showed symptoms until 17-months-old, these findings suggest that latent infection of RFAV exists among raccoon dogs and constitutes a hidden source of virus among these animals. Thus, effective measures should be taken to prevent RFAV infection on raccoon dog farms.

Acknowledgements

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Table 1. *Detection of RFAV infection in diseased and healthy raccoon dogs during the years of 2012 to 2015 using PCR, CIEP and IAT tests, China*

Year	Status	Age /month	Farm[a]	Number	Rate			
					PCR+[b]	CIEP+	IAT+	CIEP+PCR+
2012-2015	Diseased		12(Total)	161	135/161	105/158	97/152	105/158
2012-2013			5(Total)	29	26/29	26/26	20/20	23/26
2014			5(Total)	36	31/36	31/36	27/36	31/36
		3-7	LN-PJ1	10	9/10	9/10	9/10	9/10
		3-7,17	LN-HS	9, 2	9/11	9/11	11/11	9/11
		3-4	JL-QA	2	1/2	1/2	1/2	1/2
		3-4,15	JL-NA	8, 1	8/9	8/9	4/9	8/9
		6	JL-1	4	4/4	4/4	2/4	4/4
2015			4(Total)	86	78/86	48/86	50/86	48/86
		3-4	LN-HS	3	3/3	1/3	3/3	1/3
		3-4	HB-CLQ	32	26/32	15/32	11/32	15/32
		3-4	HB-CLZ	19	19/19	11/19	10/19	11/19
		7	JL-ZJ	7	7/7	5/7	6/7	5/7
		7	HB-CLZ	25	23/25	16/25	20/25	16/25
2013-2014	Healthy	3-7	3(Total)	21	0/21	2/21	0/21	0/21

[a] Farm: JL-QA, Qian'an County; JL-NA, Nong'an County; JL-1, Jilin County; LN-HS, Heishan County; LN- PJ, PanjinCounty. HB-CLQ, HB-CLZ, Changli County; Qian'an, Nong'an and Jilin Counties are located in Jilin province. Heishan and Panjin Counties are located in Liaoning province. Changli County is lo-

cated in Hebei province. The numbers indicate the sum of the farms and there were interval samplings on some farms.

[b] Blood and spleen samples were tested mainly by PCR except for the blood samples from the 3-month-old healthy raccoon dogs.

Figure 1. *Phylogenetic tree of amdoparvoviruses based on the 514nts sequence of VP2 hypervariable regions (2715-3228 nt of JN04043 AMDV-G). The neighbor-joining method was used with p-distance or Kimura 2-parameter and 1,000 bootstrap replicates, and otherwise default parameters in MEGA5. Scale bars represent estimated phylogenetic divergence. Sequences obtained from the study are shown in bold. AMDV, RFAV and GFAV indicate amdoparvoviruses from mink, raccoon dog or arctic fox and gray fox, respectively. SAV, shunk amdoparvoviruses.*

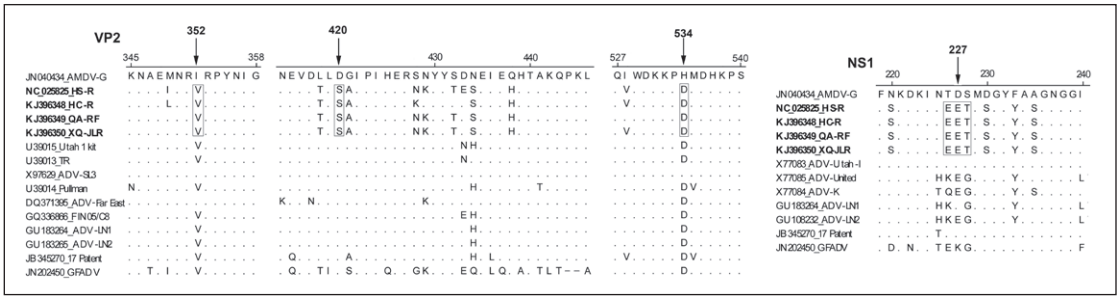
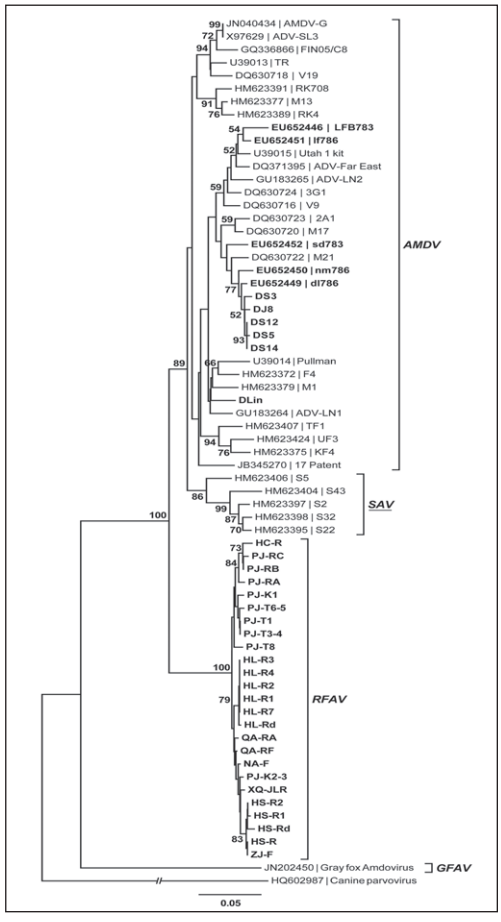


Figure 2. *Sequence analysis for some key residues on VP2 and NS1 proteins of typical RFAV isolates in comparisons with the other members within the genus Amdoparvovirus. The residues of RFAV were boxed at key sites labelled with arrows and number.*



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Canadian experience with MALDI-TOF profiling of plasma proteins in selection for breeding for Aleutian disease resistance, and comparison to iodine precipitation and AMDV-Ab ELISA

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Abstract

Recently, N. American farmers have turned to breeding for disease resistance, with 3 different testing methods in use. MALDI-TOF determination of albumin: total IgG ratio (A/IgG), iodine precipitation test (IPT), and AMDV-Ab ELISA for quantification of the specific anti-viral antibody. It is important that the test/s for selection of the 'disease resistant' breeding animals reflects both the specific antiviral and the autoimmune components of the AD induced hypergammaglobulinemia (hyper-Ig). MALDI-TOF A/IgG covers both the antiviral and the autoimmune antibody, but compared to IPT, it provides an individualized disease marker within a range of total IgG levels. AMDV-Ab ELISA does not reflect the autoimmune portion of hyper-Ig. Compared to MALDI-TOF A/IgG, the sensitivities - specificities of detection of 'disease phenotype' by AMDV-Ab ELISA, IPT, and UV spectrometry (280 nm) were 77,5% - 72% , 91.4 % - 90.6%, and 95.2% 91%, respectively (n= 1850). Pearson correlation of AMDV-Ab ELISA, IPT and 280 nm-UV spectrometry with MALDI-TOF was r = -0.385, p = <0.05; r= -0.821, p= <0.05, and r= -0.943, p= <0.05, respectively. MALDI-TOF selection of breeding animals on a commercial farm led to significant yearly improvement of production in 3 subsequent years, with full restoration of the pre-AMDV outbreak production level.

Keywords: AD control, albumin: total-IgG ratio, hypergammaglobulinemia

Introduction

Efforts to eradicate the virus from affected farms by 'individual detection/ removal' of infected animals with the use of specific antiviral antibody (CIEP or ELISA) or by virus detection (PCR), as well as through depopulation/ repopulation, have been made since 1978 (Cho and Greenfield, 1978). However, sustained eradication of the virus, at least under the North American husbandry remained elusive (Cepica and Iwamoto, 2012). That report confirmed the farmers' experience, that a) the individual 'detection/ removal' whether by CIEP or PCR, was unable to remove the virus from the infected farms in a sustainable manner, b) that depopulation/ repopulation was commonly followed by AD outbreaks soon after restocking. We argued that the widely accepted premise of the feasibility of long term, large scale sustainable AD eradication was false. This was subsequently fully accepted in N. America by both researches (Farid 2014) and by the mink industry (Hildebrant 2015). Similar trend is seen in most European countries, with notable exception of Denmark (Espregueira *et al*., 2012).

A new sensitive and quantitative test for determination of the disease and health phenotypes of AMDV infected animals has been recently introduced and validated (Cepica et al. 2012). Current co-existence of the two AD control methods among mink producing countries, i.e., 'breeding for AD resistance', and 'virus eradication', prevents the desirable exchange of breeding animals among those two groups. In N. America, where most farms have now turned to breeding for AD resistance, importation of animals from the AD-virus free farms and/or countries like Denmark, should not continue, as the

imported animals would not have been subjected to the same selection for disease resistance, and they would inevitably suffer high morbidity and mortality. Moreover, if the imported males were bred to females that had already been selected for AD resistance, which is currently the common way of introducing desirable genetic traits, the genetics of AD-resistance in the progeny would be lost. Conversely, importation of animals farmed in the presence of the virus, after the selection for disease resistance, into a country or region previously free of the virus would be unacceptable, because of the present virus. In order for the two dichotomous approaches to AD control be able to harmonize in future, there is a pressing need for a genomic test for AD resistance to be applied where eradication is practiced. Development of such test would once again be predicated on the most specific and sensitive phenotypic disease vs. health' marker among the AMDV infected animals. In addition to facilitating exchange of breeding animals between N. America and farms, regions/countries, still adhering to virus eradication, it is reasonable to expect that the selection for 'AD resistance' will improve the resistance to other infectious agents, as resistance to viral diseases is commonly a complex polygenic trait, involving genes related to components of antigen-nonspecific innate, as well as antigen-specific adaptive immunity (Mellencamp *et al.*, 2008).

As already stated, currently, in addition to MALDI-TOF A/IgG ratio, iodine precipitation test (IPT), and AMDV-Ab ELISA are also being offered for the purpose of AD resistance selection. All of these tests claim to be based on some form of assessment of hypergammaglobulinemia, the main feature of AD (Obel, 1959). However, there is no published research addressing comparison of these tests, whether based on the knowledge of the tests principles, or on the effectiveness of detection of hyper-Ig, the main pathophysiological feature of the disease.

In this report, the results of a 3 year trial with MALDI-TOF testing for AD resistance on a commercial farm are described. The issues of validity of the use of AMDV-Ab ELISA and IPT in 'AD resistance testing' are explored through the analyses of working principles of these tests, through the correlation with MALDI-TOF A/IgG ratio, and through determination of the diagnostic sensitivities and specificities of these tests in comparison to MALDI-TOF A/IgG. UV spectrometric assessment of protein quantification at 280 and 230 nm was included to verify the performance of IPT, as both tests are to measure total protein. The agreements and discrepancies among the tests are discussed, based on the review of simultaneous testing of 1,850 animals.

Materials and Methods

MALDI-TOF for Albumin/IgG.

Albumin/IgG profiling was performed as initially described (Cepica *et al.*, 2012), with the following adjustments. 0.5 uL of the 1st matrix (sinapinic acid in 85% ethanol at the concentration of 25 mg/mL, Sigma Aldrich, Germany) was applied onto the target polished stainless steel target (MSP 96, Bruker Daltonics, Germany. One microliter of plasma was diluted 1:200 with proteomic grade water (Milli-Q, Millipore Corp.), and then 1 µL of the mixture of the diluted plasma with equal volume of the 2nd matrix (sinapinic acid in Milli-Q water: acetonitril: trifluroacetic acid) was applied over the first matrix. Matrix assisted laser desorption ionization time of flight instrument (Microflex, Bruker Daltonics), in the automatic spot selection mode was used, applying Flexcontrol 3.4 software. Setting of accumulation of 360 satisfactory shots, or early termination was applied if the intensity of the highest peak reached 5000.

Measuring raster was set at 5. Data analysis was performed by Flexcontrol 3.3 (Bruker Daltonics, Ger-

many). Albumin: γ-globulin peak heights were recorded, and the ratios were calculated. As previously reported, the ratios >8 were considered associated with health, and the ratios <8 were considered to indicate hyper-IgG, and by extension Aleutian disease.

Iodine Precipitation

The working iodine solution was prepared by dissolving 2g iodine crystals and 4g potassium iodine crystals (PCCA Canada, USP grade) into 20 mL of distilled water. One drop of the tested blood plasma was placed onto a back lit glass panel, followed by a drop of the iodine solution onto the plasma drop. After mixing with a toothpick, the grading was done by visual assessment as follows: 0 = clear sample; C = cloudy but no precipitation present; 1= faint precipitation present; 2= medium to high amount of faint precipitation was present; 3= high amount of large dark precipitation was present. Animals with the test results 1-3 were recommended for culling, and samples graded as 0 and C were recommended as suitable for breeding. For the purpose of examining the Pearson's correlation coefficient between MALDI-TOF A/IgG ratios and IPT (n=80), the results were graded as 1-5, with 1 being negative (clear), and 2-5 having progressively increasing amounts of precipitation. After visual assessment, the results were verified microscopically.

Commercial ELISA for AMDV-antibody correlation with MALDI-TOF A/IgG

In this experiment, 200 mahogany mink were simultaneously ELISA tested by the laboratory of the Middleton Veterinary Services, 1163 Brooklyn Rd, Middleton, NS B0S 1P0, and by MALDI-TOF. These ELISA results were provided as actual absorbency values with the recommendation of the cutoff absorbency value of 1.02. The animals and blood collection were as described below under 'animals', except the blood was collected on collection discs.

Correlation of AMDV VP2-Ab ELISA absorbencies with MALDI-TOF A/IgG, and IPT

The VP2 was produced of local AMDV containing samples (n=80), the ELISA absorbencies were acquired essentially as described by Knuuttilla *et al*., 2009. All related work was performed in the author's laboratory. For this purpose, the samples were tested in duplicate simultaneously on two plates, repeated, and average absorbance values were used. Plasma samples over the range of MALDI-TOF A/IgG ratios (<2-13) were used, and VP2-Ab-ELISA absorbances were acquired and plotted. Pearson correlation of the VP2 synthetic peptide based ELISA absorbance and of A/IgG was calculated.

AMDV-Ab ELISA for diagnostic sensitivity and specificity

The ELISA for this experiment was done by Hoofd laboratorium, Molenweg 7, 6612 AE, Nederasselt; Postbus 488, 6600 AL, Wijchen, Netherlandse Federatie Van Edelpelsdierenhouders (www.NFE.NL). Absolute absorbency values, corresponding to the quantity of the specific antiviral antibody were not provided, rather the animals were classified according to the absorbency values into one of the 9 groups (0-8), indicating amount of specific antibody from the lowest to the highest. The laboratory's information, based on unpublished information, recommends retaining groups 1-4, and culling groups 5-8 based on the inferior reproductive performance of animals of the >5 scores.

Animals

The work was performed under the license of the Regional Diagnostic Laboratory (Atlantic Veterinary College, University of Prince Edward Island, Canada), as such was covered by the permit issued to the diagnostic laboratory of the University of Prince Edward Island's from the institution's Animal Care Committee. One thousand eight hundred and fifty, 8 months old female mink of mixed color (mahogany, demi-brown, black), housed on 3 commercial farms in Atlantic Canada were bled by nail clipping, and collected into the heparinized hematocrit tubes, followed by centrifugation of the on farms. The

samples were kept refrigerated and shipped into the laboratory within 3 days. On reception, plasma was separated from the packed cells and if not used within 2 days, it was frozen at -20C.

UV spectrometric assessment of total protein

SpectraMax Plus384 Microplate Reader (Molecular Devices, Sunnyvale, CA 94089 USA) was used to acquire absorbencies of 1:100 dilutions of plasma in proteomic grade water. The pooled samples, negative by the IPT and MALDI-TOF, of total protein concentration 6.6g/ dL, were placed on each test plate to control for plate to plate variation. The absorbance values of 10.8 at 280 nm (S.D. 1.08; n=5), and of 1.7 at 230 nm (S.D. 0.22; n=5) of the control were obtained. The tested samples were assessed at both 280 nm and 230 nm, and the cutoff point of hyperproteinemia was the mean control absorbance +1 standard deviation (S.D.).

Breeding for disease resistance.

This work was done on a commercial farm, with the owner's consent. The program was sanctioned and sponsored by the Prince Edward Island Fur Breeders' Association. A thousand of one year old AMDV infected females and 200 infected males of mixed colors (mostly mahogany and black), were initially positive for AMDV-Ab by counter electrophoresis (CIEP, /Weymouth Aleutian Disease Laboratory, Weymouth, Nova Scotia, Canada/). The animals were selected for the 'health phenotype' by MALDI-TOF (A/IgG of >8) as previously established (Cepica et al., 2012), and they were mated in 2013. The reproductive performance and productivity were monitored, and the process was repeated in 2014, and 2015. Each year's reproduction and productivity was compared to the values observed in the year before the trial. The number of born kittens/ bred female, and the number of animals reaching reproductive age were recorded. Calculations of average litter size were made from individual reproductive female records.

Results

A/IgG profiles of hyper-Ig animals (disease phenotype) had in addition to the A/IgG ratios <8 elevated IgG peak, and an additional 76 kDa peak, absent from the animals with A/IgG ratios >8 (health phenotype). The 76 kDa peak represents the laser degradation product of IgG (Mohamed 2014, unpublished). Thus, the 76 kDa peak provided an additional and unequivocal assurance that the lowered A/IgG was not due to the reasons other than overproduction of IgG, e.g., decreased albumin production (liver disease), or increased albumin degradation *(Figure 1)*.

The value of A/IgG for sensitive discrimination of infected 'disease-susceptible' and 'disease-resistant' mink, for resistance breeding has been demonstrated by the field trial, in which the tested mink were bred, followed by monitoring of the reproductive performance (average number of born min per bred female), and productivity (number of kittens reaching sexual maturity). Both criteria indicated that application of the MALDI-TOF testing dramatically improved the performance already in the first year of application (from 1.5 kittens/ female to 3.3/ female), second year (4.2 kittens/ female), and the overall performance in the third year returned to pre-AMDV outbreak period (5.2 kittens/ female). The mortality rate among the progeny was 5%, 4.5% and 2.8% in the 1st, 2nd, and 3rd year of the trial, respectively.

Compared to MALDI-TOF A/IgG, the sensitivities - specificities of detection of 'the disease phenotype' (n= 1850) by AMDV-Ab ELISA, IPT, and UV spectrometry (280 nm) were 77,5% - 72% , 91.4 % - 90.6%, and 95.2% (280 nm) 91% (230 nm), respectively. In addition, MALDI-TOF A/IgG ratios, IPT, Ab-ELISA, and UV spectrometric total protein were tabulated, and analyzed for trends. Example of the work tables is presented in Table 1. The IPT disagreed with MALDI-TOF in 8.6% of samples, with 4.3% showing negative results where MALDI-TOF was positive, and 4.3% was positive where

MALDI-TOF was negative. Analysis of Pearson's correlation of MALDI-TOF and IPT yielded rather strong negative correlation, i.e., the low A/IgG ratio corresponded to high IPT score ($r = -0.821$ $p = <0.05$). The correlation of the 280nm UV spectrometric total protein estimation with MALDI-TOF was even higher, at $r = 0.943$ $p = <0.05$.

The discrepancies between AMDV-Ab ELISA, currently the main available test used, and MALDI-TOF A/IgG, on the other hand, were significant (Table 1). Overall, compared to MALDI-TOF A/IgG, the sensitivities - specificities of detection of 'disease phenotype' by AMDV-Ab ELISA, IPT, and UV spectrometry (280 nm) were 77,5% - 72% , 91.4 % - 90.6%, and 95.2% 91%, respectively (n= 1850). Overall Pearson correlation of AMDV-Ab ELISA, IPT and 280 nm-UV spectrometry with MALDI-TOF was $r = -0.385$, $p = <0.05$; $r = -0.821$, $p = <0.05$, and $r = -0.943$, $p = <0.05$, respectively.

Discussion

By the way of general introduction to the issue of virus eradication, it needs to be emphasized that the probability of eradication of any viral pathogen from a target population depends on several key prerequisites. This is contrary to the view, that any virus can be eradicated, provided that sufficient resources and efforts are applied. In reality, not all virus infection can be eradicated from target populations in spite of efforts, as demonstrated by the existence of many serious human viral pathogens of global importance in spite of considerable efforts to eradicate them (poliomyelitis, yellow fever, measles, mumps, rubella).

These prerequisites are: absence of the reservoir species (true for smallpox, and not true for AMDV), or if such reservoir exists, e.g., for rinderpest virus, the reservoirs are easily identifiable. AMDV infects feral mink, other mustelids besides mink (weasels, badgers, ferrets, otters), as well as skunks, raccoons, bobcats, cats, dogs, mice, and humans. In addition, the closely related amdoviruses of yet not fully known pathogenesis for farmed mink: Carnivore amdoparvovirus 2 (gray fox amdovirus, GFAV), the proposed Carnivore amdoparvovirus 3 (raccoon dog and fox amdoparvovirus, RFAV) and the red fox fecal amdovirus (RFFAV), infect gray fox, red fox, Arctic fox, and raccoon dog. All of these animals have the potential of breaching biosecurity of farms either through entry into the farms, or through passive entry (fomites, vehicles, people). In addition, mosquitoes have been found to carry the virus for over a month (Canuti *et al.*, 2015).

Secondly, eradication is aided if an infection is not transmitted vertically, and if a recovery from an infection is accompanied by sterile, or at least a temporarily sterile immunity (e.g, herpesviruses during latency), rather than by chronic, persistent, or periodic shedding, as is the case in AMDV infection.

Finally, the chances for eradication are diminished when a virus is highly resistant to environmental inactivation, thus facilitating transmission. Small un-enveloped viruses like AMDV are among the most resistant, and the causative viruses of the two most spectacular successes of global eradication, in veterinary medicine the rinderpest virus, and in human medicine the smallpox virus, are within *paramyxoviridae* and *poxviridae*, respectively, which are among the largest enveloped virus families. In addition to already mentioned smallpox and rinderpest, a variety of herpesviruses, e.g., pseudorabies virus in swine, infectious bovine rhinotracheitis of cattle, were eradicated on a country wide or regional scale.

For all the above reasons, the probability of success of lasting AMDV eradication must be accepted as being extremely low. This is confirmed by the experience of almost 40 years of unsustainable AMDV eradication efforts (Cepica and Iwamoto, 2012). The cost-benefit ratio associated with the continued

short lived eradication attempts involving periodic testing, depopulation, disinfection, facility down time, and quarantine, must be now weighed against the cost-benefit ratio of the alternative, i.e., 'the breeding for disease resistance'. The feasibility of the concept of breeding for AD resistance is strongly supported by two unique natural experiments: the deliberate release of myxoma virus (MYXV) and rabbit hemorrhagic disease virus (RHDV) as biological control (biocontrol) agents against the European rabbit in Australia. The subsequent evolution of the outcomes of the two virus infection, initially of very high mortality, led to establishment of a balanced co-existence of a virus with decreasing pathogenicity with a host of increasing disease resistance (Di Giallonardo and Holmes, 2015). It is obvious that a faster co-evolution in this direction should be possible, when aided by easy virus transmission in farm environment, and by deliberate host selection with the aid of a good 'resistance marker'. Breeding for resistance has a possible promise of discontinuation of the selection process, i.e., testing, if and when the population genetics regarding AD reaches pre-AD status of wild mink. This possibility is also supported by the results of our trial, where the reproduction and productivity was improving in three successive years of the selection, and reached pre-AMDV outbreak levels in the third year. However, longer studies would be needed in order to confirm whether discontinuation of the selection process might be even temporarily possible.

The important question then inevitably emerges, which currently available test is the best indicator of 'health/ disease resistance phenotype' for the use in the 'breeding for disease resistance' program? In this respect, we see the value of A/IgG for resistance breeding for two main reasons. Firstly, the A/IgG ratio, is demonstrably a measure of clinical pathology (=disease) (Cepica et al., 2012), unlike the specific AMDV antibody (ELISA) which is only assumed to be an indicator of disease, without the necessary experimental proof. Furthermore, in this report we demonstrate directly the value of A/IgG in the breeding for AD resistance. The reproductive performance (average number of born min per bred female), and productivity (number of kittens reaching sexual maturity) dramatically improved already in the first year of the trial, and in the third year, both the reproduction and the mortality returned to the pre-outbreak levels. The use of autoimmune antibody (DNA-Ab) for assessment of AD was previously suggested by Babkina, *et al.*, 1996.

We have performed to date more than 25,000 MALDI-TOF A/IgG tests while being in continued communication with the farmers. We can conclude from the combined experience, and regardless of other possible causes of death, that mink with the A/IgG ratios <8, and particularly A/IgG <5, have high mortality within days and weeks after the testing. The lowered A/ globulin (G) ratio in AD animals compared to healthy animals has been noticed as early as 1964 (Gershbein and Spencer, 1964). However, it was not until 2012, that a quantitative, sensitive, practical test of high throughput, capable of individualized diagnosis of hyper-Ig, applicable to large scale testing became available when we reported the development of MALDI-TOF for A/IgG. While hyper-IgG is not exclusive to AD, we have previously reported that the MALDI-TOF A/IgG values <8 are extremely rare on farms where AMDV is absent. Certainly, it is conceivable that severe chronic bacterial infections, e.g., pododermatitis, might possibly induce hyper-Ig, but none of the common acute viral infections (distemper, viral enteritis, other viral enteritides, influenza, or vaccination), are known to do so. A comprehensive literature search reveals, at least to our knowledge, no report of hyper-Ig caused by the causes other than ADMV infection. When random samples of mink from farms free of the AMDV were tested, A/IgG approaching 8 was initially revealed in one out of 22 (4.5%) animals (Cepica et al., 2012). Subsequent analysis of the A/IgG profiles of 300 AMDV uninfected animals showed A/IgG ratios lower than 8 (+- 1S.D.) in less than 1% samples, with no ratios <5 (Cepica, 2014, unpublished). However, even if A/ IgG <8 of non-AMDV origin were to occur, and if it were a source of false positivity of the MALDI-TOF test, this would be acceptable for the purpose of selection of breeding animals, as these animals would be too sick to successfully breed.

The A/IgG ratio indicates quantitatively, sensitively, and directly disturbance of homeostasis, as indicated by blood protein pathology (Cepica et al., 2012). IPT and AMDV-Ab ELISA, on the other hand, are widely offered for AD resistance breeding, without any validation. The positive IPT is known to indicate hyperproteinemia, in the context of AD due to hyper-Ig. It was first described by Henson et al., 1962 as a fortuitous finding without understanding of the principle involved. Before the causative involvement of AMDV, the AMDV properties, the AD pathogenesis, and the shedding patterns became known, it was simply assumed that the removal of the IPT positive animals somehow removes the disease from the premises. The the diseased animals were being identified for pelting rather than breeding, which did not eliminate the virus, but it improved the fitness of the subsequent generation. Where IPT had been practiced for several years, the reproduction and production were improved (Peter Noah, personal communication), but when subjected to MALDI-TOF A/IgG testing, only about 60% passed, compared to MALDI-TOF second year tested animals, among which over 80% passed (Peter Peters, personal communication). The positive IPT actually represents a low efficiency phenotypic marker of AD resistance among the infected animals. Because of the positive experience with IPT test/ removal AD control method, some farmers chose IPT even after the counter-immunolectrophoresis /CIEP/ and the AMDV-Ab ELISA methods of specific AMDV-Ab detection became available (Peter Noah, personal communication).

Implementation of CIEP in the late 1970 and early 1980s, followed by ADMV-Ab ELISA, as presumably better tests, had serious inherent flaw. These tests were neither accomplishing virus eradication (Cepica and Iwamoto, 2012), nor were they identifying the disease, as IPT was. Furthermore, we demonstrated that the limitations of 'detection/removal' eradication based on the serological methods are also valid for the use of PCR in eradication, i.e., continued presence of resistant virus in the environment due to only intermittent shedding in some animals, thus making them undetectable for removal by PCR virus detection. To summarize, while the uses of CIEP, ELISA, and PCR certainly remain valid for demonstration of the AMDV entry on previously AMDV free farms, they are not suitable for AMDV eradication through 'the individual detection/ removal'.

With respect to the issue of relevance of the specific antiviral antibody (AMDV-Ab ELISA) for monitoring of the severity of Aleutian 'disease', we previously reported that in AMDV-specific antibody assessed by AMDV-Ab ELISA correlates with MALDI-TOF A/ IgG only weakly (Cepica, 2014). In this report, we confirm this from the experiment with AMDV-VP2-Ab ELISA, that the correlation was too weak ($p= -0.385$, $p <0.05$) to consider ELISA an alternative test to MALDI-TOF. This is not surprising, as the same was found for feline infectious peritonitis (FIP), when after decades of the use of 'high specific FeCV-Ab' as the disease diagnostic tool, it was determined that high levels FeCV-antibody are not diagnostic for FIP (Simons et al., 2005; Pedersen 2014). FIP is among the best studied models of a persistent viral infections accompanied by hypergammaglobulinemia, and high antiviral antibody levels were claimed to be diagnostic for the FIP disease for decades prior to 2004. It is now widely accepted that FIP can develop in association with relatively high or low antiviral antibody, or even no detectable antiviral antibody. This example of FIP false diagnosis through specific FeCV-Ab quantification is not in any way weakened by the fact that feline enteric coronavirus induced antibody are indistinguishable from FIPV-Ab. It was believed that the FIPV persistency automatically must lead to abnormally high coronavirus-specific antibody, feature not present in non-FIPV FeCV infections. In this sense it appears that AD story is following the same script.

Lack of correlation, or very low correlation of hypergammaglobulinemia, that is a consequence of a persistent viral infection, with specific antiviral antibody may seem counterintuitive. It might be expected that persistent antigenic stimulation of B-cells specific to AMDV should logically lead to

high specific anti-viral antibody production. However, this simplistic view does not take into consideration complex regulation system of antibody production. As a result, it is wrong to think of hypergammaglobulinemia accompanying the AD as having to be exclusively directed to the virus. Hahn and Hahn, reported the significant contribution to AD hypergammaglobulinemia by autoimmune antibody already in 1983. To pursue this line of reasoning against perceiving AD hyper-Ig to be exclusively, or even primarily, of antiviral specificity, one can't overlook the fact that there is, at least to our knowledge, no credible scientific report of any animal or human viral disease-induced hyper-Ig, where a certain level of the specific antiviral antibody would considered diagnostic of the disease. If, such report indeed does not exist, then it might mean that 'disease-diagnostic' AMDV-Ab ELISA absorbance values do not exist, and that such values are used, they are determined entirely arbitrarily. The use of such arbitrary cutoff points to differentiate infected animals that remain healthy from those that develop the disease in 'disease resistance' breeding programs is based on the presumption that there is a relationship between the levels of AMDV-Ab and the severity of AD. This presumption carries a very convenient extension, i.e., that one can make the criteria to satisfy the need for any proportion of disease resistant animals, simply by retaining the animals of the lower ELISA absorbency level available. However, if, as we report, the specific AMDV-Ab is poorly correlated with the disease, and it is the total IgG, levels of which are primarily driven by the AD accompanying autoimmune antibody, then it should be a parameter estimating total Ig that should be used to monitor severity of the disease. This notion is supported by previous reports of Hahn and Hahn, 1983; Bloom *et al*., 1994; Miyazawa *et al,* 1994; Cepica, 2014. Based on our data and on the above reports, it is reasonable to conclude that the evidence indicates that total Ig is reflecting severity of AD, and AMDV-Ab is not. While the mechanism of induction of autoimmune antibody in association with AMDV remains to be elucidated, polyclonal mitogenicity (James, 2001; Slight-Webb *et al*., 2015) and molecular mimicry are the main candidates (Trela *et al*., 2016).

With this in mind, a sensitive method of quantification of total immunoglobulins, particularly of the IgG, seems to hold promise for phenotypic differentiation of disease susceptibility vs. resistance in AMDV infected animals. MALDI-TOF albumin/ IgG ratio fulfills extremely well this role as an individualized in vivo phenotypic marker of disease susceptibility (A/IgG <8) and disease resistance (A/IgG >8) among the AMDV infected animals. The albumin: IgG ratio, is a more accurate indicator of the disease than the simple IgG quantification, as the ratio unlike IgG quantity, provides the 'built-in' sensitive individualized marker of the disease vs. health. This is true for the following reason: the diagnostic efficacy and significance of hyper-Ig levels is magnified by the liver's feedback-downregulation of albumin synthesis in response to maintain blood viscosity, and this is under the genetic control of an individual's homeostatic regulation. Thus, the IgG: IgG ratio enables detection of the disease within a wide range of normal IgG values. Since reference 'normal' IgG range can vary widely, as for example in humans older than 19 years, IgG values between 700-1600 mg/dL are considered compatible with health, and when children are included, the lower limit can be as low as 231mg/dL (Wade, 2016), it is obvious that simple direct IgG quantification, e.g., by electrophoresis, or indirectly through total protein estimation (IPT, UV spectrometry) misses more subtle IgG elevation.

As for the IPT, it correlated much better in our experiments with A/IgG than AMDV-Ab ELISA ($r= -0.821$ $p= <0.05$). However, the subjectivity of the test reading due to varied lighting and the readers' visual sensitivity and experience, and variation of the quality of the iodine solution due to its age, together, make this test of lower reproducibility. As for the UV spectroscopy, while not equaling the MALDI-TOF, it showed considerable promise for its rapidity, minimal labor effort for sample preparation, higher correlation with A/IgG than IPT ($r= -0.943$ $p= <0.05$), diagnostic sensitivity of 91.4% and specificity of 90.6%. This test has a potential as an in-house farm, at lease to replace the currently

used IPT, where MALDI-TOF A/IgG ratio test is not available.

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Figure 1A.
MALDI-TOF profile of 'disease phenotype' (A/IgG = 1.11)

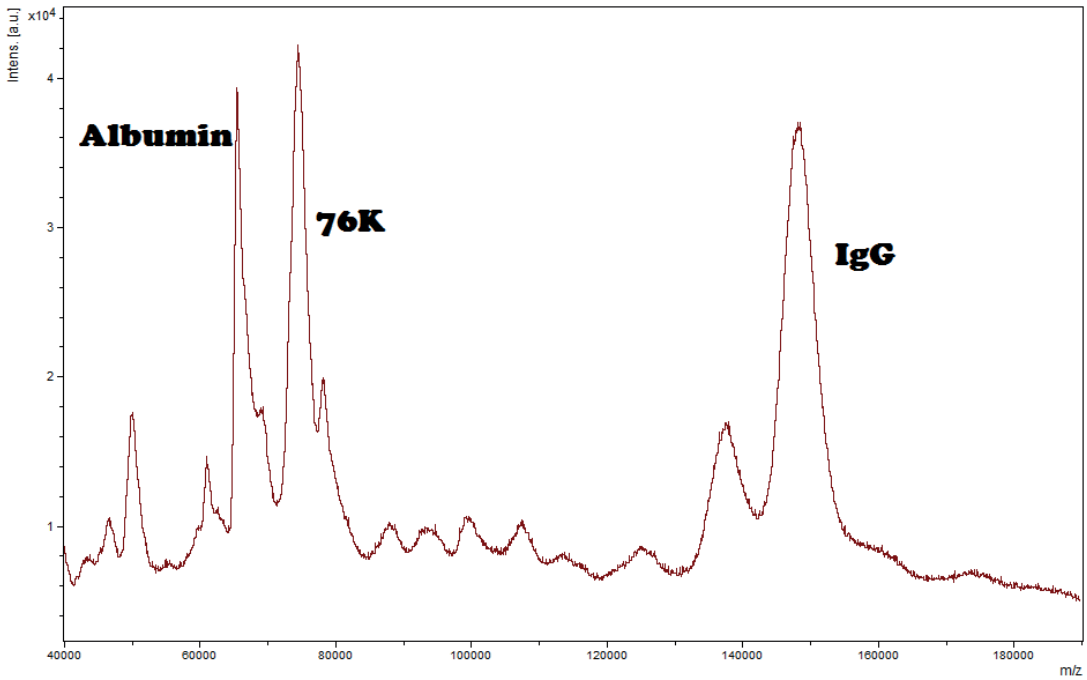


Figure 1B.
MALDI-TOF profile of 'health phenotype' (A/IgG = 9.75)

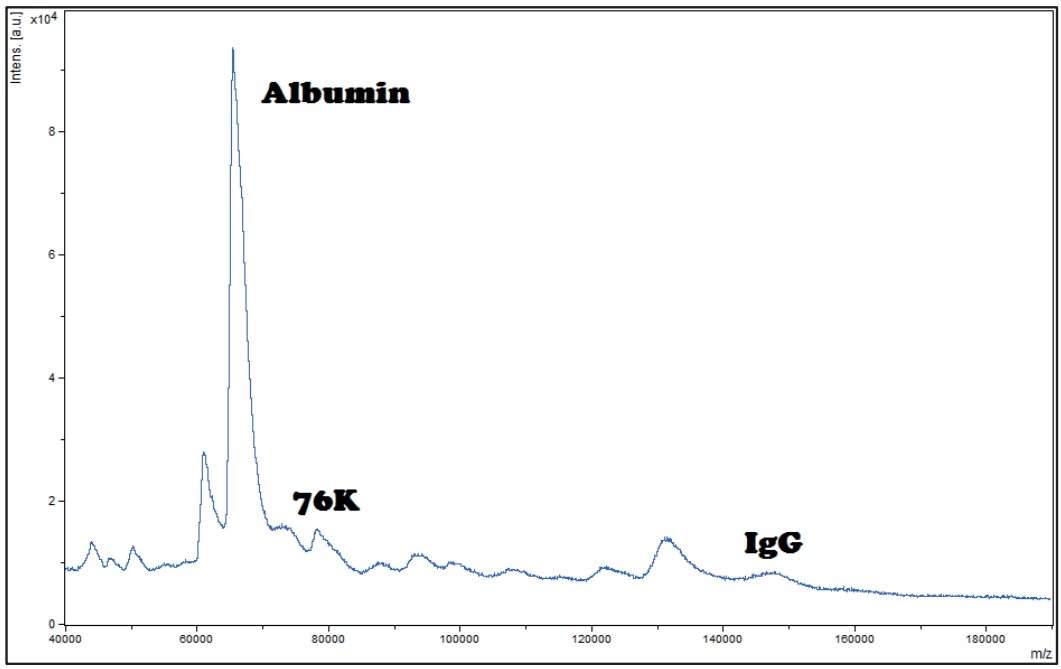
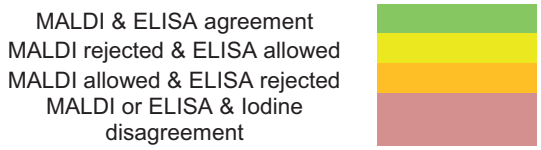


Table 1.
Example of the work sheet of test results by MALDI-TOF, ELISA, iodine, and UV 280 and 230 nm spectrometry, on a farm after outbreak without any previous selection.

Tray	#	MALDI	IP	ELISA	A230	A280
3M	1	2.6	3	4	14.8	2.5
3M	2	2.1	3	5	15.4	2.6
3M	3	5.0	3	4	12.4	2.0
3M	4	3.2	3	5	13.8	2.4
3M	5	9.5	1	3	11.1	1.8
3M	6	3.1	3	4	13.8	2.3
3M	7	4.3	3	7	13.5	2.3
3M	8	4.2	3	3	13.4	2.2
3M	9	3.6	3	7	15.3	2.7
3M	10	6.8	2	1	12.9	2.5
3M	11	4.3	3	4	12.8	2.0
3M	12	9.0	ND	3	11.7	2.0
3M	13	2.3	3	5	14.3	2.4
3M	14	1.5	3	6	15.9	2.8
3M	15	2.3	3	6	14.3	2.5
3M	16	5.3	3	3	13.2	2.1
3M	17	4.3	3	4	13.2	2.1
3M	18	8.0	2	4	11.8	1.9
3M	19	4.0	3	4	12.5	2.1
3M	20	6.0	3	3	ND	
3M	21	4.2	3	6	16.1	2.8
3M	22	4.4	3	5	15.3	2.7
3M	23	10.9	1	3	11.9	1.9
3M	24	4.7	3	6	13.4	2.2
4M	1	3.9	2	3	13.7	2.2
4M	2	3.1	3	2	13.9	2.2
4M	3	2.3	3	4	14.8	2.5
4M	4	12.2	C	1	10.4	1.6
4M	5	2.1	3	7	14.5	2.5
4M	6	3.7	3	4	13.1	2.1

Tray	#	MALDI	IP	ELISA	A230	A280
5M	1	4.9	C	0	ND	
5M	2	3.2	3	6	16.6	3.0
5M	3	3.9	C	2	ND	
5M	4	10.5	C	5	11.1	1.7
5M	5	9.6	C	4	14.2	2.5
5M	6	12.0	C	3	13.0	2.3
5M	7	5.3	3	7	15.9	3.2
5M	8	4.6	3	4	11.3	1.9
5M	9	2.9	3	4	13.6	2.3
5M	10	5.7	2	6	1.7	0.3
5M	11	10.0	2	4	14.4	2.4
5M	12	15.3	C	3	13.0	2.1
5M	13	15.5	C	1	13.0	2.0
5M	14	16.2	C	4	11.1	1.7
5M	15	16.2	C	2	10.9	1.7
5M	16	6.4	3	4	12.3	1.9
5M	17	12.7	C	2	13.3	2.1
5M	18	3.3	3	3	14.4	2.4
5M	19	14.0	C	3	10.6	1.6
5M	20	11.8	3	2	ND	
5M	21	12.2	0	2	12.0	2.0
5M	22	15.6	C	2	14.0	2.3
5M	23	14.8	C	1	12.3	1.9
5M	24	8.1	1	4	10.5	1.6
6M	1	7.0	C	1	ND	
6M	2	6.9	1	3	ND	
6M	3	5.4	C	4	17.6	3.5
6M	4	3.5	C	3	ND	
6M	5	7.1	C	2	11.5	2.0
6M	6	3.1	3	5	ND	

Legend: IP- iodine precipitation test; A280 and A230-UV spectrometric absorbance; Test results in red indicate failure of the tests. For MALDI-TOF the red indicate the A/IgG ratio below 5, and the blue data (the ratios 5-8) indicate still failed test, but the hypergammaglobulinemia is of lesser degree. ND=not done (insufficient amount of sample).



MRSA in mink *(Neovison vison)*

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important human pathogen that may be isolated from production animals, too – including mink *(Neovison vison).* Presence of MRSA in mink has not been documented before 2013 when it was found in two Danish mink submitted for routine laboratory investigation. The two isolates both belonged to CC398 and the infections were presumably caused by contamination of the feed containing slaughter offal from the pig industry. As a result of these findings an active surveillance was initiated in 2014 and all submissions were tested for MRSA by systematic sampling. The samples were collected from five body locations from all the mink in a submission and pooled across mink by sample location. In addition to this an active sampling of swabs from healthy mink raised on 50 Danish farms were carried out during pelting time in 2015. The results confirmed that MRSA can be isolated from one third of the farms irrespective of whether the mink originated from diagnostic submissions or from healthy mink at pelting. The main sites for recovering MRSA are the paws and pooled swab samples from the pharynx.

Keywords: Mink, MRSA, prevalence

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA), especially those belonging to the clonal complex CC398, have since 2003 emerged in livestock worldwide. Although CC398 is most commonly found among pigs, it has also been found in many production animals (Cuni *et al*. 2010) and animal products such as meat (Agersø *et al*. 2012). MRSA is also an important human pathogen and infections with MRSA can be severe due to treatment failure (Cuni *et al* 2015, Fitzgerald 2012). *Staphylococcus aureus* can occasionally be isolated from diseased mink even though most infections caused by staphylococci in mink are caused by other *Staphylococcus* spp., most notably *Staphylococcus delphini*.

To our knowledge MRSA has not been isolated from mink until 2013 when it was isolated from two Danish mink submitted to the National Veterinary Institute for routine laboratory investigation. These two isolates both belonged to the CC398 clonal complex. The animals may have become infected via contamination of the feed containing slaughter offal from the pig industry.

In consequence of these findings, an active surveillance was initiated in 2014 onwards and all submissions to the laboratory were in addition systematically tested for MRSA by swab or organ sampling.

Material and methods

All mink submitted for diagnostics were included in this study. Samples were collected from five locations from each mink: swab samples from nostril, pharynx, and perianal skin, a piece (5 cm) of

jejunum, and a front paw, and pooled across mink in the submission by sample location.
In addition to this, an active sampling of paws from healthy mink raised on 49 Danish farms was carried out during the pelting period in 2015.

Isolation of MRSA was done by selective procedures including pre-enrichment in Mueller Hinton Broth with 6.5 % NaCl followed by plating on selective MRSA2 agar. Identification of suspected *Staphylococcus aureus* on the MRSA2 agar was performed by MALDI-TOF, whereafter the presence of the *mecA* to confirm MRSA was done by PCR (Agersø *et al.*, 2012) and the spa-type and clonal complex affiliation was determined by PCR and sequencing as previously described (Shopsin *et al.*, 1999). Statistical significance was tested using the chi-square test.

Results

The results showed that MRSA was isolated from one third of the farms irrespective of whether the mink originated from clinical diagnostic submissions or from healthy mink at pelting *(Table 1)*.

Table 1: *Test of samples for presence of MRSA in pools from mink.*

Year	Tested (n)	Positive (n)	Origin of sample
2014	26	2	Clinical diagnostic submission
2015	58	20 (34 %)	Clinical diagnostic submission
2015	49	15 (31 %)	Active sampling from healthy mink in 49 farms

There was a statistically significant difference between clinical diagnostic submissions in 2014 and 2015 after alteration of the test procedures (p=0.01). There was no statistical difference between clinical diagnostic submission sampled in 2015 and healthy mink sampled in 49 farms in the same year (p=0.8). MRSA was most frequently recovered from the paws or from pharynx, suggesting contamination from feed. The most prevalent spa-types were t034 and t011, which are also the most prevalent in Danish pig farms.

Discussion

MRSA CC398 has been found in many different animal species, most often in pigs, but has until now not been reported in mink (Cuni et al. 2010). In pig farming, livestock associated (LA)-MRSA is a working environmental problem, and MRSA CC398 infections in humans have been increasing dramatically in recent years (Anon. 2015). The localization of MRSA primarily in the pharynx and the paws is in particular worrisome, as the primary hazards from working with mink are bites and scratches, and there are reports of humans working at mink farms who have attracted LA-MRSA infections. The two different sources of samples in 2015 – healthy and diseased mink – did not differ statistically indicating that MRSA is not specifically associated with clinical disease in mink but rather an incidental finding. Therefore, we do not attribute the isolation of MRSA to use of medicine in the clinically diseased mink but assume that the mink become infected via contamination of the feed containing slaughter offal from the pig industry. This is supported by the spa-type finding of the isolates.

Acknowledgements

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The correlation between the histological method and modern serologic methods of mink Aleutian disease diagnosis

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Abstract

A comparative investigation was carried out at a fur farm affected by aleutian disease (AD) to plasmacytosis using two methods: counter immuno-electrophoresis (CIEP) and histological analysis. When the CIEP method was used, 62% of mink were found to give a positive reaction, the histological method gave a positive reaction in 71,4% mink. The current investigation confirms that the histological method has a high diagnostic accuracy rate when used to identifying of mink plasmocytosis.

Keyterms: mink plasmocytosis, histological analysis, CIEP

Introduction

AD - aleutian disease – is a contagious, slowly progressing viral disease, characterised by a widespread plasma cell proliferation (plasmocytosis). AD was first described by Hartsough G.R. and Groham J.R. in 1956. The presence of AD in mink induces an overproduction of antibodies, however, as they are unable to neutralise it, the results is a life-long presence of both the virus and the antibodies as an immune infection complex. The inability to neutralise the virus leads to an uncontrollable increase in antibodies and hypergammaglobulinaemia. The high susceptibility of mink and resistance of the pathogen in the external environment led to a wide spread of the disease in many countries. The disease causes huge economic damage due to high mortality, fall of pelt quality, fall in female fertility and an increased sterility of males. The percentage of seropositive mink in various countries is between 40 to 90%. Due to the fact that attempts at developing cures or preventative measures have had no success, the main method for combatting the disease is early diagnosis and culling of affected animals. The disease is diagnosed using the epizootological data, clinical analysis, post mortem, serological analyses and PCR. Several types of serological methods have been developed. The CIEP method is the most commonly used at the present time, good results can also be obtained by using the ELISA and PCR tests (Knuuttila *et al*., 2009). The histological method is used in a post mortem analysis (Akulova V.P. *et al*., 1972). The purpose of the research is to prove the efficacy of the histological method of analysis.

Materials and methods

Research was carried out at a fur farm affected by AD using two methods to diagnose viral plasmocytosis: CIEP and histological analysis. A histological study of the material from 42 of the corpses

of minks, received from disadvantaged by AD on the fur farm during slaughter. In the study took a standard mink at the age of six months without sex differentiation. The animals were euthanized ditilin according by standard method. All minks at the age of 60 days were vaccinated with quadrivalent vaccine against canine distemper, mink viral enteritis, Pseudomonas infection and botulism. The material was fixed in 10% formalin solution, histological sections prepared on the freezing microtome and stained with hematoxylin and eosin. In comparison, the blood samples of all minks examined on CIEP using a standard set of antigen for this reaction. The blood of mink were taken immediately after slaughter.

Results

Histological changes at AD expressed in the generalized plasma-cell hyperplasia with a tendency to the formation of mature plasma cells in with reticuloendothelial tissue. At early stages, separate plasma cell infiltrates are noticed in the bone marrow, kidneys, liver, spleen and lymph nodes, which then become generalised. Progressive proliferative and sclerogenic glomerulonephritis, periarteritis nodosa develop. In most mink, in the presence of viral plasmocytosis, various stages of hepatodystrophy are present. Fatty liver is coupled with plasmocell infiltration in the interlobular connective tissue. In the kidneys plasmocytosis is manifested by a local proliferation of plasma cells in the interstitial tissue. Progressive proliferative and sclerosing glomerulonephritis develops. Changes in the spleen are characterised by lymphoid tissue hyperplasia and the presence plasma cell patches in the red pulp.

In all carcasses with a positive reaction to CIEP (26 heads 61%), AD was found during histological analysis. Histological analysis also found animals (4 heads) at the inapparent stage of disease, 30 heads (71.4%) in total. The histological changes in the AD of mink are characterized for this disease and represent a diagnostic test The histological changes in the PV mink are characterized for this disease and are diagnostic test. The presence of the virus plasmacytosis of mink was confirmed using PCR.

Discussion

All modern methods of AD diagnosis have both positive and negative qualities. Progressive AD is characterised by a fast increase in levels of specific antibodies, unique clinical characteristics and typical post-mortem changes. Chronic and inapparent infections differ by a moderate rise in antibody levels, a rapid fall in antibody level in kits after weaning with a gradual rise thereafter, inconsistent post-mortem changes (Slugin, 2004, Mikheev and Semikrasova, 2014). The histological method can only be carried out post mortem, which prevents it from being used to test the entire population. However, this method is effective for the entire duration of the disease, starting from the earliest stages (Akulova V.P. *et al*., 1972; Ezhkov *et al*., 2013) and can be used as an additional diagnostic method.

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Aleutian Disease

Oral presentations

Study of distribution of AMDV load in infected mink farms environment

Evaluation by qPCR of personal protective equipments for visitors against AMDV

Outbreaks of Aleutian mink disease in farmed mink *(Neovison vison)* in Denmark: molecular characterization by partial NS1 gene sequencing

Emerging infections at the interface of wildlife and livestock

Dot immunoenzyme assay for the diagnosis of Aleutian mink disease

Detection of Raccoon Dog and Fox Amdoparvovirus Infection and Viral Genetic Characteristics

Canadian experience with MALDI-TOF profiling of plasma proteins in selection for breeding for Aleutian disease resistance, and comparison to iodine precipitation and AMDV-Ab ELISA

MRSA in mink *(Neovison vison)*

Posters

The correlation between the histological method and modern serologic methods of mink Aleutian disease diagnosis

Part 3. Breeding, Genetics & Reproduction

The draft genome sequence of the American mink *(Neovison vison)* opens new opportunities of genomic research in mink

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Abstract

The American mink *(Neovison vison)* is a semiaquatic mustelid native to North America. It is an important animal for the fur industry. Although many efforts have been made to locate genes influencing fur quality and color, the lack of a reference genome impedes the search. American mink has the smallest chromosome number among studied *Carnivora* species. Genomic information about American mink is also vital to understand the evolution of *Carnivora*. Hence a reference genome of mink will facilitate genetic improvement of economic traits and will increase our knowledge about the evolution of *Carnivora*.

Here we present the draft genome sequence of American mink. In our study, a male inbred pearl mink was sequenced by Illumina paired-end and mate pair sequencing. The reads were assembled, which lead to 22,419 scaffolds with an N50 (shortest sequence length at 50% of the genome) of 646,304 bp. The assembly constituted 2.4G plus gaps, representing 90% of the estimated genome size. Repeat annotation showed that repeat sequences constitute about 25% of the mink genome. The biggest repeat family was a family of LINEs similar to LINEs found in the dog and ferret genomes. Gene annotation of our draft genome indicated our draft genome contains 87% of 1:1 *Vertebrata* genes (63.5% complete single copy genes, 0.5% duplicated genes and 22% fragment genes). We were able to map on the draft genome all the well-studied genes which are thought to be involved in the coat quality and coat color phenotypes.

Our draft genome has great potential to facilitate genomic research towards improved breeding for high fur quality and will strengthen our understanding of *Carnivora* evolution.

Keywords: NGS, genome assembly, farm animal

Introduction

The American mink *(Neovison vison)* is a semiaquatic species that belongs to the *Carnivora* family. American mink is the only species in the genus *Neovison*. With the release of genome sequences of dog (Lindblad-Toh, Kerstin *et al.* 2005), cat (Pontius *et al.* 2007) and ferret (Peng *et al.* 2014), the genome sequence of American mink will therefore provide additional valuable information into the evolution of *Carnivora*.

For decades the fur industry has used American mink as the major source of fur. The mink fur is an important industry in China, Denmark and Canada etc. (Hansen 2014). As a result, the improvement

in fur quality using genetic markers is a focus of mink breeding research. Since 1996, researchers began to develop SSR markers for mink (O'Connell *et al.* 1996, Fleming *et al.* 1999, Hansen and Jacobsen 1999, Vincent *et al.* 2003, Anistoroaei *et al.* 2006). A linkage map for these markers was released in 2007 (Anistoroaei *et al.* 2007). Subsequently, two updated versions of the linkage map were also released adding new SSR markers (Anistoroaei *et al.* 2009, Anistoroaei *et al.* 2012). Next-generation sequencing (NGS) techniques are also used to generate markers for mink. Using Restriction site-Associated DNA (RAD), 1,256 single nucleotide polymorphism (SNP) markers were identified (Thirstrup *et al.* 2013).

Fur quality is among the most economically important traits in mink production. Therefore, finding genes affecting fur quality is vital for the industry. There was limited effort to identify the QTL for fur quality (Thirstrup *et al.* 2014). A BAC library was constructed and some homology genes for fur quality and color from other mammalian species were analyzed (Anistoroaei *et al.* 2011). The transcriptome of mink was also published, and some color genes were studied using transcriptome data (Christensen and Anistoroaei 2014). But the absence of a mink reference genome hinders the creation of high density maker panels for genotyping mink in order to map genes for economically important traits. Therefore the objective of this study was to construct a mink genome assembly.

Materials and Methods

Genomic data generation and genome assemble

A male pearl American mink *(Neovison vison)* individual from Aarhus University's mink farm was used to generate the genomic sequence. The chosen one is from one of the inbred line. The spleen tissue was used to extract DNA. Extraction of DNA and sequence generation was performed by Aros (http://aros-ab.com/). In order to use the Allpaths-lg pipeline(Butler et al. 2008), we designed one overlap pair-end library (165bp, 100PE) and two long insert mate-pair libraries (3k and 5k,100PE). The total data were 163.2 Gb for pair-end sequence and 184.3 Gb mate-pair sequence. All the data were analyzed using Allpaths-lg to construct the genome assembly.

Down-stream analysis

In order to capture the species-specific repeat sequence from mink, we performed do novo prediction before running RepeatMasker (Smit *et al.* 1996). We used RepeatExplorer (Novák *et al.* 2013) and RepeatModler (Smit and Hubley 2010) with the default parameters to analyze the repeat sequence of mink. For the RepeatExplorer results, we analyzed all the clusters to remove those belonging to non-repeat sequence by blasting(Johnson *et al.* 2008) the contigs against NR (Pruitt *et al.* 2007) database. Finally, we combined results from these two sources and constructed a mink-specific repeat database and used this repeat database to annotate the repeat sequence of the genome. We used BUSCO (Simão *et al.* 2015) to assess the quality of the genome assembly and help annotation.

Results and discussion

Sequencing and assembly

A whole genome sequencing (WGS) strategy was used to sequence and assemble a male brown American mink. A total of 163.2 Gb of next-generation Illumina paired-end reads was generated by sequencing genome shotgun libraries of 165 bp insert size. 184.3 Gb of mate-paired reads were also generated with 3 kb and 5 kb insert size library. The total sequence covered 128.7 fold of the estimated 2.7 Gb genome. All the sequencing reads were assembled by Allpaths-lg (Gnerre et al. 2011), which yielded

Table 1. *The repeat sequence of mink*

Families	Numbers	Length	Percentage
SINEs	993,357	163,046,403 bp	6.71 %
LINEs	555,858	296,246,162 bp	12.19 %
LTR elements	213,839	71,725,044 bp	2.95 %
DNA elements	18,265	32,396,996 bp	1.33 %
Small RNA	919,752	155,347,649 bp	6.39 %
Satellites	6,402	3,167,324 bp	0.13 %
Simple repeats	729,614	28,893,422 bp	1.19 %
Low complexity	134,873	6,823,683 bp	0.28 %
Unclassified	7,651	860,216 bp	0.04 %
Total			24.82 %

Table 2. *The candidate fur quality and color genes*

Gene	Location	Gap	Gene	Location	Gap
FGF5	scaffold2417	780bp	MC2R	scaffold7389	NA
PMEL	scaffold1856	746bp	KITL	scaffold978	2057
MLPH	scaffold4345	NA	KIT	scaffold1621	4084
TYRP1	scaffold4069	1354bp	LYST	scaffold910	3048
MC1R	scaffold1073	NA	RSPO2	scaffold178	3378
KRT71	scaffold819	NA	HLADRB1	scaffold4491	3098
MITF	scaffold53	NA	AGRP	scaffold68	NA
ASIP	scaffold1740	NA	Atoh-1	scaffold810	NA
DEFB103	scaffold1496	NA	ITGB1	scaffold1078	NA
DEFB1	scaffold1496	592	TMIE	scaffold213	120
MC3R	scaffold1804	NA	SLC24A5	scaffold449	NA
TYR	scaffold4579	140			

a 2.43 Gb assemble plus gaps (Table 1). The draft genome consisted of 22,419 scaffolds with N50 of 646,304 bp and largest scaffold was 4.7 Mb.

Down-stream analysis

In order to construct the species-specific repeat library for mink, we used the RepeatModler (Smit and Hubley 2010) and RepeatExplorer (Novák *et al.* 2013) software to analyze repeat families, and then used RepeatMasker (Smit *et al.* 1996) to annotate the genome. Our result showed that 25% of the mink genome was repeat sequence. American mink is thereby a low-repetitive genome, compared to human and dog (Lindblad-Toh, K. *et al.* 2005). The dominant repeat sequence was a family of LINEs similar to LINEs found in the dog and ferret genomes (Peng *et al.* 2014). SINE families were the second largest type of repeat sequence in the mink genome. The detailed composition of different families is listed in *Table 1*. We found three species-specific satellite repeats of mink, which were not reported in the dog and ferret genomes. However, they only covered 0.13% of the genome *(Table 1)*.

To help the annotation of the genome and also to assess the quality of the assembly, we used BUSCO (Simão *et al.* 2015) to analyze the completeness of the *Vertebrata* single-copy orthologs. The result

showed that 87% of 1:1 Vertebrata genes (63.5% complete single copy genes, 0.5% duplicated genes and 22% fragment genes) were present in our assembly. Subsequently, we also mapped on the genome all the well-studied genes which are thought to be involved in coat quality and coat color phenotypes; results are listed in Table 2. Our result capture complete sequence of half of these genes, which complements data from previous research (Anistoroaei *et al.* 2011).

Conclusion

We used NGS technology to generate whole genome sequencing data. The data was used to assemble a reference genome of mink. Our results showed that the assembly is a high quality draft genome. Repeat annotation shows that mink has a low-repetitive genome. Our gene annotation will facilitate the fur quality genes identification. And the SNP calling using reference will provide enough markers to build the high density linkage map and show the great potential in genomic breeding, QTL mapping and GWAS analysis.

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Breeding for a better pelt size by selecting on body length

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Abstract

Over a period of 2 years the body length (BL) of minks was measured in a transparent tube and their pelt size (PS) was measured after drying. The validity of the ante-mortem tube-measurements was confirmed by post-mortem measurements of 1710 of these minks (R2=0,7). To determine whether the obtained BL or Body weight (BW) was useful as a selection criterion when breeding for a longer PS, 315 parents and their offspring were weighted and measured across 28 different groups (table with groups) at 3 moments during the growing period.

Year	Groups	Parents/group	Mating	BL-BW-PS kits
2014	4	40 females, 8 males	Cross	712
2015	24	8 females, 1 male	Line	940

Results

BL ranges from 44 to 54 cm in the males and from 34 to 44 cm in the females. The correlation between BL and BW is moderate (R2=0.37) while the correlation between both BL and BW to PS is strong (R2=0.64).

The BL of kits was positively correlated to the BL of parents in all groups. Moreover, the PS of kits was positively correlated to the BL of the parents in all groups, but not at all or less to the parents BW. Selection of breeders for BL using the transparent tube proved more efficient than selection on BW to enhance PS in offspring.

Keywords: Body length, Body weight, Pelt size, Selecting Breeders

Introduction

Numerous studies have shown that the body weight (BW) of minks is positively correlated with their pelt size (PS) and it is therefore commonly used as a selection criterion. However, if minks are grouped based on their PS, large individual variation in BW is found. We have previously shown that the heavy mink in each pelt length class have a BW which is the average for the next length class (de Rond, 2015).

Differences in body length (BL) could partly explain why minks with highly different BW have the same PS. Unfortunately good data to support the added value of BL in addition to BW are lacking, since many reports focus on either BW or BL as selection criteria (Lohi, 1990, Lagerkvist, 1994, Hansen, 1997, Møller, 1999, Nielsen, 2012).

The heritability of BL is high in minks: 0.46 (Hansen, 1997). Lohi, (1990) measured BW and BL in

October and reported a heritability of 0.54 for BW and 0.72 for BL. BW was significantly correlated with BL (0.80), and BL was significantly correlated with PS (0.74).

De Rond measured a stronger correlation from body length plus body weight to pelt size compared to the correlation from body weight to pelt size (de Rond, 2015). Considering this strong relation with PS and the high heritability, BL can be a useful selection criterion. An important reason to favour selection based on BW over selection based on BL is the difficulty to determine BL in the living animal. Edelveen Research farm has therefore developed and tested a new system to measure BL in live minks. This paper reports the results of selecting breeding minks based on BL in two consecutive years.

Material and Methods

Two projects were done, one in 2014 and one in 2015. In both projects minks were selected based on BL and BW and production parameters of their kits were measured. The BL was measured in a transparent tube, open at both ends and with a cm scale printed on the outside. During measurements, a mink was held at the base of the tail, and guided into the tube until the hand holding the mink reaches the entrance of the tube (Picture 1, 2). When the mink tries to run to the other end of the tube, it performs a stretching posture. At that moment the distance between the base of the tail at the beginning of the tube and the nose of the mink was noted as the BL (Picture 3).

Project 2014

Four groups, each consisting of 40 females and 8 males with different body length were formed (Short, Mid. Short, Mid. Long, Long). The minks of each group were cross mated and at weaning the kits were housed in a pair or a group (3-4 mink) per BL group. Litters of 3 kits or less were removed from the project. BW was measured at weaning and BL and BW were both determined ont the 22nd of September, the 6th of October and the 20th of October and again post-mortem during pelting on the 15th of November *(Table 1)*. After processing the pelts, pelt size was measured. Table 1 shows the BL and BW of the parents and number of kits per group.

Table 1: *Average BL and average BW of breeding minks and the number of their kits, for each of four groups based on BL (Short, Mid. Short, Mid. Long, Long)*

	Male Breeder		Female Breeder		N Kits Grow-Pelt*	
Groups	BL(cm)	BW (kg)	BL (cm)	BW (kg)	Male	Female
Short	46	3.2	38	1.5	98	85
Mid.Short	49	3.4	39	1.6	989	5
Mid.Long	50	3.5	40	1.6	95	70
Long	51	3.6	42	1.7	83	88

Note: not all mink were pelted

Project 2015

Twenty-four groups, each consisting of 8 females and 1 male were formed, based at the groups in 2014. Each group of 8 females had 2 females per length group (short, mid-short, mid-long and long). The males were also chosen per length group (6 males per length group). The mating system was line mating, 1-8 for young females and 1-1 for old females. This project is the first year of a 2 year project

Picture 1:
hold the mink at the base of the tail

Picture 2:
guide the mink in the tube

Picture 3:
BL of stretching mink

for measuring the heritability for BL measured by the transparent tube. This first year provides the information for judging whether the BL or BW is important in relation to the PS. Therefore the breeder minks are separated in 2 groups for BL and BW: Low and High. The information of the offspring BL and BW at the end of October (selecting period) and November (Pelting period, post mortem) and the PS are presented in this paper. Table 2 shows the information per group.

Table 2: *Average BL and average BW of breeding minks per group Low and High and the number of their kits followed in the growing and pelting period*

Groups	Sex	Breeder info		Kits Growing-Pelting*	
		BL (cm)	BW (kg)	Male	Female
Low	Male	47	3.2	265	215
	Female	38	1.5		
High	Male	51	3.5	200	260
	Female	41	1.8		

Note: not all mink were pelted

The data of the offspring of project 2015 will be presented per parent group and the combined parent groups:

1. Low (both parents Low)
2. Middle (1 parent from Low and 1 parent from High)
3. High (both parents High).

Statistical analysis of BL and BW of the offspring is done using anova. The regression is calculated between the ante-mortem tube-measurements at the end of October and the post-mortem measurements for BL in November using the data from both 2014 and 2015 (850 male and 860 female minks).

Results

Regression of tube measurement and post mortem measurement BL

The BL measured post-mortem is correlated to the BL measured ante-mortem in the tube (Table 3). The regression is high and 80% of the BL post mortem is within 1 cm of the BL measured in the tube at the end of October.

Table 3: *Regression formula and coefficient of determination (R2) of BL measured in the tube and BL measured post mortem (male and female, 2014 and 2015)*

Regression	Sex	Formula	R^2
BL Tube - BL post mortum	Male	y = 0.0051x + 37.8	0.7
	Female	y = 0.0062x + 45.4	0.72

Project 2014. For each sex, there are significant differences between the groups for BL and BW and PS (Table 4 and 5).

Table 4: *BL and BW of male minks at various time points in 2014, and their PS in December, for each of four groups (Short, Mid, Short, Mid. Long, Long) based on the BL of the parents*

Date	item	Body Length Parents			
		Short	Mid.Short	Mid.Long	Long
6-jul	BW (kg)	0.9[a]	0.9[a]	0.9[a]	1[b]
22 Sept	BL (cm)	45.2[a]	46.6[b]	46.9[b]	48.3[c]
	BW (kg)	2.6[a]	2.8[b]	2.8[b]	2.9[c]
6 Oct	BL (cm)	45.9[a]	47.3[b]	47.8[b]	49.3[c]
	BW (kg)	2.8[a]	3[b]	3[b]	3.2[c]
20 Oct	BL (cm)	46.1[a]	47.5[b]	47.9[b]	49.6[c]
	BW (kg)	2.9[a]	3.1[b]	3.1[b]	3.3[c]
15 Nov	BL (cm)	46.9[a]	48.2[b]	48.5[b]	49.9[c]
	BW (kg)	3[a]	3.2[b]	3.2[b]	3.4[c]
December	PS (cm)	90.4[a]	91.8[b]	92.5[b]	94.6[c]

Different superscript letters indicate significant differences ($p<0.05$).

Table 5: *BL and BW of female minks at various time points in 2014, and their PS in December, for each of four groups (Short, Mid, Short, Mid. Long, Long) based on the BL of the parents*

Date	item	Body Length Parents			
		Short	Mid.Short	Mid.Long	Long
22 Sept	BL (cm)	37.8[a]	39.1[b]	39.6[c]	40.8[d]
	BW (kg)	1.5[a]	1.6[b]	1.6[b]	1.7[c]
6 Oct	BL (cm)	37.9[a]	39.1[b]	39.6[c]	41[d]
	BW (kg)	1.6[a]	1.7[b]	1.7[b]	1.8[c]
20 Oct	BL (cm)	37.9[a]	39[b]	39.7[c]	41[d]
	BW (kg)	1.5[a]	1.7[b]	1.7[b]	1.8[c]
15 Nov	BL (cm)	38.5[a]	39.8[b]	40.3[c]	41.6[d]
	BW (kg)	1.5[a]	1.7[b]	1.7[b]	1.8[c]
December	PS (cm)	71.8[a]	74[b]	75.3[b]	76.7[c]

Different superscript letters indicate significant differences ($p<0.05$).

The difference in BL per group at each date was also measured at the same BW of the mink from the different groups. The male mink offspring from group Long are in the range 3 – 3.7 kg (20 October) in average 2.2 cm longer then the minks with the same BW from group Short and 1.2 cm longer than those from group Middle. The female minks from group Long were also longer than the female mink from the other groups with the same BW in the range 1.5 – 2kg.

Table 6: *BL and BW of male offspring mink at various time points in 2015, and their PS in December, for each of three groups (Low, Middle, High) based on the BL or BW of the parents*

Male offspring		Parents Selected BL			Parents selected BW		
		Low	Middle	High	Low	Middle	High
October	BL (cm)	47.6[a]	48.3[b]	49[c]	48.2[a]	48.1[a]	48.8[b]
	BW (kg)	3.1	3.2	3.2	3.1	3.2	3.2
November	BL (cm)	47.4[a]	48[b]	48.8[c]	47.8[a]	47.9[a]	48.6[b]
	BW (kg)	3.2	3.3	3.3	3.2[a]	3.3[ab]	3.4[b]
December	PS (cm)	92[a]	93.1[b]	94.1[c]	93.3	93	93.1

Different superscript letters indicate significant differences ($p<0.05$).

Table 7: *BL and BW of female offspring mink at various time points in 2015, and their PS in December, for each of three groups (Low, Middle, High) based on the BL or BW of the parents*

Female offspring		Parents Selected BL			Parents selected BW		
		Low	Middle	High	Low	Middle	High
October	BL (cm)	39.4[a]	39.9[b]	40.8[c]	39.6[a]	39.9[b]	40.4[c]
	BW (kg)	1.7[a]	1.7[ab]	1.8[b]	1.7[a]	1.7a	1.8[b]
November	BL (cm)	39.5[a]	40[b]	40.9[c]	39.7[a]	39.9[a]	40.4[b]
	BW (kg)	1.7[a]	1.7[ab]	1.8[b]	1.7	1.8	1.8
December	PS (cm)	74.5[a]	75.6[b]	76.4[c]	75.3	75.4	75.5

Different superscript letters indicate significant differences ($p<0.05$).

Project 2015

Because line mating was used, the correlation from BL to PS and from BW to PS could be calculated (Table 6 and 7). When the parents are grouped based on BL, both their male and female kits have significantly different BL and PS. When the parents are grouped based on BW, the male kits have significant difference in BW and the female kits significant different BL, but neither have differences in PS.

Discussion

Measuring the BL of live minks in the tube turns out to be a valid method, with results strongly correlated to post-mortem measurements. Additionally, practical application proved to be easily enforceable while measuring BL takes nearly the same time as weighing for BW. The variation in BL becomes visible for the farmer. Valid measurements can however only be obtained when the mink stretches to show its length. When the mink is not stretching, the measured BL will not correlate with the post mortem measurement. The results in both years show a strong correlation between the BL of the parents and the BL of the kits.

After we found a strong correlation between BL and PS (de Rond, 2015), the next question was: is BL in minks determined by nature (hereditary) or nurture (depending on good health, feeding and management)? The results of 2014 support our suggesting that it is highly heritable, and were encouraging

to go forward in this topic. The PS is much longer in kits from parents with a high BL and animals with equal BW varied greatly in BL. Still, the parents differed not only in BL, also in BW. Was the result we measured in the offspring due to the difference in BW of the parents? That is not what the project of 2015 shows. We found no relation between the BW of the parents and the PS of their offspring. By selecting breeders for high BL, we could significantly increase the PS in the offspring.

In a full grown mink, the BL is a very useful characteristic to select for in order to achieve a better pelt size in their offspring. Selection for BW does not result in the same increase in pelt size while heavy breeder mink can be short or long in BL. We describe a method to measure BL using a transparent tube, which is shown to be a valid and practical method.

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Accuracy of breeding values in herds using crossmating

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Abstract

Simulations have been used as decision support by consultants in many farm species. They can be helpful to analyze dynamics between decision making in breeding and medium to long term outcome. The aim of the project was to program, using the R programming language, a stochastic simulation of a mink farm. The simulation takes into account multi-trait genetic considerations and returns the production results as well as genetic trends of a mink farm of an arbitrary size for a certain period of years. Mink farming has a number of management decisions/practices that influence the results from artificial selection. Such practices as crossmating, stepwise selection of kits and differing selection intensities between different paths of inheritance are difficult to estimate using deterministic predictions. In the first presentation of the program, three scenarios are compared. Phenotypic selection, index selection with the true pedigree and index selection with uncertainty regarding paternity of a number of animals.

Keywords: breeding model, R, herd dynamics, decision support

Introduction

Mink breeding has a decentralized breeding strategy, with each individual farmer having a breeding stock of animals and his/her own breeding goal. A number of management practices in mink breeding, such as crossmating and differing intensities of selection that this practice invokes, makes it difficult to employ deterministic prediction of genetic gain. It was therefore proposed to develop a stochastic simulation tool using the R programming language to simulate breeding as practiced on farms with the options of changing parameters to estimate the effects of decisions taken in breeding. Some simplifications are necessary in the design of such a simulation as in some cases precise knowledge of the parameters is not known. To demonstrate the utility of the program a presentation will be made of accuracy of estimated breeding values of two scenarios of index selection and compared with the correlation between the phenotype and genotype in the context of phenotypic selection.

Crossmating is used extensively on commercial mink farms. The practice involves using different males for the first and second mating. Two bouts of matings are well known to increase litter size and decrease barren percentage (Venge, 1973). Usage of different males is done for mainly two reasons. The first is that sometimes the female does not show an interest in mating with the male again but is willing to mate with a different male. The second reason is that mink farmers wish to use their best males more in the second bout of matings. This is because mating willingness increases during the mating season, and therefore males can handle a greater number of females per time unit in the 2nd mating than in the 1st mating (Elofson *et al.*, 1989). In addition, earlier investigations have shown that ~85% of the kits from a female mated with two males, in two different ovulation events, come from the second mating, with the rest being from the first mating (Enders, 1952). The concept of superfetation is reviewed

in detail by Roellig *et al.* (2011). The mating practice introduces uncertainty to the pedigree which can potentially affect the utility of index selection.

Genetic progress in phenotypic selection is dependent on the selection intensity, heritability of the trait and additive genetic variance. In index selection the genetic progress is dependent on the selection intensity, the accuracy of the selection index and the additive genetic variance (Falconer, 1989). It has been possible to have substantial increase in body weight of mink in the last decades while litter size has stagnated (Hansen and Berg, 2008). This is a reflection of the high heritability of body weight and the negative correlation between litter size and body weight (Thirstrup et al., 2016a). Additionally, selection candidates do not have a phenotype of 3 their own for litter size when they are selected, further reducing genetic progress (Falconer, 1989).

Material and methods

The simulation was written in the R programming language (R Core Team, 2016). Various other R packages were used in the programming (Coster, 2012; Dowle *et al.*, 2015; Genz *et al.*, 2016; Højsgaard and Halekoh, 2015; Stasinopoulos *et al.*, 2015; Wickham, 2009). Calculation of breeding values was done with the DMU software (Madsen and Jensen, 2007).

For each replication of the simulation a base population of males and females is set up, mating is performed, selection of next generation of animals and the process is repeated for 11 generations. Each step will be described below, with an emphasis on mating and paternity of the kits.

Traits included

The traits included in the simulation are: litter size, body weight of males and females, skin length of males and females, quality graded on live animals, quality of skins, guard hair length and residual feed intake. Genetic correlations are from (Thirstrup et al., 2016b). It was necessary to estimate a model for litter size in order to generate a discrete litter size. The genetic correlation of this new model of litter size to the other traits was assumed to be the same as in Thirstrup (2016).

The following model was assumed to describe litter size $y \sim Poisson(\lambda)$

$$\lambda = \mu + a + pe + t$$

With **a** being the additive genetic value for litter size, **pe** being permanent environmental effect of the dam and **t** being effect of the age of the dam. The parameters for this model were estimated from data from a commercial farm using the DMU software. See Table 1 for description of data set. For this non-linear model, heritability is not well defined because the genetic and permanent environment variance components are not additive to the residual component (Perez-Enciso *et al.*, 1993).

Table 1. *Farm data from a commercial Danish farm used to estimate genetic parameters of litter size*

No. of dams with records	19,425
No. of observations	34,308
No. of age classes	2
Total number of animals in pedigree	27,411
Average litter size ± Standard deviation	7.14±2.33

For the other traits, the models assumed were those from Thirstrup (2016) and Shirali et al. (2015).

Accuracy

To estimate the effect of using the true sire or the assumed sire accuracy was calculated as the Pearson correlation coefficient between the estimated breeding values (EBV) and the true breeding value (BV) within birth cohorts.

Mating and survival

To illustrate today's management, the following method was used. Each female is assigned a mating willingness for first and second round of mating, this discrete variable takes the value one if the female is willing to mate and zero otherwise. See Table 3 for details of settings for the simulation. Each male is assigned a number of females he is willing to mate in round 1 and round 2 of mating. The number of females a male is willing to mate, is drawn from a zero inflated Poisson distribution, the lambda parameter is given a value of 6 and the amount of males with zero mating willingness is 5%. The males have a specified barren percentage and the females as well. The chance of a female being barren is related to the dam's age and if they were mated once or twice. For each animal a value is drawn from a Bernoulli distribution according to the barren percentage that indicates if the animal is barren or not. For the second round, a specified number of males are disqualified from mating. These are males that mated less than three females in the first round and then the 20% of males with the lowest live quality score and body weight. If the male does not manage to mate all of the females he mated in the first round, a different male is used in the second round. A different male is also used in the second round for females that were mated to males that were disqualified for the second round. Preference is laid upon mating yearling females twice as this is practiced on farms.

For the females not barren and not mated with a barren male, a phenotype for litter size is generated according to the Poisson model described above. For a female mated with two males, her kits have an 85% chance of being from the second mating and 15% of being from the first mating. The genetic values for the kits are generated according to the true sire while the pedigree is prepared from either the true sire or the assumed sire, which is the male used in the second mating.

Breeding values

For each kit a genetic value is calculated according to classical quantitative genetics theory, assuming only additive effects (Lynch and Walsh, 1998).

$$A_{off,i} = 0.5\left(A_{dam,i} + A_{sire,i}\right) + A_{mendel,i}$$

With $\mathbf{A}_{\mathbf{dam\ i}}$ being the additive effect of the dam for trait **i**, $\mathbf{A}_{\mathbf{sire\ i}}$ being the additive effect of the sire for trait i and $\mathbf{A}_{\mathbf{mendel\ i}}$ being the Mendelian sampling term for trait **i**. The variance of the Mendelian sampling is

$$Var\left(A_{off} \middle| F_{sire}, F_{dam}\right) = \frac{1}{2}\sigma_a^2\left(1 - \frac{1}{2}\left(F_{sire} + F_{dam}\right)\right)$$

Where $\mathbf{F}_{\mathbf{sire}}$ and $\mathbf{F}_{\mathbf{dam}}$ are the inbreeding coefficients of the sire and dam (Wright, 1922).

The phenotype used for litter size is the litter size at 1st counting after birth. To simulate random culling a survival rate from 1st counting to pelting is considered 82.5%. For each kit a value is drawn from a Bernoulli distribution that takes the value one if the kit survives and zero otherwise. To simulate the fostering that usually takes place with kits from large litters, kits that are from litters larger than 8 are discarded as selection candidates. Since males are usually fostered, the animals are arranged so that males are cross fostered in preference to females.

Phenotypic scenario – the current practice

The method used in the phenotypic selection is the following. Kits from the 40% smallest litter are discarded as selection candidates. Of the remaining, the 40% of the kits with the lowest body weights are discarded as selection candidates. The animals are then assigned a discrete quality score that takes the value of 1-5, where 5 is the best. This is done by truncating the continuous phenotype for quality (see Table 2). The animals are then ranked by the quality score and the number of animals needed picked. A specified proportion of older females is selected based on their litter size for the year in the simulation and by setting the maximum age for older females to three years.

Table 2. *Truncation of quality to obtain a discrete quality score*

Quality score	1	2	3	4	5
Percentage of kits	5%	25%	40%	25%	5%

Index selection

The index selection scenario is simulated to reflect the usage of the FurFarm software on Danish mink farms. The animals with the lowest 40% of litter size index are discarded as selection candidates. For those candidates the phenotypes for body weight and live quality are used to calculate breeding values with the DMU software. The pedigree is trimmed so that non-informative individuals are removed from the pedigree and individuals more than 5 generations from the generation that breeding values are being predicted for. This is done to avoid the cumulative errors in the pedigree to a certain extent because of cross-mating. The solutions are then used to make a combined selection index according to the weights in Table 3. The combined index is then used to select yearling males, yearling females and older females.

Table 3. *Parameter settings for the simulation*

Number of females	1000
Number of males	167
Females per male	6
Number of years per replicate	11
Number of replicates	100
Proportion of older females	40%
Probability of a yearling female being barren,	
One mating – two matings	20% - 10%
Probability of a yearling female being barren,	
One mating – two matings	10% - 5%
Weights on combined index,	
Litter size – Quality – Body weight	40% - 40% - 20%
Proportion of old females willing to mate	
1st mating – 2nd mating	98% - 98%
Proportion of young females willing to mate,	
1st mating – 2nd mating	95% - 98%
Random culling ratio	17.5%

Results

As shown in Figure 1, the accuracy of the EBVs is higher than the correlation between the dam's phenotype and the kits genotype for both selection index scenarios. The difference between the two index selection scenarios is the effect of having the true sire in the pedigree compared with the assumed sire. A t-test did not reveal a significant difference between the means of the accuracy of the selection index of the two index selection scenarios for litter size or body weight (p = 0.36, df = 99 and p = 0.23, df = 99, respectively). Across all three scenarios, the average amount of kits with false sires in the pedigree was 5% per year. The average number of females mated with the same male in the second mating as in the first mating across all scenarios was 66.8%.

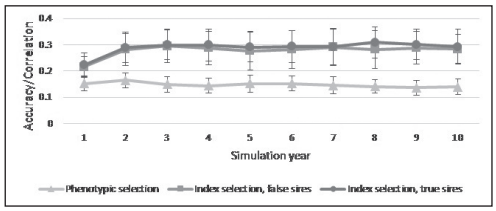


Figure 1. *Accuracy of index selection scenarios and correlation between litter size of dam to additive genetic value of litter size of offspring for phenotypic selection. Standard deviation shown with bars.*

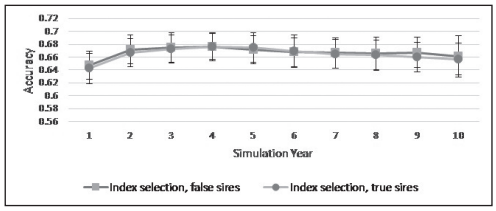


Figure 2. *Accuracy of index selection scenarios for body weight. Standard deviation shown with bars.*
*As shown in figures 3-5, the genetic trend was similar for the two index selection scenarios. There was a significant difference between the means of all traits between index selection and phenotypic selection (p < 1.22*10-9, df = 99).*

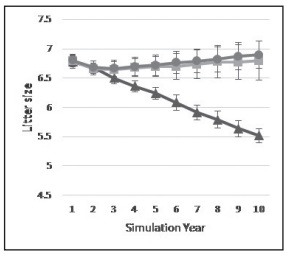


Figure 3. *Trend for litter size per littered female, for the three scenarios.*

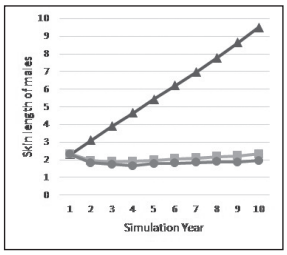


Figure 4. *Trend for the additive genetic value of skin length of males for the three scenarios*

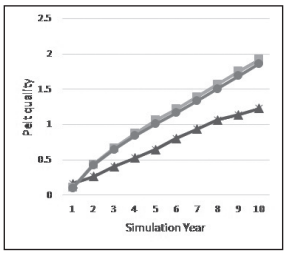
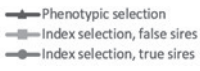


Figure 5. *Trend for additive genetic value of skin quality of the three scenarios legend*



Discussion

As shown in Figure 1, the uncertainty in the pedigree seems to have an effect on the accuracy of litter size, however the difference was not significant. The same was true for body weight. It would be better to use the true variance components for calculation of EBV and increase the number of replicates since the standard deviation of the accuracy was quite high for both scenarios. The accuracy is important in determining the genetic progress by a given selection intensity and genetic variance. Body weight has a very high heritability (Shirali *et. al.*, 2015) and therefore the animal's own phenotype weights more than the information on relatives, contrary to litter size which has a low heritability (Thirstrup *et al.*, 2016b). The outcome of the classical phenotypic selection scenario, shown in Figures 3-5, where animals are selected in an independent stepwise fashion by truncating on phenotypes, indicate that litter size will be difficult to improve or even stabilize with such a breeding design. Index selection outperformed phenotypic selection in litter size and pelt quality, while phenotypic selection outperformed in skin length. The prediction of breeding values for litter size would likely improve if a multitrait BLUP was implemented. This is because the animal's own observations for body weight and quality would contribute to the information on the animal's litter size potential, thereby mitigating the negative effect of uncertain pedigrees (Mrode, 2014).

In conclusion, this study demonstrates the benefit of using an objective decision support tool in deciding management and breeding strategies in a mink farm. Here, we have shown that even when crossmating is used, selection based on a selection index, albeit somewhat wrong, is still clearly better than ignoring pedigree information altogether as in the phenotypic selection scenario.

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New breeding value evaluation of litter size in Finnish blue fox

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Abstract

A major goal in blue fox breeding is better litter size (LS) because of its high economic value. The breeding value of LS was previously estimated with a repeatability animal model for within-farm genetic evaluation, but the new national evaluation system permits the use of more complex models. The objective of this study was to estimate variance components of the first three LS, grading size (gSI) and grading quality (gQU) using restricted maximum likelihood in a multiple-trait animal model. Data included 42,467 animals and a pedigree of 48,743 animals. Heritabilities of the first (LS1), second (LS2) and third (LS3) litter sizes were low: 0.13, 0.18 and 0.17, respectively. Fairly high genetic correlations were observed between gSI and the first three LS (-0.49 to -0.57), and between gQU and the first three LS (-0.39 to -0.56). Genetic correlations between LS1 and the next two LS were 0.76 and 0.74, and between LS2 and LS3 0.95. This confirms that LS1 is quite different from LS2 or LS3, and a multiple-trait model is better for LS than the current repeatability model. The antagonistic correlations between LS and gSI and gQU should also be considered in breeding value evaluation to increase evaluation accuracy.

Keywords: animal model, fecundity, fur animals, genetic correlation, variance components

Introduction

Breeding value evaluation of Finnish blue fox was changed from farm-based to national evaluation in the beginning of 2015. The most significant improvement was that the estimated breeding values became comparable between farms, making the selection of animals more reliable and more efficient. Further, a larger amount of data enables the development of statistical models as well as better consideration of environmental factors. This especially benefits fertility traits like litter size (LS), which has low heritability (Peura et al. 2004). The LS of Finnish blue fox has previously been estimated with a repeatability model for within-farm evaluation. However, recent studies show that it would be useful to take into account the genetic correlations between consecutive LS and grading traits, using a multiple-trait model in breeding value evaluation (Peura *et al.* 2004, Koivula *et al.* 2009, Kempe *et al.* 2010). This is essential because fertility traits in Finnish blue fox have deteriorated significantly during 1988-2002 (Koivula *et al.* 2009). The main reason for the negative trend is that selection has focused on larger animal grading size (gSI), which is known to have an unfavourable genetic correlation with fertility traits (Koivula *et al.* 2009).

The aims of this study were to estimate genetic parameters for the first three LS, gSI and grading quality (gQU), and to develop a multiple-trait animal model for national evaluation which would take into account the antagonistic effects of grading traits on fertility traits. In addition, we examined genetic trends in the studied traits.

Material and methods

The data for variance component estimation were sampled from the national data obtained from Saga Furs Oyj and comprised observations from 42,467 foxes born during 2000-2010 in 10 farms. The pedigree contained 48,743 animals. The structure of the pedigree was monitored by the RelaX2 program (Strandén and Vuori 2006). The selected farms maintained good breeding co-operation and had genetic ties between the animals.

The studied fertility traits were litter size in the first (LS1), second (LS2) and third (LS3) parities. LS was recorded three weeks after whelping. In order to include records from the second and third LS, the female was required to have a record of LS from the previous two years as well. Grading traits were based on live animal grading, which is generally used for indirect selection of pelt character traits, and were recorded by the producer on a scale of 1 (low) to 5 (top rating) with the system used in the Finnish blue fox breeding programme. Grading size was determined subjectively and scored from smallest (1) to largest (5). The recommendation was that the average gSI and gQU should be close to score 3 within farm and year.

Variance components were estimated using the DMU program (Madsen and Jensen 2012), which relies on the restricted maximum likelihood (REML) method. The used multiple-trait linear animal model was:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Wc} + \mathbf{Za} + \mathbf{e}$$

where **y** is a vector of observations, **b** is a vector of fixed effects, and **c**, **a** and **e** are vectors of random non-genetic litter effects, animal genetic effects and residual effects, respectively. Matrices **X**, **W** and **Z** are the corresponding incidence matrices.

In the multiple-trait analyses, the litter (c), animal (a) and residual (e) effects were assumed to be independent, normally distributed random effects with zero mean. The variances were:

$$\text{var}(\mathbf{c})=\mathbf{C_O} \otimes \mathbf{I_q},\ \text{var}(\mathbf{a})=\mathbf{G_O} \otimes \mathbf{A} \ \text{ and } \ \text{var}(\mathbf{e})=\mathbf{R_O} \otimes \mathbf{I_n},$$

where $\otimes$ is the Kronecker product, $\mathbf{C_O}$ is the variance-covariance matrix for litter effects between traits, $\mathbf{I_q}$ is the identity matrix of size **q**, **q** is the number of litter effects, **n** is the number of animals with an observation, $\mathbf{G_O}$ is the genetic variance-covariance matrix between traits, **A** is the additive genetic relationship matrix and $\mathbf{R_O}$ is the variance-covariance matrix for residual effects between traits.

The heritability ($\mathbf{h^2}$) and the proportion of litter variation ($\mathbf{c^2}$) for trait i were calculated as

$$h^2_i=\mathbf{G_O^{i,i}/P^{i,i}} \quad \text{and} \quad c^2 = \mathbf{C_O^{i,i}/P^{i,i}},$$

respectively, where $\mathbf{G_O^{i,i}}$ is the additive genetic variance of trait i, $\mathbf{C_O^{i,i}}$ is the litter variance of trait i, $\mathbf{P^{i,i}}$ is the phenotypic variance of trait i and $\mathbf{P=C_O+G_O+R_O}$. The fixed effects for LS1, LS2 and LS3 were farm-year, number of matings (three classes: 1, 2 or >2 matings/season) and mating method (natural or artificial insemination). In addition, LS1 had age of dam as a fixed effect in the model (three classes: 1, 2, >2 years old). For gSI and gQU, the fixed effects were farm-year, age of dam (three classes: 1, 2, >2 years old) and time of birth of the animal (four classes: 104-129, 130-144, 145-160 and 161-180 days from the beginning of the year). Moreover, the sex of the animal was included for gSI and gQU (three classes: male, female and unknown).

Genetic trends for the studied traits were assessed by examining standardized estimated breeding values (EBVs). EBVs were calculated using the MiX99 program with the same model as used in variance component estimation (Strandén and Vuori 2006). The obtained REML estimates of the variance components were used in MiX99. To make it easier to compare the EBVs of the different traits, they were standardized to mean 100 and standard deviation 10 in the reference population (animals born in 2004-2008).

Results and discussion

Means and standard deviations of the studied LS traits, gSI and gQU are in Table 1. The number of observations was largest for gQU and smallest for LS3. Yearling females produced about 2.6 kits less than 2- or 3-year-old females, which is consistent with the results of Peura et al. (2004). The standard deviation was smaller in LS1 compared to LS2 or LS3. Although the average grading scores for gSI and gQU should be close to score 3 in each farm-year, slightly higher scores are common. The means in our data were 3.86 and 4.07, respectively. Table 1 also gives the heritabilities and litter variance proportions (±s.e.) for the studied traits.

Table 1. *Number of observations (N), mean, standard deviation (s.d.), estimated phenotypic variance (σ2P), proportion of litter variance (c2), heritability (h2) and their standard errors for the first three litter sizes (LS), grading size (gSI) and grading quality (gQU).*

Trait	N	Mean	s.d.	σ^2_P	c^2	h^2
LS1	12,367	5.67	2.95	8.257	0.028±0.014	0.128±0.014
LS2	9,881	8.33	3.40	11.034	0.028±0.018	0.179±0.017
LS3	6,598	8.32	3.38	10.980	0.037±0.026	0.165±0.021
gSI	36,534	3.86	0.78	0.413	0.110±0.006	0.271±0.012
gQU	41,252	4.07	0.73	0.325	0.121±0.006	0.217±0.011

The heritability estimates for LS1, LS2 and LS3 were 0.13, 0.18 and 0.17, respectively. Our estimates are higher than in the study of Peura et al. (2004), where the corresponding heritabilities were from 0.07 to 0.08. The difference may be due to the better quality of data in our study. Furthermore, Peura *et al.* (2004) used a different, single-trait repeatability animal model for LS. The variation in common litter effects was quite low for LS traits, representing only 3 to 4% of the total variance.

In our study, the heritability for gSI was 0.27, which is higher than in the studies of Peura *et al.* (2004, 2005) and Koivula *et al.* (2009), but lower than estimated by Kempe et al. (2010). Hence, there is large variation in the heritability estimates for fox body size, from 0.16 to 0.32 (Peura *et al.* 2004, 2005, Koivula et al. 2009, Kempe *et al.* 2010). Also the heritability estimate for gQU was higher in our study than in the study of Peura *et al.* (2005). The common litter effect explained 11% of the total variation in gSI and 12% in gQU.

Genetic correlations

Genetic and phenotypic correlations and their standard errors for the studied traits are in Table 2. All pairs of trait had at least 5,156 observations. The genetic correlations between LS1 and LS2, between LS1 and LS3, and between LS2 and LS3 were 0.76, 0.74 and 0.95, respectively. These are higher and their standard errors smaller than in the former study of Peura *et al.* (2004). Although the correlations obtained in our study are very high, they show that LS1 is a somewhat different trait than LS2 and LS3. A multiple-trait model should, therefore, be a better option for LS traits than the currently used

repeatability model, which assumes that measurements are from the same trait and further presupposes equal heritability and genetic correlation of unity between all pairs of records (Mrode 2014).

Table 2. *Estimated genetic correlations and their standard errors (above diagonal) and phenotypic correlations (below diagonal) between the first three litter sizes (LS), grading size (gSI) and grading quality (gQU)*

	LS1	LS2	LS3	gSI	gQU
LS1		0.76±0.06	0.74±0.08	-0.54±0.05	-0.56±0.05
LS2	0.19		0.95±0.06	-0.57±0.05	-0.52±0.05
LS3	0.13	0.24		-0.49±0.06	-0.39±0.07
gSI	-0.09	-0.05	-0.06		0.66±0.02
gQU	-0.09	-0.05	-0.05	0.53	

Genetic correlations between the grading traits gSI and gQU and the LS traits were unfavourable and fairly high (-0.39 to -0.57), indicating that larger animals with excellent pelt quality have smaller litters if the main focus in selection is on these grading traits. In their earlier study, Peura et al. (2004) reported lower genetic correlations (-0.23 to -0.40) between gSI and the first three LS, and also higher standard errors than in our study. Antagonistic genetic correlations between gSI and the first three LS have also been reported in mink, but these correlations were somewhat lower (-0.17 to -0.38) than found for blue fox (Koivula *et al.* 2010).

In this study, gSI and gQU showed a high genetic correlation (0.66), whereas Peura *et al.* (2005) reported a corresponding correlation of only 0.23. Given the antagonistic relationship of both gSI and gQU with LS traits, it is important to add these into the multiple-trait model of litter size as correlated traits.

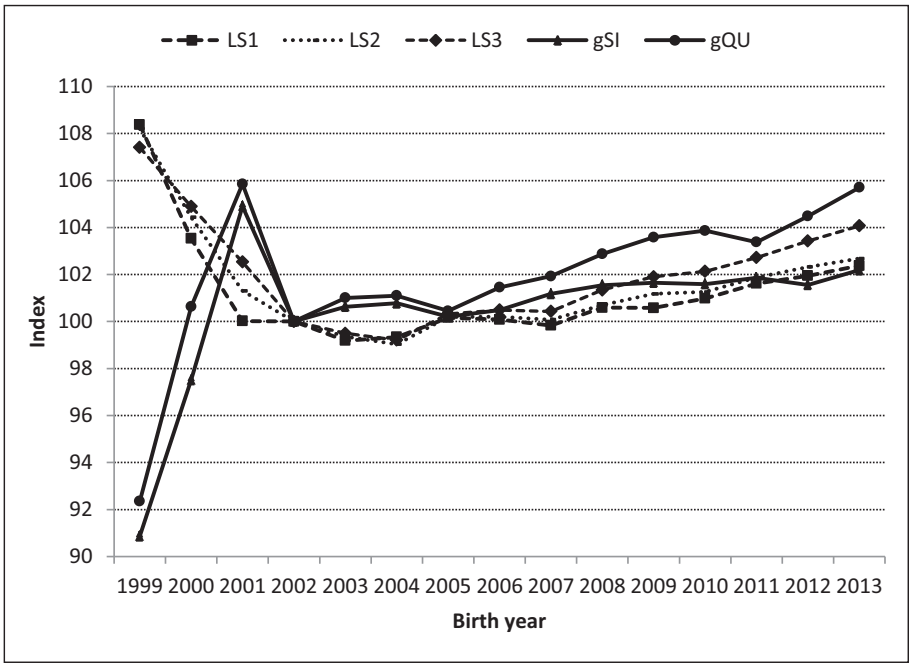

Figure 1. *Average genetic level by birth year according to the mean of the standardized estimated breeding values for the first (LS1), second (LS2) and third (LS3) litter sizes, grading size (gSI) and grading quality (gQU)*

Genetic trends

Figure 1 demonstrates the genetic trends of standardized breeding value estimates for the first three LS and the grading traits. LS1, LS2 and LS3 display a quite similar trend between 1999 and 2013. The negative trend for LS traits turns positive after the year 2003, and from then on these traits show a moderately increasing trend. From 2002 onwards the genetic trend for gSI has been fairly restrained. The sharpest increase in gSI was seen during the years 1988-2001 (Koivula et al. 2009). Grading quality exhibits a similar genetic trend as gSI until 2005, after which the genetic gain in gQU has shown good progress in line with the goals of the Finnish blue fox breeding programme. The main objectives of the programme are to improve the fertility traits and fur quality of the animals and to keep their body size at the current level. Indeed, the genetic trends indicate that the current breeding programme of Finnish blue fox works as expected.

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Cloning, Sequencing Analysis and Prokaryotic Expression of the IGF-1 Gene from mink

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Abstract

This study was carried out to obtain and analyze the sequence of mink IGF-1 gene, and to produce the fusion protein of the IGF-1 in E.coli. By RT-PCR, liver IGF-1 from mink were isolated. The IGF-1 cDNAs encoding 153 amino acids,and showed high degree of homology (>90%) with other reported sequences (gold monkey, lesser panda, giant panda, A. tiger, horse etc) at nucleotide and amino acids levels. Two differences in mink amino acids sequences were found from sequence alignment. Whether these differences affect the molecular conformation or function for the growth factor's processing or these changes in the molecular structure may be responsible for the abnormal ability of growth and reproductive by influencing the function of IGF-1 have not yet been determined. The DNA fragment encoding the mature peptide of mink IGF-1 were subcloned to the pGEX-6P-1 expression vector and highly expressed in E.coli. BL21 with IPTG induction, The expression fusion proteins(pGEX-6P-1-IGF-1)were mostly existed in soluble form and them were about 34KD.The induced cells culture were proved to be active IGF-1 antigens. The mink IGF-1 gene was successfully cloned and expressed. Furthermore,the structure and function of this gene were analyzed and predicted. These result provided a good basis for further biological research activity.

Keywords: mink, insulin-like growth factor-1, clone, prokaryotic expression

Introduction

In the zoological classification, mink belongs to mammals, carnivore, skunk, skunk genera. The main economic value is from the use of fur, the products of which are warm, light and beautiful, with the honor of "the king of fur". In the breeding industry of mink, the level of fur is important factor affecting economic benefit directly, and the size of fur is an important indicator of fur level. So far, there are several ways to improve the size of mink fur, such as enlarging the body type of mink, increasing the extensional ratio of fur and improving the nutritional level of mink. But boosting the express level of IGF-1 is thought to be most effective method for promoting growing and enlarging the body type of mink[Liu 2004; Upton *et al.* 1998] . Therefore, research in IGF-1 gene is significant for breeding industry of mink.

In 1953, Ellise *et al.* found in rat growth hormone cannot act directly on target organism, instead it induces organism to generate some factors, which can accelerate phosphorylation of the cartilage,

consequently mediated growth promoting effect of growth hormone and is named as somatomedin or sulfation factor. Then researcher found these factors are structural homologous with insulin and competitively bind with insulin receptor, furthermore whose function is similar to insulin. So these factors are also called (insulin-like growth factors, IGFs)[Daughaday WH., *et al.* 1987;Froesch ER., *et al.* 1985]. So far, IGF-1 has been demonstrated with a wide range of biological functions, and mainly focused on promoting growth of the bone, insulin-like function, promoting growth and development of fetus, promoting the growth of hair and deposition of protein [Cohik and Clemmons 1993;Jones and Clemmons 1995;Upton 1998]. The IGF-1 is widely expressed in body, and liver was whose uppermost site of expression and synthesis [Lund 1986]. The IGF-1 genes in various species of mammals are highly homologous according to many sequences which have been reported, but the sequence of IGF-1 in mink is unknown. Because IGF-1 is very important for animal growth, study on IGF-1 is necessary for mink breeding industry.

In this research, we cloned and analyzed the cDNA of IGF-1 from liver of mink. By expressing the IGF-1 with biological activity through prokaryotic expression system, the function of IGF-1 in mink can be further understood, which is fundamental for gene function research and application in breeding industry of mink in future.

Materials and methods

Sample collection

In the fur harvesting period, liver of the mink was picked and put into cryogenic vials dealt with DEPC immediately after the mink was dead, then immersed into liquid nitrogen.

Primers

Primers R1 which amplified partial fragment of IGF-1 in mink were designed according cDNA sequence of IGF-1 in dog with Primer Premier 5.0 software. Then the sequence of this fragment was Blast in NCBI. According the conserved region among the high homologous sequences, Primers R2 were designed and R3 was primers for expression (Table 1).

Table 1. *PCR primers for amplification of cDNA sequences of mink IGF-1 gene*

Primer name	Primer sequences	Length of fragment (bp)	Annealing temperature °C
R1	5'TGGTGGACGCTCTTCAGTTCG3' 5'CCTTGGGCATGTCGGTGTG3'	222	58
R2	5' TTGCCTCATTATTCCTGCT 3' 5'CTTCCTACATTCTGTAGTTCTTGT 3'	521	56
R3	5'CGCGGATCCGGACCAGAGACGCTCTGTG 3' 5'CCGGAATTC GCGGACTTGGCAGGCT3'	210	58

Extraction of total RNA

The total RNA from liver of mink was extracted according to the instruction in the kit of total RNA extraction, and was tested with agarose gel at concentration of 1%.

cDNA clone of IGF-1 in mink

Reverse transcription was carried out with two-step method according to the instruction of the kit. After TA cloning PCR product amplified by R1 and R2 primers, the positive clone was sequenced by Sangon Biotech (Shanghai) Co.,Ltd.

Construction of recombinant expression plasmid

Based on the sequence of PCR product amplified by R2, the sequence of IGF-1 mature peptide was identified with BLAST and software of signal peptide prediction.to ensure the right direction of inserting fragment into expression vector, restriction enzyme cutting sites of BamH1 and EcoR1 were added to the 5' and 3' end of primers R3, respectively. After the double digestion with BamH1 and EcoR1, the fragment amplified with primers R3 and pGEX-6p-1 were linked with T4 DNA ligase, then this recombinant expression plasmid was transformed into BL21. 14 h later, the clones were identified with PCR and double digestion, and the positive clones were sequenced by Sangon Biotech (Shanghai) Co., Ltd to make sure the correctness of the insertion direction.

Expression of recombinant protein

In pGEX-6p-1 expression system, the expression of recombinant protein was control by ptac promotor and induced by IPTG. single BL21 colony containing pGEX-6p-1-IGF-1 were inoculated into LB liquid culture medium(Amp+), and cultivated at 37. until OD600 reached 0.6.0.8, then IPTG was added with final concentration of 1mmol/L, continued to cultivate at 37. for 3-5h.

SDS-PAGE and Western blot

1 ml cultivated and induced bacterial liquid was centrifuged and precipitate was collected. Then added 1 x loading buffer to the precipitate and boiled the mixture for 5 min, followed by centrifuge for 1 min. load the sample when supernatant get cooled.

After western blotting at 30mA overnight, blocked the NC membrane with block buffer (TBST added 5% skim milk powder), then washed block buffer off. Incubated NC membrane in primary antibodies for 1h, then washed primary antibodies off. After that, incubated NC membrane in second antibodies for 1h, washed primary antibodies off and colorated horseradish peroxidase (HRP) on NC membrane with DAB.

Results

Clone of cDNA of IGF-1 gene in mink

Product of RT-PCR was electrophoresized in 1.5% agarose gel (figure 1), then the target fragment was recycled and linked with pMD19-T. After transformation, cultivation and identification, positive clone was cultivated in LB culture medium overnight. Then plasmid was extracted and identified with PCR and double digestion (Figure 2 and 3).

Analysis of nucleotide and amino acid sequence

Fragment amplified with primers R1 (Genbank ID: EU869268) was aligned with BLAST on NCBI, whose homologies with lesser panda, golden monkey, giant panda, human, south china tiger, wild horse and dog were 96%, 96%, 96%, 95%, 95%, 95% and 93%, respectively (Table 2). Based on the eight sequences above, primers R2 were designed according the conserved region to amplify the IGF-1 fragment (Genbank: FJ472818). The open reading frame was identified (black body in Figure 4), which encoded 153 amino acid residues. Same result was reached with both Neural network diagram and the hidden markov model (figure 5 and 6, http.//www.cbs.dtu.dk/services/SignalP-3.0). The signal

peptide of IGF-1 was comprised 48 amino acid residues, and the mature peptide contained 70 amino acid residues, molecular weight of which was 7.65 KD and whose pI was 7.76.

Table 2. *Homology comparison of IGF-1 with that of other species in nucleotide and amino levels*

The levels of comparison	Golden Monkey	L. Panda	G. Panda	A. tiger	horse	pig	fox	cattle
The sequence of open reading frame (%)	96	96	95	95	94	92	93	91
The mature peptide coding sequence (%)	97	96	94	95	93	91	92	90
The amino acids of precursor.%.	98	98	98	97	98	96	96	95
The number of amino acids of precursor	153	153	153	153	153	153	153	154

3.3 Expression of mink IGF-1 fusion protein

After recombinant expression plasmid pGEX-6p-1-IGF-1 was identified to be correct with PCR, double digestion and sequencing, recombinant expression plasmid was transformed into BL21 and induced with IPTG. Tricine-SDS-PAGE demonstrated there was specific band at 34 KD (Figure 7), which was equal to the theoretical molecular weight of IGF-1 fusion protein (GST tag was 26KD and mature peptide of IGF-1 was 7.65 KD).

Because of the high similarity of IGF-1 sequence between mink and primate, the rabbit anti-human IGF-1 antibody was as primary antibody. Western blotting demonstrated there was specific band at 34 KD (figure 8) and recombinant IGF-1 had biological activity, which was basis for the further analysis of function.

Discussion

Along with the development of genomics and bioinformatics, research contents in special economic animal becomes constantly rich, and exploring the basic biological characteristics from molecular level is more possible. Carrying out the cloning and research of functional gene in special economic animal has laid the foundation for protecting the resources of special economic animal and developing the special economic animal breeding industry. IGF-1 is an important functional gene concerning growth and differentiation of cell and individual development, therefore the research of IGF-1 gene in mink is significant for the mink breeding industry.

In this research, we cloned the cDNA of IGF-1 gene in mink successfully. Double digestion, PCR and alignment demonstrated the fragment was coding region of IGF-1 gene, and ORF of which was comprised of 462bp base. Alignment with seven amino acid sequences of IGF-1 in mammals indicated that sequence of ORF in mink is highly homologous with golden monkey and lesser panda (both were 96%), the sequence of mature peptide in mink is highly homologous with golden monkey and lesser panda (97% and 96% respectively), and sequence of IGF-1 precursor in mink is highly homologous with golden monkey, lesser panda, giant panda and horse (all 98%).

According to alignment results of amino acid sequences in figure 9, only the 9th amino acid residue of IGF-1 in mink was Asp and in other mammals were all Thr, and only the 24th amino acid residue of IGF-1 in mink was Leu and in all other mammals is Met. So far, we do not know if the two differentions could influence the conformation and even function of IGF-1 in mink, these need the further research.

The mature peptides among nine species aligned were all comprised of 70 amino acids whose sequences were identical. In IGF-1 precursor of mink, there were six conserved Cys (in 54th, 96th, 66th, 109th, 95th and 100th residue respectively). Gly-49 was important for removal of signal peptide, while Glu-51 and Glu-57, Thr-52, Glu-63 and Phy-64 residue participated in interaction process between IGF-1 and IGF-1 BP [Von Heijne G.,1986]. Arg-69 residue and Phe71-Tyr72-Phe73 motif were highly conserved in all mammals, which may play an important role in maintaining the stability of spatial structure of IGF-1 and influencing the activity of combining with IGF-1 R [Hodgson DR.,et al.1996;Cascieri MA.,et al.1988;Nagagawa SH and Tager HS.,1987;Bayne ML.,1990]. Tyr-79 and Arg84-Arg85 mainly participated interaction between IGF-1 and IGF-1 R/ IGF-1 BP [Hu 2005], while Lys-116 was essential for the process of IGF-1 [Stephen *et al.* 2001]. To sum up, the amino acid in mink demonstrated the conservation of IGF-1, which indicated important role in growth and development of biont.

As shown in cladogram (Figure 10), all mammals could be divided into two clusters. Evolutional relationship among mink, lesser panda and golden monkey is close, while evolutional relationship between mink and fox is relatively far.

The result of western blot indicated the recombinant IGF-1 is expressed successfully and has immunological competence. Moriyama et al. 1993 demonstrated recombinant trout IGF-1 expressed by E. coli was activated, which can significantly stimulate the perturbation sulfur capacity of cartilage dose-dependently in the range of 3.9-250ng/mL [Moriyama S., *et al.* 1993; Hua YM and Lin HR.,2001]. Zhang 2005 determined the activity of recombinant gallinaceous IGF-1 in NIH3T3 cells and chicken embryo fibroblast with MTT method, and the result indicated IGF-1 was dose-dependent highly activated at 100~800ng/mL [Zhang 2005]. At present, we are harvesting recombinant mink IGF-1 for the research of function and mechanism of action.

Acknowledgements

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Figure 1. *PCR product of R1*

Figure 2. *PCR product of R2*

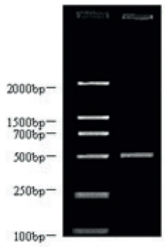


Figure 3. *Identification of recombinant plasmid pMD19-T-IGF-I by double restriction endonuclease digestion*

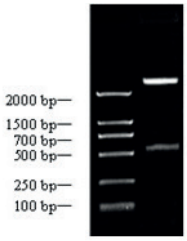


Figure 4. *Nucleotide sequences and extrapolated amino acid sequences of mink IGF-1 cDNA clone*

```
                                                          |→
TTGCCTCATTATTCCTGCTAACCAATTCATTTCCAGACTTTGCACTTCAGAAGCAATGG
                                                          M
GAAAAATCAGCAGTCTTCCAAACCAATTATTTAAGTGCTGCTTTTGTGATTTCTTG
G  K  I  S  S  L  P  N  Q  L  F  K  C  C  F  C  D  F  L
AAGGTAAAGTTGCACATCATGTCCTCTTCTCATCTCTTCTACCTGGCCCTGTGCTT
  K  V  K  L  H  I  M  S  S  S  H  L  F  Y  L  A  L  C  L
                signal peptide      →|→     mature peptide
GCTCACCTTCACCAGCTCCGCGACAGCTGGACCAGAGACGCTCTGTGGGGCTGA
  L  T  F  T  S  S  A  T  A  G  P  E  T  L  C  G  A  E
ACTGGTGGATGCTCTTCAGTTCCTGTCTGCGAGACAGGGTTTTTATTTCAACAAG
  L  V  D  A  L  Q  F  V  C  G  D  R  G  F  Y  F  N  K
CCCACGGGGTATGGTTCAAGCAGTCGGAGGGCACCTCAGACAGGCATTGTGGAC
  P  T  G  Y  G  S  S  S  R  E  A  P  Q  T  G  I  V  D
GAGTGCTGCTTCCGGAGCTGTGATCTGAGGAGGCTAGAGATGTACTGCGCACCC
  E  C  C  F  R  S  C  D  L  R  R  L  E  M  Y  C  A  P
                   →|
CTCAAGCCTGCCAAGTCCGCCCCGCTCTGTCCGTGCCCAGCGCCACACCGACATG
  L  K  P  A  K  S  A  R  S  V  R  A  Q  R  H  T  D  M
CCCAAGGCTCAGAAGGAAGTACATTTGAAGAACGCAAGTAGAGGGAGTGCAGGA
  P  K  A  Q  K  E  V  H  L  K  N  A  S  R  G  S  A  G
AACAAGAACTACAGAATGTAGGAAG
  N  K  N  Y  R  M  .
```

Figure 5. *Signal peptide analysis result of Artifical Neural Network*

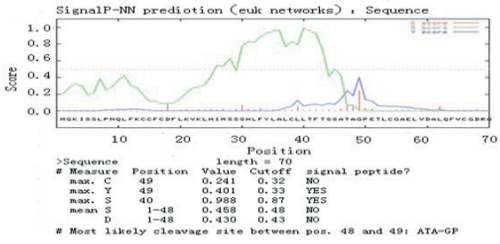


Figure 6. *Signal peptide analysis result of Hidden Maharanobis Model*

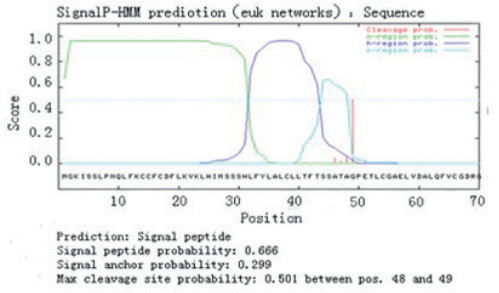
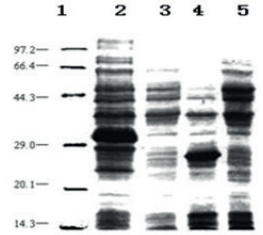


Figure 7. *Tricine-SDS-PAGE analysis of mink fusion protein GST-IGF-1*



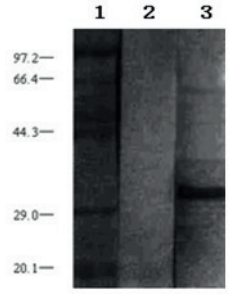
1:protein molecular weight marker

2:Induced strain containing recombinant plasmid

3:Strain containing recombinant plasmid but without inc

4:Induced Strain containing pGEX-6p-1

Figure 8. *Detection of mink rekombinant fusion protein GST-IGF-1 by Western-blotting*



1: protein molecular weight marker

2:Strain containing no recombinant plasmid

3:Strain containing recombinant plasmid

Figure 9. *Homology comparison between mink IGF-1 and other mammals at amino acid level*

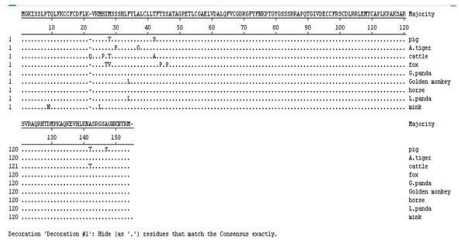
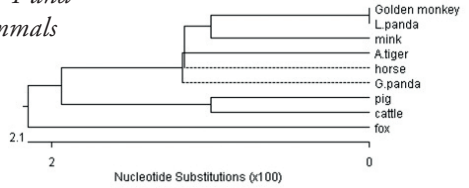


Figure 10. *The phylogenetic tree of the nucleotide sequences of the deduced amino acid sequences of mink IGF-1 and other mammals*



Towards genomic selection – stochastic simulation of alternative blue fox selection strategies

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Abstract

This study compares three strategies for blue fox breeding: a) basic scenario: selection based on BLUP evaluation using pedigree information and current selection criteria, b) selection based of BLUP evaluation using pedigree information and new selection criteria, and c) selection based on genomic selection and new selection criteria. The current selection criteria include animal, pelt and litter size. New selection criteria include animal size, pelt quality, litter size, leg conformation and feed efficiency. In all three scenarios the selection objective was the same and included animal size, pelt quality, litter size, front leg conformation and feed efficiency. Study was done using stochastic simulation with the genetic evaluation done using multitrait model. Genomic selection was simulated using pseudo-genomic simulation with three (low, medium and high) levels of accuracy for breeding value. Both the new selection criteria and genomic selection resulted in higher total economic gain than the basic selection scenario. Also the inclusion of information on feed efficiency and front leg conformation increased the total economic genetic gain. For some traits the genetic gain was negative and therefore adjustment of selection index weights is needed if the genetic gain in all traits should be positive.

Keywords: Genetic gain, rate of inbreeding, stochastic simulation

Introduction

Globally Finland has the highest blue fox production. In the current breeding scheme, selection is done within farm using BLUP breeding values. Finnish fur industry established the first BLUP evaluation schemes in 1990s. At the beginning of 2015 a national database and national breeding value evaluation were launched. The latest development does not use the full potential of the national breeding value evaluation. For example, male selection is still mainly done within farm. Several cattle and pig breeding organizations are using genomic tools in their selection programs. The main difference between conventional BLUP selection and genomic selection is that the conventional evaluation is based on pedigree data whereas genomic evaluation is based on variable DNA markers, mainly single nucleotide polymorphism (SNP) (Meuwissen *et al.* 2001). At the moment there are no genomic selection programs for fur industry. Currently it is possible to screen approximately 1400 SNP in blue fox with commercial SNP chip (Illumina Canine HD BeadChip). Unfortunately this is not enough to build an effective genomic evaluation. Genomic tools are being developed also for fur industry and it is likely that these tools will be part of the future breeding programs. Now the fur industry needs studies on how the breeding schemes should be organized to ensure improved genetic gain with reasonable costs.

At the moment, the most important selection traits in the Finnish blue fox breeding scheme are fertility traits (litter size, conception rate and whelping success), pelt quality and pelt size. There is, however,

increasing discussion whether feed efficiency and leg conformation should be included in the breeding scheme.

The hypotheses of the study were: a) The genetic gain of selection based on traditional BLUP breeding values gives lower genetic gain than selection based on genomic selection, b) Both the selection and use of breeding males across farms gives higher genetic gain than selection and use of males within farms and c) The rate of inbreeding in selection based on traditional BLUP breeding values is higher in selection based on genomic selection.

Materials and methods

To test the hypotheses five different selection strategies were compared. All scenarios included five farms with equal herd size (1352 females and 140 males) and the same mating ratio of 10 females per male. The age distribution of males and females and the litter size of females are presented in Table 1.

Table 1. *Number of breeding females, litter size and number of males in different age classes*

Age (years)	Females		Males
	n (%)	Litter size at birth	n (%)
1	600 (44)	7	92 (66)
2	353 (26)	9	34 (24)
3	200 (15)	9	14 (10)
4	128 (9)	9	-
5	72 (5)	8	-

Five different selection scenarios were compared (Table 2). Scenario I simulates current selection strategy and scenarios II-V simulate alternative selection strategies.

Table 2. *Alternative selection scenarios in the study*

		Selection method information	Traits with breeding value	Accuracy of genomic
	Current traits, selection and mating within farm			
Scenario I		BLUP	3[1]	-
	Scenarios with new traits, selection and mating within farm			
Scenario II		BLUP	5[2]	-
Scenario III	A	Pseudo-genomic	5[2]	0.30
	B	Pseudo-genomic	5[2]	0.50
	C	Pseudo-genomic	5[2]	0.80
	Scenarios with new traits, selection and mating of males across farms			
Scenario IV		BLUP	5[2]	-
Scenario V	A	Pseudo-genomic	5[2]	0.30
	B	Pseudo-genomic	5[2]	0.50
	C	Pseudo-genomic	5[2]	0.80

[1]Animal size (scale 1-5), pelt quality (scale 1-5), litter size at birth, [2]Same as [1] and front leg conformation (scale 1-5) and feed efficiency (g growth / kg DM feed).

The genetic parameters and economic values used in the simulations are presented in Table 3. Stochastic simulation with 10 years of selection with 50 replicates using ADAM software (Pedersen *et al.* 2009) was used to study alternative breeding scenarios. Breeding value evaluation was done using multi-trait animal model with DMU software (Madsen *et al.* 2006). In scenarios III and V pseudo-genomic (Buch *et al.* 2009) selection was used. In both these scenarios three accuracies of direct genomic breeding value were compared. Moreover, in all pseudo-genomic scenarios 50 % of male pups were genotyped. The total gain (EUR), genetic gain in each trait and the rate of inbreeding were estimated.

Table 3. *Economic values (EUR/product unit), heritability (h2), and genetic correlations (off diagonal)*

	EUR /unit[a]	h^2	Pelt quality	Litter size	Front leg conf.	Feed efficiency
Animal size	8.43	0.32[d]	0.17[d]	-0.10[c]	-0.51[c]	-0.09[c]
Pelt quality	26.08	0.28[d]		-0.05[e]	0.00	0.05[b]
Litter size	14.91	0.12[f]			0.00	0.00
Front leg conf.	0.00	0.22[c]				-0.11[c]
Feed efficiency	0.40	0.25[b]				

[a]Peura *et al.* 2016, [b]Kempe *et al.* 2013, [c]Kempe *et al.* 2010, [d]Peura *et al.* 2005, [e]Peura, Unpublished data, [f]Peura *et al.* 2007.

Results

The genetic gain in the traits included in the selection objective, total genetic gain (EUR) and rate of inbreeding in alternative selection scenarios are presented in Table 4. Scenarios with pseudo-genomic selection resulted in the highest total gain in euros. Litter size, which has low heritability, did benefit most from the inclusion of genomic information whereas especially in animal size the genetic gain decreased. In scenarios where feed efficiency was included in the phenotypic information, the genetic gain of feed efficiency was clearly higher that when there was no recording on it. In all scenarios the genetic change of front leg conformation was negative. In other words, front leg conformation is impaired.

Table 4. *Genetic gain and rate of inbreeding / year in each selection scenario*

			Genetic gain					
		Total gain (EUR)	Animal size[a]	Pelt quality[a]	Litter size[b]	Front leg conf.[a]	Feed efficiencyc	Rate of inbr. / year
Scenario I		8.5	0.13	0.15	0.23	-0.05	0.09	0.017
Scenario II		9.5	0.10	0.14	0.21	-0.08	4.92	0.015
Scenario III	A	10.4	0.09	0.14	0.24	-0.08	4.95	0.011
	B	11.8	0.10	0.14	0.22	-0.08	4.86	0.007
	C	14.6	0.09	0.14	0.28	-0.08	4.79	0.004
Scenario IV		9.8	0.09	0.13	0.38	-0.08	4.66	0.004
Scenario V	A	10.7	0.08	0.13	0.59	-0.07	4.48	0.003
	B	12.0	0.09	0.14	0.28	-0.08	4.75	0.002
	C	14.7	0.09	0.13	0.40	-0.08	4.71	0.001

[a] Scale 1 to 5, [b]Number of pups [c]g growth / kg DM

Rate of inbreeding was higher in scenarios where selection was done within farm than in scenarios where selection was done across farms. Moreover, scenarios, which were based on BLUP selection, resulted in higher rate of inbreeding than scenarios that used also genotyping information.

Discussion

According to this study, the best economic results are achieved with selection strategies that use genomic information. However, we assumed that 50 % of male pups are genotyped, which is a higher proportion than usual for other species. In the next stage of the study the effect of lower proportion of genotyped pups will be tested. We also assumed that the number of females / male is the same both in across and within farm scenarios. However, it is likely that in across farm scenarios some males are used more intensively. This may also be the reason why the difference in total gain between across and within farm scenarios was small. Different number of mates / male will be tested in the next stage of the study.

The negative genetic gain in front leg conformation was caused by the used unfavorable genetic correlations between front leg conformation and animal size and feed efficiency, and the economic value of zero for front leg conformation used in the simulation. It is difficult to calculate an economic value for front leg conformation. However, if front leg conformation is preferred to remain stable or improved then a substantial economic value is needed if the correlations used in this simulation are true.

Accuracy of genomic breeding values had a clear effect on the results. In this study it was simply assumed that all the traits had the same accuracy in genomic breeding values. In real life this is not the case, but since the accuracy of genomic breeding values with real data is unknown, low (0.30), intermediate (0.50) and high (0.80) accuracies were used to see the effect of accuracy on the results.

To conclude, all the hypotheses of the study could be accepted. According to this study, the best gains are achieved with a selection scheme where selection of males is done across farms and genomic information is used. The next stage of the study is to compare the costs of alternative selection scenarios and compare them to economic benefits of each scenario.

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18 generations of mink selection for defensive reaction towards man. What are the consequences?

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Abstract

This paper considers the main results of the long-lasting experimental domestication of mink *(Neovison vison)*. The following important conclusions have been made. Fundamental to the domestication process is the intensive selection of animals for human-tolerant behavior and the capability to adapt to the emerging social structure "man – domestication object". Intensive selection for behavior and, therefore, for the central regulatory systems, which controls the function of the entire organism, leads to large amounts of variability in the population under domestication. Stress caused by rapid environmental changes, with its neurohormonal mechanisms of regulation of genetic processes, has an important role in the induction of variability. These considerations prompted Dmitry Belyaev to single out the kind of selection that accompanies domestication into a category of its own, which was termed "destabilizing selection". Under stabilizing selection, mutational variability is rendered neutral. Under directional (and destabilizing) selection, hidden mutational variability becomes exposed. If the regulatory circuits with negative feedback are lost, hidden genotypic variability becomes exposed and individuals with major phenotypic aberrations occur.

Keywords: American mink, *Neovison vison*, selection for behavior, domestication

Introduction

Animal domestication started out about 15 thousand years ago and in fact marked a transition from hunting and gathering to a new period of human evolution, the modern civilization. The development of the modern society proceeded side by side with the emergence and inclusion of domesticated animals in human work activity and civil life, and the importance of these processes for human welfare is still as great. At the same time, the genetic mechanisms of domestication remain to be poorly studied. Pondering the matter of animal domestication, Charles Darwin assumed that domestication is a result of selection, which can be seen as a model of evolution (Darwin, 1868). It is therefore no wonder that the reconstruction and study of the history of domestic animals may represent quite an efficient tool for studying the main factors and mechanisms of the evolutionary process. These considerations made Dmitry Belyaev conceive and initiate in the 1950s a daunting experiment on the domestication of farmed silver foxes *(Vulpes vulpes)* – one of the century's most intriguing systematic investigation of the nature of evolutionary processes (Belyaev, 1969, 1979).

Performed on different animal species, selective breeding experiments allowed Belyaev and his co-workers to identify and state some of the common patterns of the evolutionary process that runs under strong selection acting to induce and fix the so-called domesticated type of behavior. The important result was the creation and development by D. Belyaev of the concept of a destabilizing function of selection for domestic behavior, the feature that marks the function of the main neuroendocrine

regulatory systems, and about the role of stress responses in the evolutionary process.

Looking at the data obtained from this long and extensive experiment on the domestication of mink – the following conclusions can be made:

1. The key element in the domestication process is the intensive selection of animals for tame behavior.
2. Intensive selection for behavior during the domestication of the wild animal leads to considerable rearrangements of the most basic nervous pathways fixed in the instincts. This involvement of the central regulatory systems in selection explains the specific character of evolutionary transformations that go along with domestication.
3. The main feature of the evolutionary process that leads to animal domestication at first stages in a burst-like exposure of variation in the population under domestication. Noteworthy, this variation often appears to be non-stochastic, which suggests that it has always been there, albeit in a hidden form, and has, under selection for the central mechanisms of homeostatic regulation, eventually "gone phenotypic", as Belyaev put it. Disruptions of homeostatic regulations during domestication occur physiologically, genomically, and in the regulation of morphogenetic processes during ontogenesis. These considerations promoted D. Belyaev to single out the kind of selection that accompanies domestication into a category of its own, which was termed "destabilizing selection".
4. The most important component and mechanism of destabilizing selection is stress response, which serves as a factor of mobilization of hidden genetic variability and its induction by exerting influence on various genetic processes such as mutagenesis, recombination, mobilization of mobile genetic elements and others. 5. A special place in the emergence of rapid evolutionary transformations during domestication should probably be given to hereditable epigenetic regulations. The study of this mechanism of genetic variability is now widely considered as being of critical importance. Stress, too, can act as the main mechanism that triggers on epigenetic regulations of genome function.

Because Belyaev had a great interest in the effects that selection for domesticated behavior might produce on animals in different taxonomic groups, he initiated a selective breeding experiment on the American mink (Neovison vison) bread on the Experimental Farm of the Institute of Cytology and Genetics, with the aim to induce and fix non-aggressive and aggressive behavior towards humans (Trapezov, 1987, 1997 a, b, 2000, 2013). These work are still ongoing.

Materials and methods

The selection of farm-bred mink for defensive behavior towards human had been realized for 18 generations. Fig. 1 and 2 demonstrates two lines by selection in the opposite directions of two lines of tame and aggressive standard (+/+) minks for 18 generations. The main effect of selection for aggressiveness to humans has been obtained already in third generation. Course of selection for tameness has multiple pattern – graph has sinuous view.

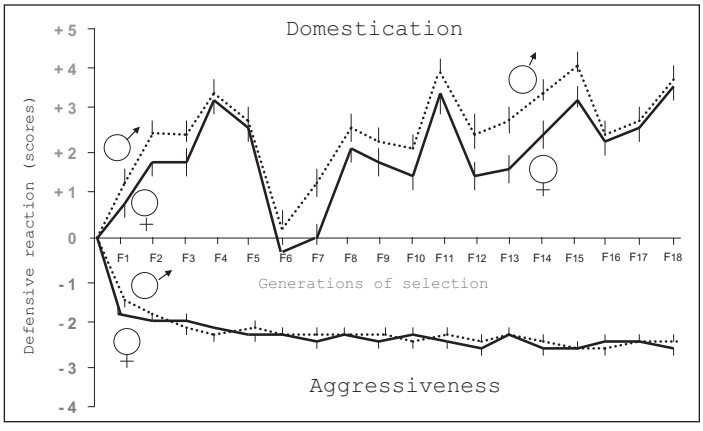

Figure. 1. *Changes in mean behavioral score in the populations of minks under selection for domesticated and aggressive behavior for 18 generations.*

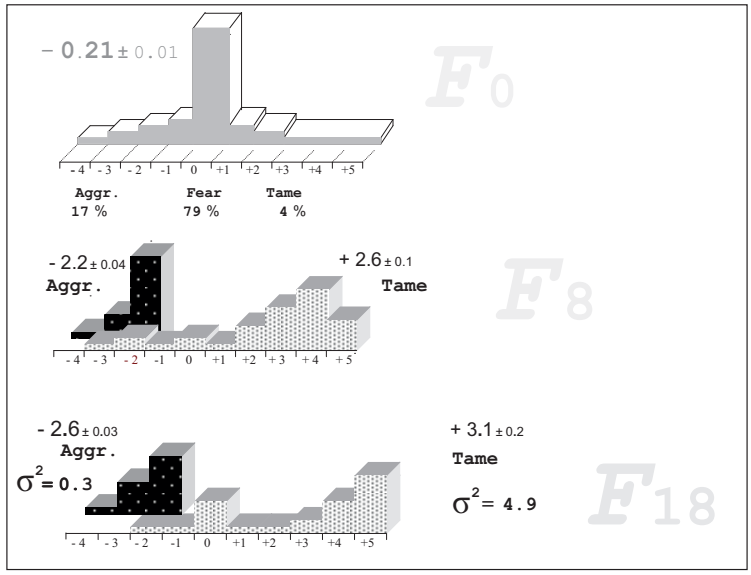

Figure 2. *Reorganization of the defensive responses to human in mink in the course of the breeding program designed to the study the effects of selection on aggression and tameness.*

Results and discussion

As can be seen from Fig. 1, 2 the course of selection in two opposite directions is different. The main effect of selection for aggression becomes observable over the first two generations, with little variation in the mean afterwards. Selection for domesticated behavior is not that easy: the curve is saw-toothed in shape. Additionally, in contrast to the animals under selection for aggression, those under domestication had throughout 15 generations under selection sex-dimorphic tame behavior (males were tamer than females on average ($p < 0.001$)). These results demonstrate the efficiency of that selection first of all. What is even more important, the value of the phenotypic variance of the trait under selection, behavior, was 10 times as high in the population under domestication as in the population under selection for aggression. The effort resulted in two mink populations with different defense responses to humans. A rapid divergence of the populations under selection suggests that the presence of aggressive responses in the original mink population was likely to be controlled by a small number of loci with a strong additive effect.

A complex pattern of change of the mean and phenotypic variance of the trait was demonstrated. The phenotypic variance is higher under selection for domesticated behavior than under selection for aggression. One of the first correlated responses in the American minks under selection for domesticated behavior was, as in many other animals domesticated previously, increase in depigmentation (white spots). By contrast, the correlated response under selection for aggression was epidermal and hair hyperpigmentation (Fig. 3).

As was demonstrated by molecular-genetic analysis, the hyperpigmented animals have large duplications of genomic regions with various genes in them. One of them produces the neuropeptide neurotensin, which has relevance to aggressive behavior. It can be hypothesized that aggressive behavior can be enhanced in animals that possess additional copies of this gene. Additionally, this duplication contains the KITLG gene implicated in pigmentation, which may account for hyperpigmentation in aggressive animals. Individuals with color phases never seen in the original population were observed to occur in the group under domestication at a frequency of 10–3. A hybridological analysis of these phenotypic innovations demonstrated that they are semidominant (Figs. 4, 5).

Figure 3. *Changes in pigmentation during the selection-driven transformation of behavior in minks: (a) a control animal, which was under no special selection for behavior; (b) black-and-white minks following selection for tame behavior have well-observed domestication markers; (c) epidermal and hair hyperpigmentation in the minks under selection for aggressive behavior.*

Figure 4. *The depigmentation pattern in a new coat color form, Silvery (S^x/S^x), is a monogenic trait.*

Figure 5. *A new coat color form, Black Crystal (CR/CR), is a monogenic autosomal trait.*

Selection for behavior was accompanied not only by the emergence of new color phases, but also by morphological aberrations. Minks with helically curled tails were observed to occur (Fig. 6). The genetic control of that trait is complex and has yet to be studied.

Figure 6. *Helically curled tail.*

It can be concluded that color and morphological innovations emerge due to selection for domestication. Not a single case of the color aberrations as described above has been recorded in the control population, which was under no special selection for behavior.

The experimental material let us know that the genes acted upon by selection for domesticated behavior possess a function that influences developmental rates. The experiment on the selection-driven transformation of behavior in American minks in two opposite directions – towards aggression and towards domestication – demonstrated that eye-opening was an early event in the former case and belated in the latter. The difference in eye-opening between young aggressive and tame minks is 4.1 days ($p<0.001$). The prolonged maintenance of this juvenile feature during early postnatal ontogenesis in the minks under domestication should be regarded as a case of neoteny.

Acknowledgements

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The mink embryo and embryonic diapause

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Abstract

Embryonic diapause is an evolutionary strategy where the embryo undergoes an arrest in development. In the American mink, diapause begins at the blastocyst stage, approximately six days after fertilization. In this species, the regulation of diapause and reactivation is influenced by photoperiod, which then acts to regulate the secretion of pituitary prolactin. Prolactin in turn regulates ovarian steroid function. Reciprocal embryo transplant studies indicate that embryonic arrest is conferred by lack of specific factors necessary for continued development. Global gene expression analysis has revealed reduced expression of a cluster of genes that regulate the abundance of polyamines in the uterus during diapause in the mink. In addition, in the mink, in vivo inhibition of the ornithine decarboxylase-1 (ODC1), the enzyme that regulates conversion of ornithine to the polyamine, putrescine, induces a reversible arrest in embryonic development and an arrest in both trophoblast and inner cell mass proliferation in vitro. We studied whether polyamines can mitigate escape from embryonic diapause by incubation of mink embryos in diapause in vitro in the presence of putrescine at 0.5, 2 and 1000 µM concentrations. Embryo survival in culture was increased three fold by the lowest and highest doses of putrescine. We also observed that we could induce the embryos to escape from diapause by treatment with putrescine. Resumption of development was confirmed by increases in embryo volume, embryo cell proliferation, and by hatching from the embryonic capsule. Control embryos remained in the diapause state. To determine whether the uterus was the target of the pituitary prolactin that terminates diapause, mink uterine epithelial cells were treated with varying doses of this hormone. We observed that prolactin induced the expression of ODC1 in the uterus, thereby regulating uterine polyamine levels. In summary, our findings indicate that the polyamine, putrescine, whose synthesis is under the control of pituitary prolactin, is the uterine factor whose absence is responsible for embryonic diapause in this ranch mink. These findings may lead to treatment regimes that increase embryo survival, and therefore litter size on mink farms.

Keywords: mink, embryo, diapause, prolactin, polyamines

Introduction

The American mink, *Neovison vison*, exhibits an obligatory diapause, breeding in early to mid-March (Murphy and James, 1974). Following fertilization, the mink embryo takes approximately six days to form a blastocyst, at which time it undergoes a period of near arrest of the cell cycle and exhibits low metabolic activity, lasting from a few days to two or more weeks (Llerena-Vargas, 2011). At the vernal equinox (21st March in the Northern hemisphere), the decreasing duration of nocturnal melatonin secretion resulting from the increasing photoperiod permits secretion of prolactin from the pituitary (Lopes *et al*., 2004). The first signs of reactivation of the mink blastocyst are observed three days later by an increase in mitoses and protein synthesis, followed by expansion at day 5, with implantation oc-

curring after a further 8 days (Desmarais *et al.*, 2004). Papke et al. (Papke *et al.*, 1980) first showed that treating mink with prolactin during diapause results in precocious termination of embryonic delay, whereas treatment with dopamine agonists prevents implantation. And prolactin, acting as a luteotropin (Murphy and Rajkumar, 1985), is the main factor essential for both reactivation from embryonic diapause and embryo implantation (Murphy *et al.*, 1981). In other mammalian species, progesterone, estradiol or a combination of both, are the factors responsible for implantation. However, in the mink, neither progesterone nor estradiol can reactivate the diapause blastocyst, despite progesterone secretion increases at reactivation and elevation in the levels of both progesterone and estradiol around implantation (Murphy *et al.*, 1981, Stoufflet *et al.*, 1989). It has been shown that the mink uterus has receptors for prolactin (Rose *et al.*, 1983) but the role of this hormone in induction of implantation at the uterine level has not been explored.

Our recent study demonstrated that a class of low molecular weight polycations, the polyamines, regulate embryonic diapause in the mink (Lefèvre *et al.,* 2011b). In de novo synthesis, putrescine can be produced either from the amino acids arginine or proline through ornithine by the highly regulated, rate-limiting enzyme ornithine decarboxylase (ODC1), spermine and spermidine can then be formed from putrescine via methionine and adenosylmethionine decarboxylase 1 (AMD1) (Wallace *et al.*, 2003). The polyamines are essential for cell growth and can interact with nucleic acids, hence they have a plethora of biological actions, including a role in embryonic development (Igarashi and Kashiwagi, 2010, Lefèvre *et al.*, 2011c). In the mink, polyamine related genes and polyamine content in the uterus are significantly lower during diapause (Lefèvre *et al.*, 2011a). ODC1 is up-regulated during the reactivation phase and preventing polyamine synthesis by treating in vivo with an inhibitor of ODC1 activity, difluormethylornithine (DFMO) during reactivation returns the blastocyst to a diapause-like state (Lefèvre *et al.*, 2011b). However, there is no information yet about whether polyamines have any direct role in reactivating the diapause blastocyst in the mink, nor what the proximal signal is that initiates their activation.

We explored the endocrine control of the reactivation process from diapause in the mink and examined expression of the prolactin receptor (PRLR) in the uterus(Fenelon et al., 2016). To determine whether prolactin regulates ODC1 expression in the uterus, thereby regulating uterine polyamine levels, mink uterine epithelial cells were treated with different doses of prolactin and examined for ODC1 expression. We collected embryos in diapause and cultured them in vitro with different doses of putrescine. We also treated a fourth group with arginine, to determine whether the diapause blastocyst is able to synthesise polyamines from arginine via ODC1. Finally, we treated an inner cell mass (ICM) cell line to determine the effect of putrescine and DFMO on cell proliferation.

Materials and Methods

Tissue collection and treatment

Female mink were bred to two fertile males according to usual farm mating practices. Embryo reactivation was synchronized among females by daily injection of 1 mg/kg/d ovine prolactin (Sigma-Aldrich, Oakville, Canada) beginning on the 21st of March and for the following 14 days, as previously described (Murphy *et al.*, 1981). Uterine horns from mated females were collected before diapause (4 days after the final mating), during diapause (7–9 days after the final mating and before March 21) and after prolactin-induced reactivation on d 3, 5, 9, with implantation and non-implantation sites collected on d 13 (n= 5 mink per stage). Embryos in diapause were flushed from the uterus using pre-warmed TC-199 medium (Life Technologies, Inc., Burlington, Canada) containing 10%

(vol/vol) fetal bovine serum (Invitrogen) and cultured in M16 medium (M7292, Sigma-Aldrich). Diameters of recovered blastocysts and the observation of blastocyst expansion after putrescine treatment were measured by ocular micrometer.

Quantitative RT-PCR in mink uterine tissue and mink uterine epithelial cell line

Total RNA was isolated from uteri with the RNeasy Mini Kit (Qiagen) and DNase-treated using DNase I free (Ambion, Austin, TX, USA) according to the manufacturer's instructions. An aliquot of 1 µg of total RNA was reversed transcribed in a 20 µl reaction using SuperScript III kit (Invitrogen, Carisbad, CA, USA) with oligo(dT) priming, according to the manufacturer's instructions, and diluted 1:20 with nuclease free water. Quantitative amplification of cDNA (qPCR) was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) with 500 nm primers using the Bio-Rad provided plastics and reactions run in triplicate on an CFX96 Real-Time System with a C1000 Touch Thermal Cycler (Bio-Rad) and results analysed using the CFX Manager software. The mink genome has been partially sequenced, hence, where available, primers were designed based on homologous sequences. If no mink sequence was available, cross-species primers were designed based on sequences from either ferret or dog. Primers were initially chosen based on their lack of secondary structures and their specificity.

Immunocytochemistry of prolactin receptors in mink uterine tissue

Paraffin cross-sections of uterine tissues were used to localize PRLR expression before diapause, during and at reactivation from diapause. Briefly, after deparaffinization and hydration, the sections were blocked in 5% (wt/vol) BSA at room temperature for 1h. After blocking the tissues were incubated overnight at 4°C with mouse prolactin receptor antibody (20 µg/ml, ab2772, Abcam), in a humid chamber. After three washes in PBS containing 0.1% Tween 20, sections were incubated for 1 h with goat anti-mouse (1:300 115-165-146, Jackson laboratory) cy-3 secondary antibody. Nuclei were counterstained with DAPI (D9564, Sigma-Aldrich) and slides were mounted in Permafluor (Thermo Fisher Scientific). Negative control sections were submitted to the same procedures, except that the first antibody was replaced by blocking solution or the relative IgG control at the same concentration as the primary antibody. The specificity of all antibodies was confirmed by the lack of staining in the IgG controls.

In vitro culture of mink uterine epithelial cells

The immortalized mink uterine epithelial cells, previously established in the laboratory (Moreau *et al.*, 1995) were cultured in phenol-red free DMEM-F12 (21041-025, Invitrogen), 10% heat inactivated steroid stripped FBS (Invitrogen), 10 µg/ml insulin (I1882, Sigma-Aldrich), 100 U/ml penicillin G/100 µg/ml streptomycin (15140-148, Gibco], and 1.25 µg/ml fungizone (15290-018, Gibco). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 in air. The uterine epithelial cells were treated with either 100 ng/ml, 250 ng/ml or 500 ng/ml prolactin and the cells collected in a time-dependent manner with groups harvested after either 0h, 1h, 3h, 6h, 9h and 24h or 0min, 15 min, 30 min and 45 min of prolactin treatment.

Embryo culture

Blastocysts in diapause were collected by uterine flushing and randomly allocated to treatments that consisted of one of three doses of putrescine (0.5 µM, 2 µM and 1 mM, D13208 Sigma-Aldrich) or a single dose of arginine (10 mM, Sigma-Aldrich) based on doses used in previous studies (Cui and Kim, 2005, Lefèvre *et al.*, 2011a). All chemicals were diluted in M16 media. For each treatment, embryos were cultured in groups of 4-5 in 200 µl M16 medium in 5% CO2 and 95% air at 37°C for a period of ten days. Medium was changed every two days and embryo diameters were measured by ocular micrometer after 5 days. Embryos were observed and measured until their death as determined by the

occurrence of embryo collapse and the onset of opacity of the cells. All the experimental treatments included 4-6 embryos each and were repeated three times except the group, putrescine 1 mM, which was repeated twice, due to scarcity of mink embryos.

In vitro culture of inner cell mass cells (ICM)

Cells from an immortalised ICM cell line previously established in the laboratory (Desmarais *et al.*, 2004) were cultured in a mixture of media from mouse embryonic stem (ES)-cell medium conditioned medium (30%) and M16 medium (70%) with leukemia inhibitory factor (LIF, Sigma Aldrich, 1 µg/ml). The medium was changed daily. In cultures that proliferated, cells were passaged at confluence by scalpel or by trypsinization (0.25% trypsin EDTA supplemented with 10% chicken serum; Gibco). To examine the effect of putrescine and DFMO on cell proliferation the ICM cells were treated with or without putrescine (0.5 µM or 1 mM) and DFMO (10 mM) based on doses used in a previous study (Lefèvre et al., 2011b). DFMO was kindly provided by Dr. Patrick Woster (Wayne State University, Detroit, MI, USA).

Cell Proliferation assay

Evaluation of cell proliferation in diapause blastocysts and the inner cell mass (ICM) cell culture was carried out using the Click-iT EdU imaging kit (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. Briefly, blastocysts and ICM cells were incubated overnight at 37°C with 100 µM of EdU diluted in M16 medium or ICM cell medium, respectively. After fixation in 3.7% formaldehyde and permeabilization of the samples in 0.5% Triton X-100, EdU was detected by incubation of the samples in the Click-iT reaction cocktail, which included CuSO4 and the Alexa Fluor 595 azide. Nuclei were then counterstained by Hoechst 33342 before mounting the slides using the mounting medium Permafluor (Thermo Fisher Scientific, Fremont, CA). The same staining was used to demarcate the apoptotic blastocysts as evident from the fragmented nuclei compared to the normal blastocysts.

Statistical Analyses

Data are expressed as mean ± standard error. Differences in embryo survival, diameter and proliferation rate among groups was determined by one-way analysis of variance (ANOVA) and if significant, post-hoc analysis was performed using the Student Newman-Keuls test. For the immunoblot and qPCR analysis, a Shapiro-Wilks test was performed to establish normal distribution, if the distribution was skewed, the data were either arcsine (immunoblot) or log transformed (qPCR) for analysis and normality confirmed by Shapiro-Wilks test. The data were then analysed by either one-way (qPCR) or two-way (immunoblot) ANOVA, with individual comparisons of means compared using Tukey contrasts (only if ANOVA was significant). A probability level of $p<0.05$ was chosen as the measure of significance.

Results

In other mammals with diapause, progesterone and/or estradiol are the key regulators for embryo reactivation and implantation (Fenelon et al., 2014), whereas in the mink, prolactin is the proximal signal and neither progesterone nor estradiol are able to sustain reactivation, despite both being present around reactivation (Stoufflet et al., 1989, Lagerkvist et al., 1992). It was therefore of interest to explore the gene expression and protein localisation of PRLR in the mink uterus. The abundance of PRLR mRNA was low during diapause and early reactivation but increased significantly at d9 of reactivation (Figure 1). In contrast, PRLR protein was consistently expressed in the luminal and glandular epithelium throughout all stages, with strongest expression before diapause (Figure 2). At d9 of reactivation, specific focal expression in the glands was also observed (Figure 2L) whilst at implantation sites, staining was strongest on the edge of the luminal epithelium (Figure 2).

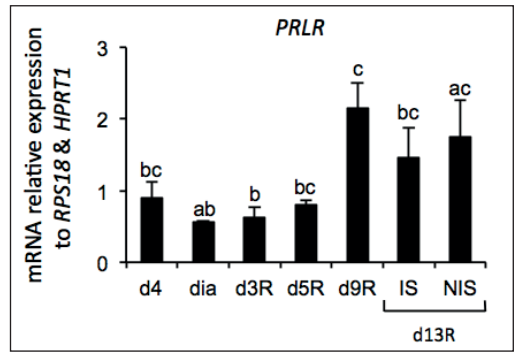


Figure 1. *The abundance of mRNA of the prolactin receptor (PRLR)) in the mink uterus during the stages of diapause, reactivation and implantation. The PRLR signal was low during diapause and early reactivation but increased significantly at d9 reactivation. (From Fenelon et al. 2016)*

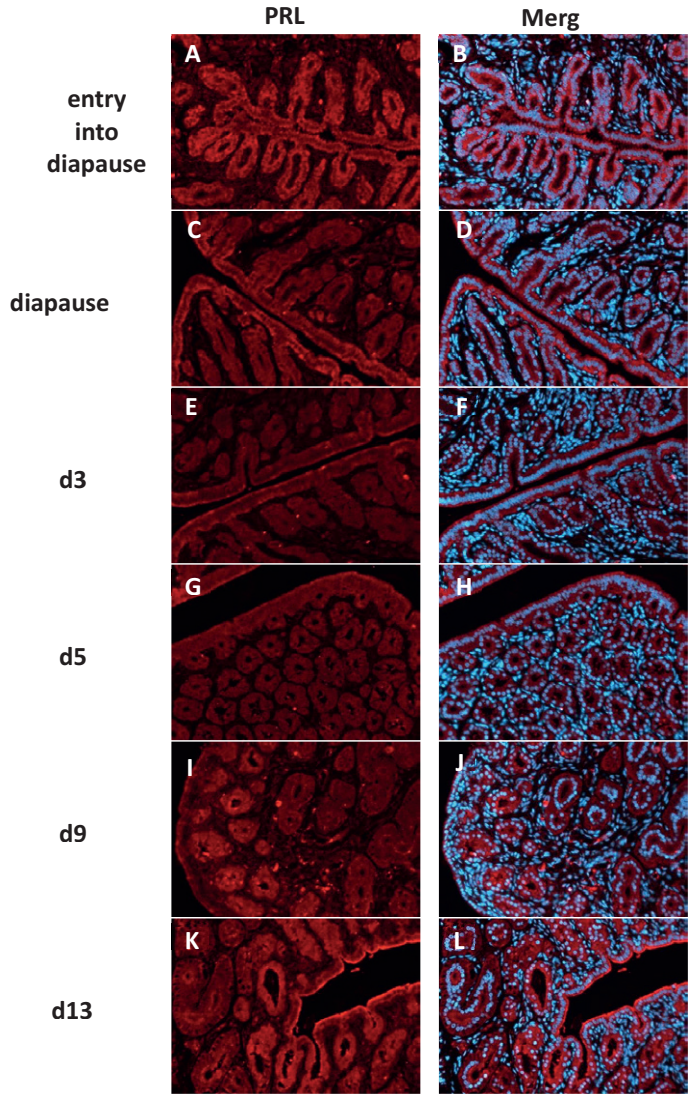
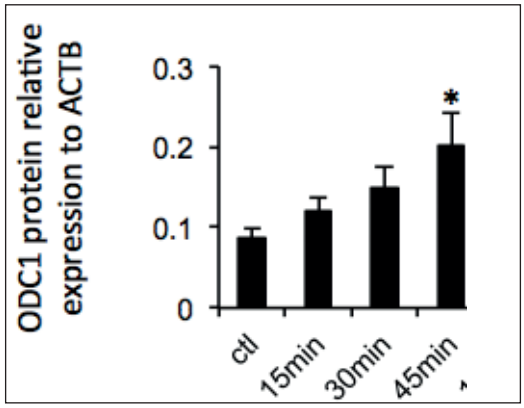
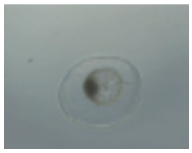


Figure 2. *Immunolocalization of the prolactin receptor (PRLR) in mink uterine cross-sections during diapause, after reactivation of the embryo at d3, d5 and d9 and at d13 in the implantation site. PRLR expression at the protein level was nearly undetectable in the uterus during diapause whereas it increased during d3 after embryo reactivation in the endometrial luminal and glandular epithelium. A similar distribution of PRLR protein in cross-sectioned uteri collected on d5, and d9 after reactivation of blastocysts was observed. However, in d13 implantation sites (IS) after reactivation the expression was higher in the luminal epithelium. (From Fenelon et al. 2016)*

To explore the role of PRLR in the uterine epithelium, we cultured mink uterine epithelial cells with or without prolactin (100 ng/ml, 250 ng/ml or 500 ng/ml) and measured subsequent ODC1 expression. Over a long (0-24h) time period we found no effect, regardless of prolactin concentration. However, over a short time period (0-45 min), ODC1 protein expression increased in the mink uterine epithelial cells in a time dependent manner, being most intense after 45 min of prolactin treatment in comparison to the control (Figure 3).

Figure 3. *Protein expression of ornithine decarboxylase 1 (ODC1) in the mink uterine epithelial cells after culture with or without prolactin (250 ng/ml) in a time dependent manner (0 min, 15 min, 30 min and 45 min respectively). (From Fenelon et al. 2016)*





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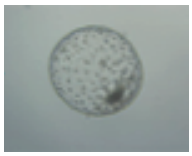


19 Mar

Figure 4. *Mink blastocyst collected during diapause (March 18) and cultured in vitro. Soon after immersion in culture medium, the embryos collapsed (upper panel), but they expanded to fill their capsule within 24 h (lower panel).*

Figure 5. *Mink embryos recovered in obligate diapause and cultured either in presence of vehicle (control), or different doses of putrescine (0.5 µM, 2 µM, 1 mM) and arginine (Arg, 10 mM) respectively. A: Putrescine treated blastocysts were enlarged in comparison to the control and arginine treated embryos. B: After five days of culture the percent of blastocysts treated with putrescine was elevated relative to control or argenine treated blastocysts. C: The mean diameter of the blastocysts treated with putrescine increased gradually over a period of 5 days, open bars: day 1, grey: day 5, black: day 10. *Significant at p<0.05. Data are mean ± SEM. From Fenelon et al. 2016)*

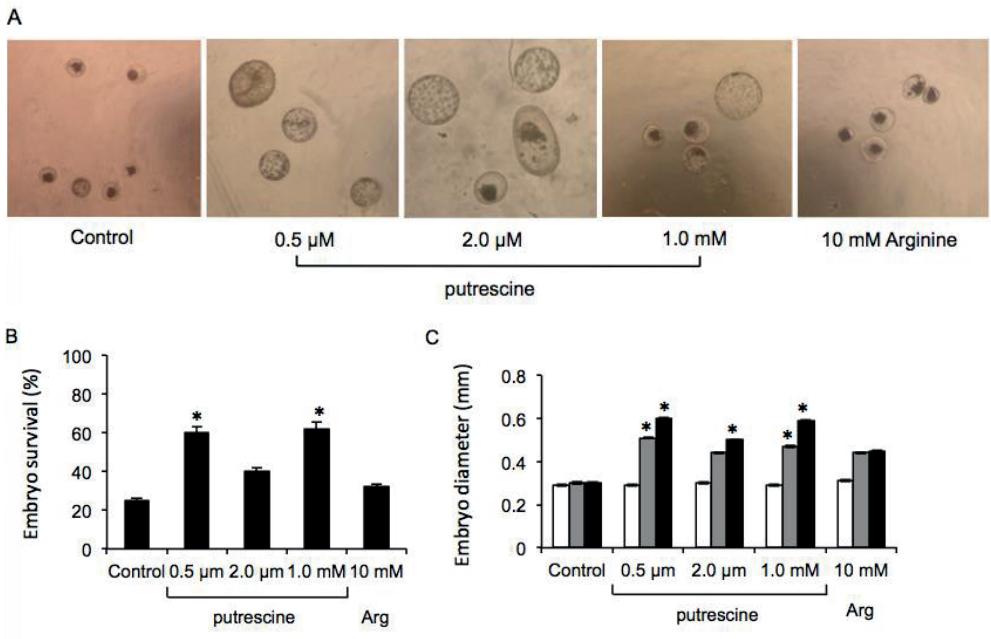
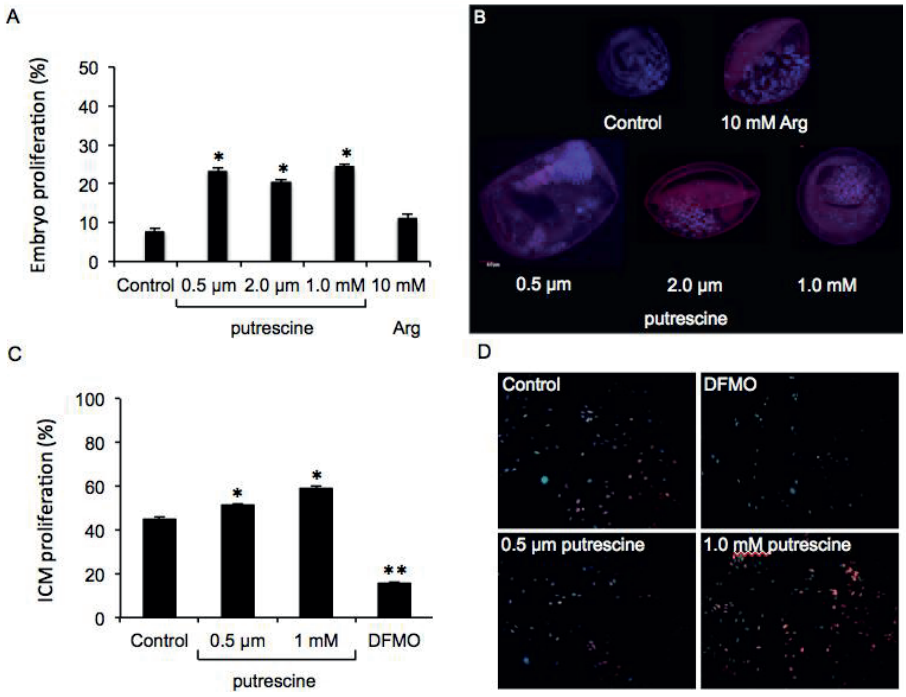


Figure 6. *The effect of putrescine and difluormethylornithine (DFMO) on cell proliferation. A: Percent proliferating cells in embryos collected after culture in vitro with or without putrescine (0.5 µM, 2 µM and 1.0 mM) and arginine (Arg, 10 mM). B. Whole blastocysts were visualized using differential interference contrast microscopy and cells labelled with nucleoside 5-ethynyl-2-deoxyuridine (EdU, in red) and counterstained by Hoescht. C. Percent proliferating cells of the inner cell mass collected after in vitro culture with or without putrescine (0.5 µM and 1 mM) and DFMO (10 mM). D. Cells of the inner cell mass were labelled by the modified nucleoside 5-ethynyl-2_-deoxyuridine (EdU, red) and counterstained by Hoescht. * indicates a significant difference (p<0.05) from control. (From Fenelon et al. 2016)*



To establish the effect of polyamines on reactivation from diapause, mink blastocysts were cultured with varying concentrations of putrescine and arginine. When first flushed out from the uterus, the mink blastocyst collapsed, but after a few hours in culture, the majority recovered their spherical shape (Figure 4), consistent with previous reports (Polejaeva et al., 1997). After five days, the majority of putrescine treated blastocysts were alive whereas control blastocysts and those treated with arginine were dead, indicated by their opaque cytoplasm (Figure 5). The survival was best in the groups treated with putrescine 0.5 um and 1 mM (Figure 5). More importantly, an increase in embryo diameter and in some cases, embryo hatching from the capsule, the earliest indicators of emergence from diapause, were induced by the putrescine treatments (Figure 5). The lowest and highest doses of putrescine were more potent in increasing the mean diameter of blastocysts. In addition, we assessed the effect of putrescine and arginine on the rate of cell proliferation of the diapause blastocyst (Figure 6). Whereas the diapause blastocysts in the control group showed only a 6-7% proliferation rate, the putrescine-treated blastocysts displayed an increase in proliferation of approximately 20%.

An earlier study from our laboratory showed that polyamine deprivation and/or putrescine supplementation affects the reactivated proliferation and growth of a mink trophoblast cell line in vitro (Lefèvre

et al., 2011b). Therefore, in the present study, we focused on the effect of putrescine on the proliferation of the mink inner cell mass cells. Putrescine treatment for 24 hours increased the proliferation rate of the reactivated inner cell mass cells in vitro in comparison to the control (Figure 6). There were more EdU positive cells in the putrescine-treated groups and the increases were dose dependent. In contrast, treatment of the inner cell mass cells with the inhibitor DFMO decreased the cell proliferation rate.

Discussion

The results reported herein demonstrate the first known in vitro induction of reactivation of carnivore embryos in obligate embryonic diapause and confirm and extend our findings of the role of polyamines in this process (Lefèvre *et al.*, 2011b). They further provide new insight into the mechanisms of polyamine activation of embryos. In the mink during diapause, blastocysts undergo nearly complete cell cycle arrest (Desmarais *et al.*, 2004). Putrescine can stimulate cell proliferation (Miller-Fleming *et al.*, 2015) and our study showed that putrescine treatment significantly increased proliferation in both the diapause blastocysts and in isolated inner cell mass cells. The increase in percentage of proliferating cells due to putrescine treatment in the viable blastocysts confirmed that the increase in the mean diameter of the blastocysts was due to reactivation of the capacity to proliferate, rather than an increase due to accumulation of fluid within the blastocyst, consistent with previous in vivo studies (Desmarais *et al.*, 2004, Lefèvre *et al.*, 2011b). In addition, when the inner cell mass cells were treated with DFMO there was a significant decrease in the rate of proliferation, as evident from the significant decrease in EdU positive cells. This extends the findings of our previous study that showed that in mink trophectoderm cells, polyamines stimulate proliferation, whilst polyamine deprivation causes a precipitous decline in cell proliferation (Lefèvre *et al.*, 2011b). Together these results provide solid evidence that polyamines dictate reactivation from diapause and begin the progression of events that lead to implantation.

The increased survival of embryos cultured from diapause is consistent with a previous study showing that addition of all three polyamines prevents apoptosis and increases viability in porcine parthenotes (Cui and Kim, 2005). Putrescine at the lowest dose tested (0.5 μM) appeared to be sufficient to elicit this effect with the higher doses having no increased benefit. The polyamine biosynthesis pathway is tightly regulated to control the levels of polyamines present hence higher doses may have resulted in compensatory pathways to prevent the detrimental effects of excess polyamines (Miller-Fleming *et al.*, 2015). Arginine, a precursor to the polyamines, was unable to induce any significant changes in the blastocysts at the doses employed. Arginine requires ODC1 to be converted to putrescine (Wallace *et al.*, 2003) and there is less ODC1 expression during diapause in mink in both the uterus and the blastocyst (Lefèvre *et al.*, 2011b). Presumably, this prevented sufficient polyamine synthesis from arginine to support normal embryonic development. It is possible the dose of arginine used was too low or alternatively, putrescine can also be converted via ODC1 from proline and ornithine, which may have been more effective. Due to constraints of the brief annual mating season in this species and the availability of animals, it was not practical to observe the effects of additional amino acids, spermine or spermidine on mink diapause embryos. However, previous studies have suggested that putrescine is the most effective in the fate of development of the blastocyst and trophoblast outgrowth in rodent models (Winkle and Campione, 1983, Zwierzchowski *et al.*, 1986), whilst spermine and spermidine are more important during development of the cleavage stage embryos (Zwierzchowski *et al.*, 1986). Results of gene expression analysis suggest that the embryo itself can contribute to the polyamine pool. Polyamine synthesis enzyme genes were at the lowest ebb in diapause and were up-regulated once the blastocyst was reactivated. Similar up-regulation of the polyamine genes in the uterus was reported by our laboratory (Lefèvre *et al.*, 2011a).

Earlier studies have shown that prolactin receptor is expressed in the uterus of the mouse (Reese *et al.*, 2000), the marmoset monkey (Dalyrymple and Jabbour, 2000), sheep (Stewart *et al.*, 2000), pig (Young *et al.*, 1990) and the human endometrium (Jones *et al.*, 1998). Prolactin is synthesized during decidualisation of the human stroma and in the uterus has been implicated in glandular epithelial cell proliferation, angiogenesis and immune regulation, particularly during implantation (Jabbour and Crichley, 2001). In the mink, prolactin is the main regulator of embryo implantation and substantial increases in prolactin secretion during the vernal equinox are required for both the ovarian and embryonic reactivation from diapause (Papke *et al.*, 1980, Murphy *et al.*, 1981, Murphy *et al.*, 1983). This study showed that the prolactin receptor is expressed in the uterine endometrial and luminal epithelium and its expression is consistently expressed during diapause and reactivation, with increases around the time of implantation.

The detection of the prolactin receptor in the mink uterus raised the question of its role in the uterus during reactivation of the embryo from diapause. To answer this question we examined whether variation in ODC1 expression could be regulated by prolactin. The results of the present study showed that prolactin up-regulated ODC1 expression in a time dependent manner in the mink uterine epithelial cells. The increase in ODC1 protein expression after only 45 min was unexpected.

Thus, the present study showed that putrescine treatment resulted in reactivation of the mink blastocyst from diapause, as evident from the increase in cell proliferation rate and diameter, and that prolactin is a regulator of ODC1 in the mink uterus. Thus, this report, together with our previous publications, shows the significance of the polyamines in regulating early embryo development.

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Use of physiological indicators and blood cortisol level for assessing temperament in young polar vixens

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Abstract

In young vixens (blue foxes) the body temperature is higher ($P < 0.05$) in aggressive individuals compared to those with trustful temperament. There were no differences in respiration or pulse rate between animals of different temperament types. The level of blood cortisol was higher (not significant) in aggressive and fearful foxes than in trustful animals. In conclusion, in young vixens the body temperature and the blood cortisol level can be a complementary method to select individuals for breeding. The full verification of observed differences require experimentation with a larger number of individuals.

Keywords: blue fox, physiological parameters, cortisol

Introduction

Previous studies have helped to identify basic types of foxes' temperament, referred as a reaction to the presence of man: aggressive, fearful and trustful. The foxes' temperament is particularly important during the breeding period, because the reproduction results of distrustful animals (aggressive, fearful) are poorer than those of the trustful ones (Brzozowski *et al.,* 1997; Gacek, 1999; Zoń *et al*., 1998b). The aim of this study was to examine whether there is a correlation between temperament of young female foxes determined with the behavioral tests, and physiological indicators and the level of blood cortisol. The confirmation of such relationship would give breeders an additional opportunity to identify trustful animals before the selection of breeding animals.

Material and methods

The study was conducted at The National Research Institute of Animal Production, Experimental Station Chorzelów on polar foxes. Young females at the age of 4 months were selected for the study. Using 'hand test' (Kaleta, 1982, Gacek, 1999), animals were divided into experimental groups, depending on their temperament:

- A – clear aggressive animals (17 vixens),
- N – clear trustful animals (18 vixens),
- S – clear fearful animals (7 vixens)

The examination was conducted in August for animals born in April. The body temperature, the pulse and the respiration rate were measured. The study was conducted on the treatment table. The animal was captured and placed on the treatment table and after 3-4 minutes the rectal temperature was

measured. The pulse rate, expressed with the number of heart beats per 1 minute, was palpated with finger tips on the animal's chest under the left front limb. The respiration rate was determined by the movements of the abdominal wall at the inhalation and exhalation of the animal per minute.

The sample from the arm vein was used to determine the level of cortisol. The concentration of cortisol in the blood serum was tested by radioimmunoassay using a set of cortisol (ORION DIAGNOSTICA) with a measurement range of 0 to 2,000 nmol/l and resolution of 4 - 7 nmol/l.

The statistical analysis of variance (SPSS, 2006) was used for investigating the obtained results. Fisher test (F) and Duncan test were used to establish statistically significant differences.

Results and discussion

The obtained results concerning analyzed indicators are presented in Table 1.

Table 1. *The mean and standard deviation (sd) value of selected physiological measurements and the cortisol level in serum blood in young polar vixens with different temperament.*

Indicator	Group of vixens					
	Aggressive		Trustful		Fearful	
	mean	sd	mean	sd	mean	sd
Heart rate/minute	84,82	10,93	80,89	5,95	81,71	6,26
No.respirations/min						
	40,82	9,05	41,44	6,61	35,14	3,44
Body temperature (^{o}C)	39,89[a]	0,37	39,45[a]	0,55	39,70	0,19
Cortisol level (nmol/l)	57,88	31,35	49,44	25,65	56,05	19,45

a classes with statistically significant differences at level p<0,05

In young females there were no differences in pulse or respiration rate depending on temperament. The parameters are consistent with the physiological norms for the species (Mononen *et al.*, 1999; Pedersen, 1996). There have been reports on an increase in heart rate and respiration rate in aggressive and fearful young foxes in comparison with the trustful contemporaries (Zon *et al.*, 1998a; 1998b). Similar findings have been made for grown up foxes (Bakken *et al.,* 1994).

There was a statistically significant difference in the body temperature of aggressive and trustful foxes. Animals classed as aggressive, had 0,44oC ($p<0.05$) higher body temperature higher than in the trustful. Similar relationships were described by Zon et al. (1998b) who observed an increased body temperature of aggressive foxes.

We also observed a higher (not significant) level of cortisol in the blood serum of young females classified as aggressive, especially when compared with trustful foxes. The values obtained are within the norms considered to be physiologically correct for the blue foxes, comprising values between 49.9 nmol/l and 140.9 nmol/l, found by Mononen et al. (1999) and Rekila (1997) who considered the difference in the level of cortisol to be possibly related not to temperament, but to seasonal changes in animal physiology.

The observed tendency confirms the results by Kowalski (1996) that the fearful animals have an increased levels of corticoids in blood serum. Aggressive and fearful animals are considered to be less adapted to the farm environment with suboptimal adaptive processes (Kowalski, 1998).

The results indicate the existence of relationship between foxes temperament, specified by using behavioral tests, and selected physiological indicators and the level of cortisol in the blood.

In young females foxes these relationships have proven to be statistically significant in relation to body temperature, when comparing foxes aggressive and trustful. Other dependences were not so clear, although similar trends were observed when comparing the results obtained from animals of different type of temperament.

In conclusion, the applied parameters may be useful as an additional method for assessing foxes' temperament; however, full verification of the observed dependences requires confirmation with a larger number of individuals.

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Breeding colored foxes in FSUE Russian sable

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Abstract

On a farm near Moscow, Federal State Unitary Enterprise Russian sable in Russia is breeding a large number of fox color forms. Some of them have the status of the breed, others are bred due to the demand by furriers, and others are an outcome of experimental crosses. Many colors, such as white face, platinum, marble arctic fire and ice, burgundy, kolikott, pearl have been introduced at different times from abroad. Others have been obtained from Russia or the Soviet Union. The foxes with a red cherry with a hint of color ognëvka has been brought from Kamchatka. Their fur is unique, as it is not found anywhere else in the world. The representatives of this form have the highest demand among buyers who want to keep the fox at home as a pet, as they are most sociable and responsive. Another type is snow white fox with a black stripe along the ridge, it is also known as the snow fox in Georgia (Bakuriani). The work carried out with all these stocks is presented in this article.

Keywords: fox, color mutation, color types of foxes, Russian fox breeding

Introduction

Federal State Unitary Enterprise Russian sable is located in the Moscow region of the Russian Federation and is the oldest (founded in 1928) and one of the largest farms breeding fur-bearing animals in Russia. In Soviet times, the fur farms were called Pushkinsky. Today the stock consists of sables, minks, foxes, raccoon dogs, ferrets (Fitch) and marmots. Of foxes, there are 525 females 105 males, who have been the object of research since 2010.

The main colors of foxes bred in the Federal State Unitary Enterprise Russian sable area silver-black and red ognevka (from the Russian word огонь- fire). Silver black and red colors are dominant mutations. Own work is carried out in foxes with recessive mutations causing pearl, burgundy, kolikott, creamy, fire and ice colours.

Materials and methods

Basic colors

The work on breeding silver-black fox began with the founding of animal breeding farm in 1928. The first silver-black and black-brown foxes taken to breeding were from Canada, Germany and Russia and later, in 1946, from Estonia, Germany and Finland. In 1976 a group of silver-black foxes was recognized as a pedigree type Pushkinsky, which is characterized by thick, lush, scalp with long guard hair (71-75 mm). In 2005 from Finland it was imported 66 female and 11 male silver foxes of Finnish type, 2 silver and black male of a Norwegian type and type 2 black and brown males. We have crossbred animals that combine reproductive qualities of the Pushkinsky type and the fur close to the Scandinavian type.

Breeding of a red fox started in 1970 after capturing some local red foxes. In 1975 it was crossed with Yakutian red foxes to combine low-quality color and a lot of silver, dull color, low very soft hair. Hybrids derived from the local red fox greatly surpassed them in all respects. 13 red foxes were imported from Kamchatka in 1988. They have bright saturated colors. In 2004 animals were introduced from, altogether 146 animals (120 females, 26 males) with a dark red color with almost no silver. This type was established from the population of wild Kamchatka red fox at the fur farms in Vyatka and approved in 1988. In 2005 6 males were brought from Finland: 3 red gold, 2 selected red American, 1 red European type. Combining these types, we had a herd of red foxes characterized by strong bones, thick and lush bright red saturated color with cherry hue with minimally amount of silver. While working with these animals revealed that they are more responsive and tame when dealing with humans and showing their delight with wag tail and giving sounds to express their mood. Hence the animals in this group are most popular among people who want to keep at home an exotic animal like a fox.

Dominant mutations

Dominant mutations in foxes suppress pigmentation of hair in different parts. Currently, the Russian bred foxes have known dominant mutations of **white beaked, platinum, snow** and **marble**. **White** beaked breeding has been going on in fur farms since 1946. To refresh the blood in 2005, 2 males were brought from Finland. It differs from the **silver-black** in density and shorter hair. **Platinum** emerged first in Norway in 1933 and has evolved with us in the postwar years. Also in 2005, 2 males were imported from Finland and 2 males from different Russian farms. Currently it is in high demand. **Snow mutation arose** in 1943 in Georgia (with another withdrawn name of Bakuriani), also called Georgian (to attach the name of the origin to it) snow-white fox with a strip of basic color on the back, the spots on the face and legs. The farm breeding has been going since the 1970s. Currently, there are efforts to develop thinner belt and a minimum number of spots. The type is in high demand, as it is easy to dye the fur. **Arctic marble** type was produced in 1945 from Norway and introduced into farming only in 2005 with of 3 females and 3 males. It is very popular among Russian buyers in the black or in the red (Sunglow) versions.

Recessive mutations

Pearl by common knowledge originated in the 1940s in the United States. It was introduced to Russia in 1988-1989 from Canada and Norway. In this process, the fox type was transferred in 1998 from another farm with breeding for 10 years. **Kolikott** is also known to first appear in the United States in 1940s, and was brought to Russia in 1988-1989 from Canada and Norway. The farm came from imported silver-black foxes heterozygous for the gene kolikott. **Burgundy** is also known to have appeared in the United States in 1940s, and also was brought to Russia in 1988-1989 from Canada and Norway. The farm was derived from silver-black foxes, heterozygous for the gene burgundy introduced from other farms. Pets are characterized by chocolate color. There is a pleasant color of steel work. In 2014, 6 females were imported from another farm, which bred foxes this color. In order to prevent inbreeding. also silver-black foxes, and pearl and kolikott have been used. **Cream** is a result of several recessive mutations with manifestations in the crossbred foxes as pearl colors, kolikott, Burgundy. **Fire mutation** (which has similarities with the appearance of color in the Siamese) appeared in the 1980s and was delivered to the farm in 2005 for the first time in Russia from Finland. This mutation together the main colors results in a wide variety of color pelts.

Polygenic mutation

Smoky fox was first brought to the farm for the first time in Russia in 2005 from Finland. It goes well with the color ognëvka and burgundy.

Results

Working with a variety of different color types has shown that with proper selection and breeding work we can achieve producing high-quality products. There is a stable high among the fur buyers and also for selected females with high rates of reproduction. The small livestock species and mutant coat colors make the breeding of rare colors profitable. The pelts of foxes, grown in the Federal State Unitary Enterprise Russian sable are annually awarded championship status and certificates of 1st degree quality in the Fur Skin Contest «Farmed Fur» Russia and have a high demand in the domestic market.

Discussion

Breeding foxes of different colors has prospects, as in connection with the improvement of processing technologies and tailoring long-haired fur is again in high demand. The popularity of foxes as pets is growing, as among animals raised on fur farms there are specimens often found friendly to communicate with huamans. A wide range of products always has an advantage.

Genetic variability within the Polish population of farm mink *(Neovision vison)* – preliminary results

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Abstract

The aim of the study was to detect genetic variation within the Polish population of mink bred in production farms. 200 randomly sampled mink of three colour types: brown (n=74), black (n=120) and sapphire (n=6) were studied. The panel containing 30 microsatellite markers with possible highest coefficients of PIC (>0.70) and Het (>0.70) was used for the study. PCR products were detected for 26 microsatellites. The results obtained indicate high genetic variability in the studied sample. The number of alleles per locus varied from 3 to 11, while the number of genotypes ranged from 4 to 45 (some genotypes were detected only in a single individual). The average expected heterozygosity (H_s) was 0.70 (ranging from 0.15 to 0.89), while the average observed heterozygosity (H_o) reached 0.69 (ranging from 0.15 to 1.00). H_o for majority of microsatellite loci was higher than H_s. Furthermore, significant differences in allele and genotype frequencies were found between colour types. Out of 26 microsatellite loci, 18 differed significantly in allele frequency. The preliminary results suggest that despite intensive selection conducted by breeders, the Polish population of farm mink is still genetically variable.

Keywords: microsatellites, heterozygosity, coat colour

Introduction

Mink genetics has been widley studied for microsatellite and candidate gene polymorphism(Anistoroaei et al. 2006, 2007; Thirstrup et al. 2014). In mink genetics there is l tendency to sequence and explore in detail the whole genome (Anistoroaei at al. 2007, 2011). There are also studies on the genetic diversity in mink populations (Belliveau at al. 1999, Kidd at al 2009, Lecis at al. 2007).

The aim of the study was to detect genetic variability within the Polish population of mink in production farms using microsatellites and taking into account the colour variant of animals.

Materials and methods

200 randomly sampled minks of three colour variants: brown (n=74), black (n=120) and sapphire (n=6) were studied. The panel containing 30 microsatellite markers (Anistoroaei et al. 2006, 2007; Thirstrup et al. 2014) with possible highest coefficient of PIC (>0.70) and Het (>0.70) was used for the

study. Genotyping was done using the multiplex PCR reaction in six pools (Table 1).

The population genetic analyses were performed using R package (R Core Team 2015). The observed (H_o) and expected (H_s, H_t, H_{tp}) heterozygosity, inbreeding coefficient (F_{is}), fixation index (F_{st}, F_{stp}), index for the amount of gene diversity among samples (D_{st}, D_{stp}), index of genetic differentiation (D_{est}) for each microsatellite marker were estimated using hierfstat package (Goudet and Jombart 2015). The genetic distances by principal coordinates' analysis (PCoA) were calculated using ape package (Paradis *et al.* 2004).

Table 1. *The six pools of microsatellites for multiplex PCR reaction.*

	Size Range			Size Range	
Pool 1	Minimum	Maximum	Pool 2	Minimum	Maximum
MVi87	76	89	Mvi111	82	107
Mvi192	106	159	Mvi2243	131	162
Mvi1272	160	200	Mvis002	174	190
Mvi1302	203	228	Mvi4052	200	260
Mvi4031	279	291	Mvis022	275	289
Mvi4025	380	409			
Pool 3			Pool 4		
Mvi1321	80	120	Mvi57	91	108
Mvi1341	140	180	Mvi232	139	164
Mvi1381	181	197	Mvi1354	175	220
Mvi4026	238	251	Mvis072	253	270
Mvi4060	286	302	Mvi4066	312	335
Pool 5			Pool 6		
Mvis075	90	140	Mvi219	140	200
Mvis020	140	200			
Mvi4001	200	250			
Mvis099	300	360			

Results

Table 2 presents the characteristics for all the analyzed microsatellite markers. All the loci were polymorphic and the number of alleles per locus ranged from 3 (Mvis002) to 11 (Mvi321). The major allele frequency for most of the loci was lower than 0.5. Only in six loci this frequency was higher than 0.5 with the highest value being 0.89 (Mvis002). The major genotype frequencies ranged from 0.1 to 0.44, only for Mvis002 this was very high (0.79).

Table 3 presents the basic population genetics statistics. The majority of microsatellites were characterized by high, both observed and expected heterozygosity (except Mvis002). Big discrepancy between observed and expected heterozygosity was observed for Mvis020 with corresponding high Fis (0.61). No inbreeding was found for half of the loci. The observed values of Fst and Dst indicate low population diversity while the observed Dest values indicate moderate diversity for a few loci.

Table 2. *Characteristic of the microsatellite loci.*

Locus	Number of genotyped individuals	Number of alleles	Allele range	Major allele (frequency)	Number of genotypes	Major genotype (frequency)
Mvi111	199	6	87-106	87 (0.40)	20	87/87 (0.18)
Mvi1302	188	10	204-226	222 (0.41)	27	222/222 (0.25)
Mvi1341	197	10	150-170	156 (0.31)	43	156/166 (0.13)
Mvi1354	128	8	177-196	194 (0.55)	24	194/194 (0.34)
Mvi1381	197	7	184-196	184 (0.32)	24	184/187 (0.15)
Mvi192	197	7	126-142	132 (0.35)	21	126/132 (0.19)
Mvi219	195	7	162-177	162 (0.22)	25	162/174 (0.10)
Mvi2243	199	6	132-161	159 (0.40)	18	139/159 (0.22)
Mvi232	197	8	143-162	146 (0.39)	23	146/151 (0.24)
Mvi272	194	9	162-179	173 (0.30)	27	173/173 (0.14)
Mvi321	198	11	91-108	97 (0.40)	30	97/97 (0.14)
Mvi4001	64	4	221-227	221 (0.46)	9	221/225 (0.25)
Mvi4025	184	10	384-407	399 (0.36)	38	394/399 (0.14)
Mvi4026	198	4	239-248	242 (0.41)	9	242/245 (0.26)
Mvi4031	198	4	281-287	281 (0.40)	10	281/287 (0.30)
Mvi4052	196	5	211-240	230 (0.63)	11	230/230 (0.39)
Mvi4060	196	6	288-300	296 (0.65)	16	296/296 (0.44)
Mvi4066	195	9	314-335	320 (0.39)	27	320/326 (0.17)
Mvi57	189	6	94-104	104 (0.40)	19	104/104 (0.18)
Mvi87	198	5	79-88	88 (0.32)	11	88/88 (0.20)
Mvis002	199	3	176-187	187 (0.89)	4	187/187 (0.79)
Mvis020	197	8	160-185	173 (0.45)	26	173/173 (0.34)
Mvis022	198	6	276-286	282 (0.50)	14	282/282 (0.27)
Mvis072	197	6	253-268	260 (0.63)	16	260/260 (0.39)
Mvis075	196	10	112-132	118 (0.29)	36	118/126 (0.15)
Mvis099	196	10	315-345	339 (0.22)	45	330/339 (0.10)

Table 3. *Basic statistics for all the studied loci.*

Locus	Ho	Hs	Ht	Dst	Htp	Dstp	Fst	Fstp	Fis	Dest
Mvi111	0.89	0.89	0.90	0.02	0.91	0.02	0.02	0.02	-0.01	0.19
Mvi1302	0.57	0.67	0.70	0.03	0.72	0.05	0.05	0.07	0.14	0.15
Mvi1341	0.88	0.82	0.83	0.01	0.83	0.01	0.01	0.01	-0.06	0.06
Mvi1354	0.63	0.66	0.65	-0.01	0.65	-0.01	-0.01	-0.02	0.05	-0.04
Mvi1381	0.79	0.77	0.78	0.00	0.78	0.01	0.01	0.01	-0.03	0.03
Mvi192	0.70	0.73	0.75	0.02	0.76	0.03	0.03	0.04	0.04	0.11
Mvi219	0.73	0.78	0.80	0.02	0.82	0.04	0.03	0.04	0.06	0.16
Mvi2243	0.69	0.72	0.71	-0.003	0.71	-0.005	-0.005	-0.01	0.04	-0.02
Mvi232	0.73	0.74	0.73	-0.01	0.73	-0.01	-0.01	-0.01	0.01	-0.04
Mvi272	0.70	0.78	0.82	0.04	0.84	0.06	0.05	0.07	0.10	0.26
Mvi321	0.98	0.88	0.89	0.01	0.89	0.02	0.01	0.02	-0.11	0.13
Mvi4001	0.52	0.59	0.63	0.04	0.65	0.06	0.07	0.10	0.11	0.16
Mvi4025	0.86	0.82	0.81	-0.01	0.80	-0.02	-0.01	-0.02	-0.04	-0.09
Mvi4026	0.60	0.64	0.64	0.001	0.64	0.001	0.001	0.001	0.07	0.003
Mvi4031	0.65	0.64	0.68	0.04	0.70	0.06	0.05	0.08	-0.01	0.16
Mvi4052	0.63	0.57	0.57	0.003	0.57	0.004	0.005	0.007	-0.11	0.01
Mvi4060	0.60	0.52	0.53	0.01	0.54	0.01	0.01	0.02	-0.15	0.02
Mvi4066	0.76	0.74	0.74	0.01	0.75	0.01	0.01	0.02	-0.03	0.05
Mvi57	0.69	0.83	0.84	0.004	0.84	0.01	0.005	0.01	0.17	0.04
Mvi87	1.00	0.70	0.72	0.02	0.73	0.03	0.03	0.04	-0.42	0.10
Mvis002	0.15	0.15	0.16	0.005	0.16	0.01	0.03	0.05	0.02	0.01
Mvis020	0.28	0.73	0.74	0.01	0.74	0.02	0.01	0.02	0.61	0.06
Mvis022	0.67	0.64	0.65	0.01	0.66	0.02	0.02	0.03	-0.05	0.06
Mvis072	0.56	0.57	0.56	-0.001	0.56	-0.001	-0.001	-0.001	0.005	-0.002
Mvis075	0.92	0.83	0.85	0.02	0.86	0.03	0.02	0.03	-0.10	0.18
Mvis099	0.89	0.83	0.85	0.02	0.86	0.03	0.02	0.03	-0.07	0.18
TOTAL	0.69	0.70	0.71	0.01	0.72	0.02	0.02	0.02	0.01	0.06

Genetic distances between the colour variants showed that the brown and black variants are genetically different (Fig 1).

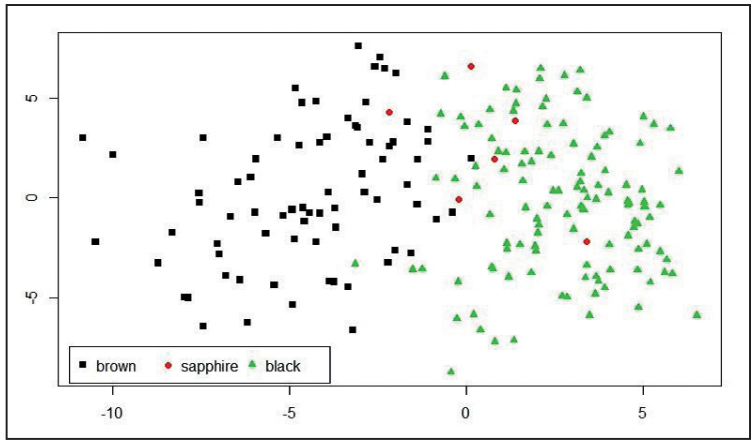

Fig. 1. *PCoA plot between pairs of individuals in the colour variants based on all microsatellites*

Discussion

Belliveau *at al.* (1999) used seven microsatellites to compare four groups of animals from different farms, with animals also belonging to different colour variants. They found expected heterozygosity comparable to our findings, with noticeably smaller values for two markers (Mvi87 and Mvi232). They also reported significant differences between farms and colour variants at the five studied loci. Hammershoj *et al* (2005) and Kidd *et al.* (2009) stated that with microsatellite variation we can assign animals (with high accuracy) to a given population – either wild or farm population. Hybrid animals can also easily be detected.

The diversity of mink population in Spain was studied by Lecis *et al.* (2007). The results differed in the level of inbreeding between populations and markers. The similar observation was made by Vincent *et al.* (2003).

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Increasing rabbit productivity using DNA-markers

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Abstract

Using the effective scheme of inter-breed crossing, a group of high-yielding rabbit hybrids has been created at the rabbit farm 'Nauka" with the involvement of FSBSI NIIPZK laboratories. Average live weight of hybrid young at 77-day age is 2.6 kg; at 90 days – 3,1 kg, and is sufficiently higher than the control group of agemates of other crosses by 130-350 g and 180-370 g ($p \leq 0.05$) respectively, according to age.

Live weight of 90-day old rabbits with the genotype TT and Ms-A mutation in the myostatin gene is 110 and 90 g higher than in individuals with the genotypes CC and CT, respectively; Ms-T mutation live weight of rabbits with the genotype GG is higher by 170 and 120 g than in individuals with genotypes AA and AG, respectively. SNP-mutations - Ms-A and Ms-T in the myostatin gene are used to carry out selection and breeding work for increasing live weight using molecular genetic information. The high effectiveness of growing hybrid young was established – expenditure on feed for 1 kg of live weight gain in hybrid rabbits, when compared to the same in pure breeds (parental breeds), was lower by 27.9-55.8% when growing to 77 days and 9.3-23.8% – to 90 days.

Keywords: rabbit breeding, high-yielding hybrid, live weight

Introduction

From the moment of its founding, the Scientific Research Institute of Fur-Bearing Animal and Rabbit Breeding Industries has dedicated a lot of time to stock breeding and selection of rabbit breeds (Kharlamov, 2004). One of the molecular-genetic markers that can be used in rabbit breeding for large body mass (from 90 days and earlier) is the myostatin gene (Fontanesi *et al*, 2008).

The stimulus for developing rabbit breeding is the year-round supply of rabbit meat with a high profit indicator. One of the faster methods of increasing the growth power of rabbits and, as a result, increasing the economic efficiency of rabbit meat, is crossing different breeds using the heterosis effect. An important condition for this is the ability to save and fix the high productivity characterstics throughout the different generations.Work was carried out on creating a group of high-yielding rabbit hybrids using DNA markers based on cross-bred animals from three domestic breeds (Kharlamov *et al*., 2014).

Materials and methods

Research was carried out over six years and took place at the rabbit farm 'Nauka' and the laboratories of the Scientific Research Institute of Fur-Bearing Animal and Rabbit Breeding Industries. The development of a high-yielding hybrid was done though a complicated cross-breeding process made of

five stages, with three to five test groups of males and females at each stage. The characteristics taken into consideration were: productivity indicators (fertility, livability), the number of weaned young, live mass of young at 20, 45, 60, 77 and 90 days of age. For each of the five stages the best combination of male and female parents was found to ensure the best possible characteristics.

To investigate the DNA polymorphism for two SNP-mutations of the myostatin gene PCR-RFLP was used (Fontanesi, 2011). DNA was extracted from the blood of three-breed hybrids (n=92) with varying live mass. Visualisation of the restriction results was done with electrophoresis in 3% agarose gel in a 1xTBE buffer at a voltage of 8 V/cm for 60 min, coloured with ethidium bromide. The results of the study were processed in Excel using the criteria Student.

Results

A group of hybrid rabbits was created with significantly better properties than their pure-bred parental breeds. The hybrids exhibited a difference of live mass at the age of 45 days by 100 - 230 g, at 60 days by 100 - 270 g, at 77 days by 130 - 350 g, at 90 days by 180 - 370 g ($p \leq 0.05$), and with the carcass weight at 90 days of the difference 120 - 150 g ($p \leq 0.05$). The average live mass of the hybrid young at the age of 77 days reaches high slaughter condition and is 2.6 - 2.9 kg; at 90 days 3.1 - 3.44 kg.

Live weight of 90-day old rabbits with the genotype TT and Ms-A mutation in the myostatin gene is 110 and 90 g higher than in individuals with the genotypes CC and CT, respectively; Ms-T mutation live weight of rabbits with the genotype GG is higher by 170 and 120 g than in individuals with genotypes AA and AG, respectively. SNP-mutations Ms-A and Ms-T in the myostatin gene are used to carry out selection and breeding work for increasing live weight using molecular genetic information. The high effectiveness of growing hybrid young was established, especially until the age of 77 days because the expenditure on feed for 1 kg of live weight gain in hybrid rabbits, when compared to the same in pure breeds (parental breeds), was lower by 17.2 - 34.4 roubles (or 27.9…55.8%) when grown from 61 to 77 days and by 6.1 - 15.7 roubles (or 9.3…23.8%) when grown from 61 to 90 days.

Discussion

The use of modern molecular genetic methods as well as traditional ones in the selection process resulted in the development of a rabbit hybrid with high productivity properties in a short space of time.

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Raising Mink Males Sexual Potency in Application of Surfagon

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Abstract

The aim of the investigations was to assess sexual activity of males stimulated with the synthetic nanopeptide 'surfagon'.

The trials were made at the breeding farm 'Saltykovsky' (Russia) on young standard dark brown minks with 42 males and 253 females selected by a principal of analogues by body weight in 3 experimental (named groups 2,4 and 6) and 3 control (named groups 1,3 and 5) groups. Surfagon was daily inoculated to the males of the experimental groups over the prepubertal period (December) for 5 days intramuscularly at the dosage of 0.7 micrograms active substance per head (2n-d and 6-th groups) and over the pubertal period (February) – to the males of 4-th and 6-th groups for 3 days.Selection of pairs for mating was made by the principle "the best with the best".

The degree of male sexual behavior manifestation was assessed by the number of placings of females, which are receptive for mating with males and number of copulations (placings of females).

The positive effect of surfagon is expressed as a decrease in a quantity of placings by 20.2 % (4-th) and 18.4 % (6-th) in males and by 13.5 % (4-th) and 14.3 (6-th) in famales as compared to the control ($p < 0.05$). In conclusion, stimulating young mink males by surfagon promotes increase in their sexual activity over the mating period.

Keywords: female, sexual behavior, copulation

Introduction

Improving reproductive qualities of animals has always been one of important tasks in animal husbandry. The process of pubertal period determines the formation of a reproductive system and its subsequent function as well.

Gonadotropic hormones and their analogues have been extensively applied for influencing farm animal gonad function. Mink breeding is rather a young branch of industry. The method of synchronizing ovulation in females with the help of chorionic gonadotropin was developed and introduced into practice (Bernatsky 1993).

The problem of improving a reproductive function of males remains rather urgent. Mink farmers have to cull almost all male stock after the first year of use, with 50% out of them - due to a low reproductive function. About 10% males do not show sexual activity at all or do it late [Demina 1984, 2001].

In animal husbandry surfagon is applied intramuscularly for advancing sexual cycle, treating ovary hypofunction and follicular cysts, improving functional activity of gonads, rising gonad functional

activity and female fertility [Prokofyev 2002]. There is a lack of literature on the dosage and schemes of hormone application for stimulating the male sexual function. One of the hormone preparations used on a large scale in animal husbandry is a hormone-like substance 'surfagon' (C56H78N16O12), which is a synthetic nanopeptide and analogue of the gonadotropin - releasing hormone LG-RG-luliberin. Surfagon stimulates release of gonadotropic hormones of the hypophysis to blood, such as-luteinizing (LH) and follicle - stimulating (FSH) gormones which influence functional activity of peripheral gonads. It is known that the quantity of Leidig cells in rabbits increases and their functional activity rises under the influence of gonadotropic hormones [Brindak 1989]. In males the application of surfagon had a positive effect on ejaculate volume and sexual activity. Therefore the aim of our investigations was to assess male sexual activity stimulated by the synthetic nanopeptide 'surfagon'.

Materials and methods

The trials were made at the breeding farm 'Saltykovsky' (Russia) on young standard dark brown minks with 42 males and 253 females selected by a principal of analogues by body weight in 3 experimental (named groups 2,4 and 6) and 3 control (named groups 1,3 and 5) groups. Surfagon was daily inoculated to the males of the experimental groups over the prepubertal period (December) for 5 days intramuscularly at the dosage of 0.7 micrograms active substance per head (2n-d and 6-th groups) and over the pubertal period (February) – to the males of 4-th and 6-th groups for 3 days. Selection of pairs for mating was made by the principle "the best with the best". The degree of male sexual behavior manifestation was assessed by the number of placings of females to males and number of coituses. Statistical processing of the data was made according to Student criterion (t-test) (Plokhinnsky1988).

Results and Discussion

The scores of male sexual activity stimulated by surfagon are given in Tables 1,2.

Significant decrease in the total number of placings of females as compared to the control was determined per male by 20.2% ($p < 0.05$) in surfagon application over a pubertal period (4-th) and by 18.4% ($p < 0.05$) in surfagon application over the prepubertal and pubertal periods (6-th).
Sexual activity scores per female were similar to those of males. The number of placing in the experimental group 4-th and 6-th was significantly lower as compared to the control by 13.5 and 14.30%, respectively ($p<0.05$).

The number of placings per copulation per male in the 4-th experimental group in the application of surfagon in the pubertal period decreased by 27.5% and comprised 2.14 against 2.95 in the control (Table 3).

In the 6-th experimental group the application of surfagon provided decrease in the number of placings per copulation per male by 21.5% as compared to the control. The application of surfagon in the period of puberty (4-th) and for two periods - prepubertal and pubertal (6-th) decreased the number of placings per copulation per female by 22.6% (4-th) and 22.5% (6-th), respectively.

Conclusions

The data obtained show a real possibility of improving sexual activity of young males stimulating with surfagon. A positive effect of surfagon application is shown to be a significant decrease both in the number of placings and the number of placings per copulation per male and per female. The significant

Table 1. *Sexual activity scores per male (mean ± sd)*

Group	number of males	number of females	Per male				
			number of placings	number of coituses	number of mated females	percentage to control	
						placings	coituses
Control 1	7	41	25.0±1.3	12.9±1.2	5.1±0.6		
Trial 2	7	40	25.3±0.8	12.0±1.2	4.9±0.3	101.2	93.0
Control 3	7	44	35.1±2.3	11.9±2.0	4.9±0.7		
Trial 4	7	46	28.0±2.1*	13.1±1.9	5.6±0.7	79.8	110.1
Control 5	7	42	29.4±1.5	12.4±1.1	5.0±0.3		
Trial 6	7	40	24.0±1.8*	12.9±1.1	5.1±0.3	81.6	104.0

* — $P < 0.05$

Table 2. *Sexual activity scores per female (mean ± sd)*

Group	number of males	number of females	Per female			
			number of placings	number of coituses	percentage to control	
					placings	coituses
Control 1	7	41	4.2±0.3	2.0±0.1		
Trial 2	7	40	4.0±0.3	2.0±0.2	95.2	100.0
Control 3	7	44	5.2±0.2	1.7±0.2		
Trial 4	7	46	4.5±0.2*	1.9±0.1	86.5	111.8
Control 5	7	42	4.9±0.1	1.9±0.2		
Trial 6	7	40	4.2±0.3*	2.1±0.1	85.7	110.5

* — $P < 0.05$

Table 3. *Number of placings per 1 coitus*

Group	males		females	
	number	% to control	number	% to control
Control 1	1.94		2.10	
Trial 2	2.11	108.8	2.00	95.2
Control 3	2.95		3.06	
Trial 4	2.14	72.5	2.37	77.4
Control 5	2.37		2.58	
Trial 6	1.86	78.5	2.00	77.5

difference in the sexual activity of the males stimulated once in a pubertal period (February) versus twice (December and February) was not determined. There is a real possibility of surfagon application only in a in a pubertal period.

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Genetic parameters and genetic trends for growth and fur quality trait in silver blue mink in China

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Abstract

Knowledge of genetic parameters is essential for efficient breeding programs. The present study was performed to estimate genetic parameter for growth and fur quality traits in Chinese silver blue mink. Recordings of body weight (BW), body length (BL), guard hair length (GL), underwool length (UL), and the rate of guard hair and underwool length (nap) from 1686 silver blue minks spanning six generations were obtained from Dalian Ming Wei Marten Industry Company. The phenotypes were analyzed using a model with year and sex as fixed effects. Genetic parameters were estimated using a multi-trait animal model with year and sex as fixed effects. The heritability estimate for BW, BL, GL, UL and nap was 0.41, 0.53, 0.53, 0.52, and 0.52. The phenotypic correlation between nap and BW, BL, GL and UL were negative (.0.218, .0.178, .0.074, .0.425). The phenotypic correlations between BW and BL, GL and UL and between BL and GL and UL and between GL and UL were positive (0.928, 0.882, 0.869, 0.806, 0.788, 0.930, respectively). The genetic correlation between BL and UL and nap was negative (.0.941, .0.983) as was the genetic correlation between nap and GL and UL (-0.074 and -0.425).The genetic correlation between BW and GL, UL and nap was 0.731, 0.972 and 0.981, between BL and GL it was 0.622, between GL and UL and nap it was 0.992 and 0.641 and between UL and nap it was 0.987. The genetic trends were estimated by regressing breeding value on year. The genetic trend for body weight and body length was close to zero and positive, respectively. The genetic changes for GL, UL, and nap were negative and parallel. Our results herein form a practical basis for designing optimal breeding schemes in Chinese silver blue mink.

Keywords: Silver blue mink; genetic parameter; breeding value; BLUP

Introduction

The American mink *(Neovison vison)*, a semi-aquatic carnivore originating from North America, is the most widespread animals used in fur production. In the breeding of mink, proper quality of hair coat and animal's body size are the utmost important production traits from the economic point of view (Peura et al., 2004). A thorough understanding of the genetic variation of these traits in mink is key to genetic enhancements in production performance. Building upon advances in animal and plant breeding, genetic improvements in fur animal have been successfully implemented during the past three decades (Liu et al., 2012; Cai et al., 2008). In particular, advances have been made and are still in progress in farming of mink (Scocha. S, 2006). Silver blue is the first color mutant known on the mink farm, which named *splatinum* and *maltese*. The first silver blue appeared in 1931 on a farm owned by W. Whittingham in Arpin, Wisconsin. The silver blue mink was first introduced into China in 1980s for fur farming, but the mink breeding was initiated in 2002.

Recently, much estimation of genetic parameters and breeding values for productive traits has been

published for fur animal (Wierzbicki, 2006; Peura 2004; Nielsen 2012). But there is a large variation in heritability and correlation estimates among different mink populations. This variability is most probably caused by natural and artificial selection (Scocha 2004). Another problem concerns the definition of traits within populations. Thus, a genetic estimation for growth and fur quality traits in the breeding program for mink is needed for the common fur animal farming environment in China.

In the present study, data were collected on production traits of silver blue mink from six generations. The objective of this study was to estimate genetic parameters and breeding values for production traits of silver blue mink in China by Multiple Trait Derivative Free Restricted Maximum Likelihood program (MTDFREML).

Material and methods

Data collection

Samples were obtained from Dalian Ming Wei Marten Industry Company. The company with its equipment is well suited to mink production. The kits were randomly selected from a litter with similar age at birth. The second and third time evaluation of body dimensions was done in October and December. Throughout this period, the kits were fed *ad libitum* twice a day with a variety of commercially available, nutritionally complete diets, which are formulated for kit growth, at a level designed to maintain optimum body composition. After weaning, minks were kept in individual cages. From mating until pelting, animals were paired, with one male and female full-sib pair in each cage.

Traits measured

The individual body weight (accuracy 0.1g), body length (accuracy 0.1 cm), guard hair length (accuracy 0.1 cm), underwool length (accuracy 0.1 cm), and the rate of two hairs were recorded in December (the third evaluation) throughout the seven generations. The mink was weighed in the morning before feeding. The body length was measured from nose to the root of tail. The natural unstretched length of guard hair and underwool was measured from skin to the top of hair by ruler. These traits for each mink were available for 1686 individuals over six generations between 2005 and 2011. The individuals were arranged according to the family tree (pedigree) and requirement of MTDFREML.

Statistical analyses of data

Simple descriptive statistics for data collected are summarized in Table 1. Variance components and heritability for each trait were estimated with single trait and two-trait animal model by derivative-free REML with a simplex algorithm using the MTDFREML.

Result

Genetic parameter

The estimated genetic parameters from the single trait animal model is given in Table 2. Body weight, body length, guard hair length, underwool length and the rate two hair lengths showed high heritability (0.41, 0.53, 0.53, 0.52, and 0.52, respectively). The genetic correlations between each pair of the above traits were estimated by using two-trait animal model and phenotypic correlations between each trait pair were estimated by using Pearson correlation method (SAS 2004). The results showed that the phenotypic correlation between the rate of two hair lengths and body weight, body length, guard hair length and underwool length was negative (. 0.218, . 0.178, . 0.074, . 0.425), while the other phenotyp-

ic correlations were positive (0.298, 0.882, 0.869, 0.806, 0.788, 0.93). The genetic correlation of guard hair length with underwool length and the rate of two hair length was negative (. 0.941, . 0.983), while the rest of genetic correlatiosn were positive (0.983, 0.731, 0.972, 0.981, 0.622, 0.992, 0.641, 0.987).

Phenotypic and genetic tendency of growth and fur traits

The phenotypic trends of all traits in male and female animals between 2005 and 2011 are reported in Fig 1. For the body weight and body length, the phenotypic changes from 2005 to 2010 appear to be flat and then decline (female) or rise (male) until 2011. The phenotypic trend of other three traits was unchanged during six generations with fluctuations in 2007 and 2008.

The annual genetic trends for all traits are given in Fig 2. For all the traits, the genetic trend from 2005 to 2011 appears flat. The genetic trend of body weight and body length was close to zero and positive, respectively. For the body length, the curve of genetic trend showed upward trend. The slope of genetic trend for GL was steeper (-0.00395±0.00126 cm/yr; $P<0.05$) than for UL (-0.00258±0.0000846 cm/yr; $P<0.05$) and RATE (-0.0000385±0.00011/yr; $P>0.05$) (Table 3).

Discussion

Heritability estimates for growth and fur quality traits

The pelt length, fur quality, color type and other pelt charactertics have had marked influence on the price of mink pelt. There is now a general consensus that body size and fur traits are the most important economic traits in mink pelt, and selecting and breeding in modern animal production has led to an increase in the mature size and improvement in the fur quality (Liu *et al.*, 2011).

A number of researches show that the parameters of growth trait are highly heritable (Sengul & Kiraz, 2005). In this study, the estimation of heritability for body weight and body length was 0.41 and 0.53, which showed moderate-to-high heritability. The value was similar to the estimates of 0.42±0.07 and 0.45±0.07 reported by Hansen (1997) on standard and mahogany type, and 0.478 for standard and pastel color type for body size and conformation, but lower than 0.54 for body weight found by Pingel et al. (1986) and 0.72 for body length found by Lohi and Hansen (2004).

Estimates of heritability of guard hair length and underwool length were 0.520 and 0.521, respectively. Genetic parameter for fur quality has been rarely reported, and the assessment for the fur quality is based on subjective scoring, which is commonly expressed in scores or referred to as grading trait (Peura et al., 2005). Heritability estimate of fur quality is usually moderate by this kind of method. Heritability estimates for fur quality was 0.235 in the study by Socha et al. (2006); between 0.10 and 0.38 for fur quality (estimates related to the gender and color type, Bosting and Clausen, 1986); between 0.24 and 0.93 for hair length, between 0.33 and 0.49 for hair density. The measurement method of hair what we used was more accurate by measuring from skin to the top of hair by ruler. The assumption is that transformation of a continuous quantitative trait to a categorical trait leads to smaller heritability because the transformation introduces inaccuracy or poorer resolution in the measurements.

Phenotypic and genetic correlations

Estimates of phenotypic correlations between RATE and BW, BL, GL, UL were negative and moderate. The negative correlations between body weight and fur quality of minks have been detected also by Lohi and Hansen (2004). The high positive phenotypic correlations were found among BW, BL, GL, and are in the general range of values cited in literature (Kempe *et al.*, 2013; Peura *et al.*, 2005).

Most of the genetic correlations were positive, for example, between the BW and BL (0.983), between BW and GL (0.731), between BW and UL (0.982), respectively, implying that getting bigger size of mink leads to longer pelt and better fur quality. Negative correlations were found between BL and GL (-0.941), between BL and RATE (-0.983). These results are in agreement with the findings by Socha et al. (2006). The majority of the genetic correlations were negative, for instance, between fur quality and body size (-0.543).

Phenotypic and genetic tendency of growth and fur traits

The genetic trends of BW and BL downward during 2003 and 2006, the trend of BL was steep upward after 2006. The annual genetic trend for BW and BL found in this study was smaller than that reported by Socha (2006) for standard and pastel color type mink body size and conformation (0.1) between 1996 and 2004. In mink selection, high body weight has shown a positive response within a few generations. This is the case both with selection for early body weight at four weeks after birth and selection for body weight in September and in November (Nielsen et al., 2009). Thus, the main reason for the differences in the genetic trends between these color type mink might be the result of different breeding model and feeding management. Our results show that there has been small genetic improvement in BW and BL and indicate that as the important role of breeding goal, BW and BL particularly for BL, will be effective in silver blue mink in long term selection. This is in agreement with the heritability estimates for BW and BL obtained in the current and in a previous study for mink and fox (Nielsen *et al*., 2012; Peura *et al*., 2004b). The genetic changes of GL, UL and RATE between 2003 and 2010 appear to be flat, which was in agreement with the studies of Socha et al., (2006) on fur quality of mink. The low and negative values of these trends may prove the difficulties in the selection on traits of fur quality in mink population.

Acknowledgements

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Fig 1. *Phenotypic change tendency of growth and fur trait in silver blue mink*

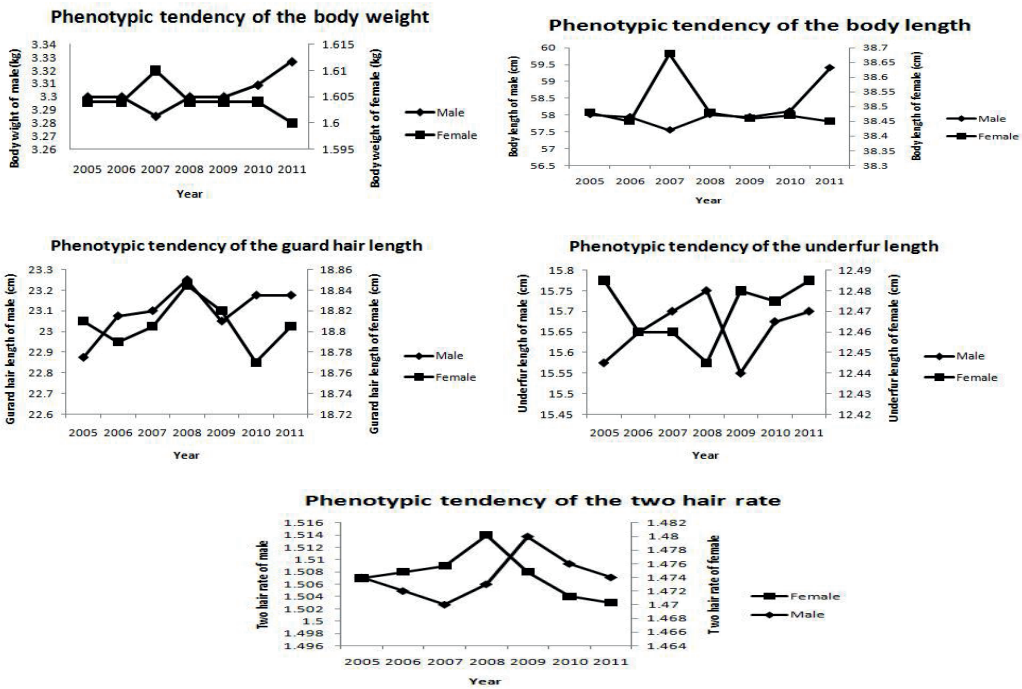

Fig 2. *Genetic change tendency of growth and fur trait in silver blue mink*

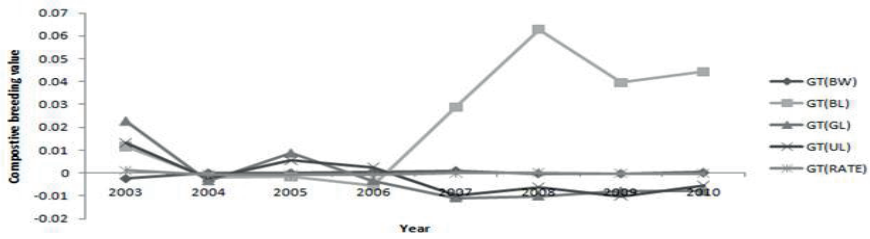

Table 1. *Total number (N) of observation's means and standard deviation for the studied traits in the silver blue mink from 2005 to 2011*

Year	Sex	N	BW	BL	GL	UL	RATE
2005	**M**	40	3.30±0.119	58.01±3.675	22.88±0.822	25.58±0.636	1.48±0.054
	F	200	1.61±0.075	38.48±3.415	18.81±0.883	12.49±0.657	1.52±0.054
2006	**M**	40	3.30±0.119	57.94±3.697	23.08±0.829	15.65±0.700	1.47±0.050
	F	198	1.61±0.074	38.45±3.430	18.79±0.820	12.46±0.640	1.51±0.058
2007	**M**	40	3.29±0.105	57.55±3.935	23.10±0.841	15.70±0.723	1.47±0.044
	F	200	1.61±0.072	38.68±3.560	18.81±0.830	12.46±0.630	1.51±0.059
2008	**M**	40	3.30±0.119	58.01±3.675	23.25±0.776	15.75±0.707	1.47±0.054
	F	200	1.60±0.075	38.48±3.415	18.85±0.833	12.45±0.623	1.51±0.060
2009	**M**	40	3.30±0.119	57.94±3.690	23.05±0.815	15.55±0.677	1.48±0.055
	F	200	1.60±0.074	38.46±3.420	18.82±0.829	12.48±0.649	1.51±0.056
2010	**M**	40	3.31±0.120	58.11±3.808	23.18±0.812	15.68±0.694	1.48±0.053
	F	200	1.60±0.074	38.47±3.415	18.77±0.825	12.48±0.657	1.50±0.053
2011	**M**	40	3.33±0.129	59.40±4.630	23.18±0.812	15.70±0.723	1.47±0.054
	F	200	1.61±0.733	38.45±3.510	18.81±0.801	12.49±0.672	1.50±0.057

M: male, F: female; BW: body weight; BL: body length; GL: the length of guard hair; UL: the length of underwool; RATE: the rate of two hair length

Table 2 . *Estimates of genetic and phenotypic parameters among growth and fur quality traits in silver blue mink (heritability on diagonal, phenotypic correlations above diagonal and genetic correlations below diagonal)*

	BW	BL	GL	UL	RATE
BW	0.410	0.928**	0.882**	0.869**	− 0.218**
BL	0.983	0.530	0.806**	0.788**	− 0.178**
GL	0.731	0.622	0.530	0.93**	− 0.074**
UL	0.972	− 0.941	0.992	0.520	−0.425**
RATE	0.981	− 0.983	0.641	0.987	0.521

h^2: direct heritability; BW: body weight; BL: body length; GL: the length of guard hair; UL: the length of underwool; RATE: the rate of two hair length

Table 3. *Genetic trends measured as regression of yearly mean EBV for body weight, body length, guard hair length, underwool length, the rate of two hairs*

	Genetic trend	significance
BW	0.000198±0.0001	0.205
BL	0.007920±0.0027	0.278
GL	-0.003950±0.0013	0.0295
UL	-0.002580±0.00008	0.0225
RATE	-0.000038±0.00011	0.7259

Genetic markers associated with economically important traits in mink (Neovision vison) - preliminary results

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Abstract

The aim of the study was to find association between microsatellite polymorphism and economically important traits in mink, and then selecting markers which could be useful in breeding programs. Genotypes of 26 microsatellites reported to be highly polymorphic were detected in 200 minks. The study took into account a few morphological traits, of which three: body length, body mass and body circumference are important for the economics of mink farming. The association between marker genotypes and three studied traits was tested using the Kruskal-Wallis test. Significant association was found between 6 microsatellites (Mvi111, Mvi1302, Mvi4001, Mvi4060, Mvi87, Mvis020) and the studied traits. Four markers (Mvi1302, Mvi4060, Mvi87, Mvis020) were associated with all the studied traits, Mvi111 was associated with body mass and body circumference, whereas Mvi4001 was associated with body mass and body length. Interesting results were obtained for Mvi87 and Mvis020. The markers significantly associated with the studied traits showed statistically significant differences between genotypes. Reported microsatellite polymorphism and its association with economically important traits may be potentially used in marker assisted selection.

Keywords: microsatellites, body weight, body length, body circumference

Introduction

Understanding the genetic background of traits that play an important role in animal breeding allows to carry out effective selective breeding. In case of fur animals, in addition to coat colour, a significant impact on final product – the pelt – is by the shape and size of animals. Searching for associations between microsatellite polymorphism and economically important traits in mink (especially fur quality traits), has been investigated by many authors. Associations between candidate genes and known QTLs important for fur and reproductive traits and microsatellites have been studied by Anistoroaei [2011], Thirstrup [2014], and Demontis [2011].

The aim of the study was to find association between microsatellite polymorphism and economically important traits in the American mink, and then selecting markers which could be useful in breeding programs.

Materials and methods

200 American minks (100 males and 100 females) were studied. The animals represented mostly brown and black colour variety, only a few were shaphire. Three morphological traits important in fur breeding were analysed: body weight (BW, g), body length (BL, cm) and body circumference (BC, cm). Each individual was genotyped for 30 microsatellites. The multiplex PCR reaction and analyses of fragment size on the sequencer were applied for genotyping. To determine the possible associations between microsatellite genotypes and the studied traits the Kruskal-Wallis rank sum test with multiple comparison was used. The statistical analyses were performed using R statistical package (R Core Team 2015).

Results

Genotypes of 26 microsatellites (out of 30 microsatellites studied) reported to be highly polymorphic were detected. For the remaining four we did not get any products of PCR reaction. Genotypes were obtained only for 64 up to 199 animals, depending on the analysed sequence.

Significant association between six microsatellites (Mvi111, Mvi1302, Mvi4001, Mvi4060, Mvi87, Mvis020) and the studied traits was found. Four markers (Mvi1302, Mvi4060, Mvi87, Mvis020) were associated with all the studied traits, Mvi111 was associated with BW and BC, whereas Mvi4001 was associated with BW and BL. Interesting results were obtained for Mvi87 and Mvis020. These markers significantly associated with the studied traits showed statistically significant differences between genotypes. The results obtained are shown in Tables 1 – 4.

Table 1. *Descriptive statistics of traits which were significantly influenced by the Mvi 1302 microsatellite genotypes*

trait	genotype	descriptive statistics						
		n	minimum	median	maximum	mean	sd	VC [%]
BW	218/220	7	1400	2200	2900	2271.43[a]	492.32	21.67
	222/222	47	750	1200	2800	1423.40[b]	518.76	36.45

n = number of individuals, SD = standard deviation, VC = variation coefficient (%)
Mean values differing statistically significant between the groups designated by each genotypes were marked by different letters (p-value < 0.05).

Table 2. *Descriptive statistics of traits significantly influenced by the Mvi 4001 microsatellite genotypes*

trait	genotype	descriptive statistics						
		n	minimum	median	maximum	mean	sd	VC [%]
BW	221/221	15	1000	1800	2750	1840.00[a]	563.53	30.63
	221/223	8	2200	2500	3600	2618.75[b]	464.40	17.73
	225/225	13	750	1900	2500	1807.69[a]	512.29	28.34
BL	221/221	15	37	43	47	42.87[a]	3.23	7.53
	221/223	8	43	48	50	47.50[b]	2.20	4.64
	225/225	13	37	44	47	42.77[a]	3.14	7.34

n = number of individuals, SD = standard deviation, VC = variation coefficient (%)
Mean values differing statistically significant between the groups designated by each genotypes were marked by different letters (p-value < 0.05).

Table 3. *Descriptive statistics of traits significantly influenced by the Mvi 87 microsatellite genotypes*

trait	genotype	descriptive statistics						
		n	minimum	median	maximum	mean	sd	VC [%]
BW	81/81	22	1500	2200	2500	2109.09 [a]	302.62	14.35
	81/84	11	1000	1250	1500	1290.91[bc]	168.55	13.06
	81/88	9	750	1200	1700	1205.56 [b]	306.64	25.44
	84/84	8	1000	2225	3600	2090.28 [a]	617.35	29.53
	84/86	17	700	1100	1200	1075.00 [b]	166.90	15.53
	84/88	16	1000	1200	1700	1214.71 [b]	202.92	16.71
	86/88	36	750	1225	1800	1215.63 [b]	253.46	20.85
	86/86	24	750	2250	3100	2125.00 [a]	593.26	27.92
	88/88	39	750	2100	3000	2014.10[ac]	533.60	26.49
BL	81/81	22	42	45	49	45.09 [a]	2.07	4.59
	81/84	11	37	39	43	39.64[bc]	1.86	4.69
	81/88	9	37	40	41	39.22 [b]	1.30	3.32
	84/84	36	38	45	50	44.14 [a]	3.45	7.81
	84/86	8	36	38	40	38.00 [b]	1.20	3.15
	84/88	17	33	38	43	38.82 [b]	2.30	5.92
	86/86	24	36	46	49	44.50 [a]	3.68	8.28
	86/88	16	36	39	48	39.19 [b]	2.74	6.99
	88/88	39	37	45	50	44.21[ac]	3.31	7.49
BC	79/88	6	14.0	16.3	18	16.08[a]	1.50	9.31
	81/81	22	17.5	20.8	24	20.57[b]	1.55	7.55
	81/84	11	15.0	17.0	20	17.23[a]	1.29	7.50
	81/88	9	9.0	16.0	19	16.67[a]	1.41	8.49
	84/84	8	14.0	21.0	25	20.67[b]	2.79	13.51
	84/86	17	14.0	16.0	18	16.00[a]	1.31	8.18
	84/88	16	15.0	17.0	20	17.18[a]	1.39	8.10
	86/86	36	14.0	21.0	25	20.83[b]	2.62	12.59
	86/88	24	13.0	17.0	19	16.91[a]	1.57	9.30
	88/88	39	13.0	20.5	25	20.27[b]	2.52	12.44

n = number of individuals, SD = standard deviation, VC = variation coefficient (%)

Mean values differing statistically significant between the groups designated by each genotypes were marked by different letters (p-value < 0.05).

Table 4. *Descriptive statistics of traits significantly influenced by the Mvi 020 microsatellite genotypes*

trait	genotype	descriptive statistics						
		n	minimum	median	maximum	mean	sd	VC [%]
BW	160/160	13	1000	2250	3000	2150.00[ac]	523.21	24.34
	160/173	16	750	1225	1500	1212.50 [b]	206.16	17.00
	160/179	5	1000	1200	1400	1180.00[ab]	148.32	12.57
	172/173	5	1000	1200	1250	1150.00[ab]	100.00	8.70
	173/173	67	750	2000	3100	1916.42[ac]	579.41	30.23
	173/175	8	750	1100	1700	1143.75 [b]	306.40	26.79
	173/179	7	750	1200	1300	1092.86 [b]	188.04	17.21
	175/175	8	1400	2250	2500	2100.00[ac]	433.42	20.64
	177/177	10	1800	2100	2600	2120.00[ac]	265.83	12.54
	179/179	20	1100	2225	3600	2187.50 [c]	581.49	26.58

BL	160/160	13	42	46.0	49	45.62 [a]	1.98	4.34
	160/173	16	36	39.0	42	38.88 [b]	1.54	3.97
	172/173	5	33	37.0	39	36.60 [b]	2.30	6.29
	173/173	67	37	43.0	50	43.28[ac]	3.87	8.95
	173/175	8	37	38.5	41	39.00[bc]	1.51	3.88
	173/179	7	36	38.0	40	38.00 [b]	1.41	3.72
	179/179	20	38	46.0	50	45.00 [a]	3.01	6.69
	181/181	6	43	46.0	48	45.67[ac]	1.97	4.31
BC	160/160	13	14	21.5	24.5	20.88 [a]	2.62	12.52
	160/173	16	13	17.0	20.0	16.69 [b]	1.67	10.02
	173/173	67	13	20.0	25.0	19.87 [a]	2.59	13.04
	173/175	8	14	16.0	18.0	16.25[bc]	1.39	8.55
	173/179	7	14	16.0	18.0	15.93[bc]	1.43	8.96
	175/175	8	18	20.5	24.0	20.88[ac]	1.89	9.03
	177/177	10	19	20.8	23.0	20.80 [a]	1.42	6.82
	179/179	20	16	21.8	25.0	21.23 [a]	2.66	12.55

n = number of individuals, SD = standard deviation, VC = variation coefficient (%)
Mean values differing statistically significant between the groups designated by each genotypes were marked by different letters (p-value < 0.05).

It can be noted that heterozygous genotype at loci Mvi1302 and Mvi4001 is associated with higher value of the studied traits, whereas at the other two loci (Mvi87 and Mvis020) the homozygous genotypes were advantageous.

Discussion

Reported microsatellite polymorphism and its association with economically important traits may be potentially used in marker assisted selection. Thirstrup *et al.* (2014) investigated 104 microsatellite sequences and found QTLs which were associated with guard hair thickness, guard hair length, wool density, surface, quality and skin length. The QTLs' positions on the chromosomes were adequately determined by the studied microsatellite markers. Lei et al. (2009) reported significant differences between genotypes of Mvi4013 and Mvi586 which were associated with awn diameter. The authors also found significant differences between Mvi4013, Mvi4015 and Mvi4001 genotypes as regards vellus length and reported associations between vellus diameter and Mvi4006, Mvi4001 and Mvi2243 genotypes. A significant association of microsatellite markers and exterior traits have also been found in the silver fox population (Zatoń-Dobrowolska et al., 2014). The results indicated associations between ten microsatellites and body weight (BW), body length (BL), body circumference (BC) and tail length (TL). The FH2613 microsatellite was associated with BW and BC, FH2097 with BL and BC, ZUBECA6 with BW and BC, whereas marker REN75M10 was associated with BL and TL. The estimated linkage disequilibrium (LD measured using r^2 ranged from 0.15 to 0.33) was strong between nine loci with significant effect on the analyzed economically important traits.

Acknowledgements

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Are there more alleles in the b-locus (TYRP1) in the American mink?

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Abstract

In American mink *(Neovison vison)*, a total of 12 phenotypes display a brown appearance in a wide range. All brown phenotypes are inherited as a simple recessive trait, thus only homozygous individuals display the phenotype. One important colour in the brown spectrum of mink is American Palomino (AP) symbolized as *kk* in the Scandinavian nomenclature and $b^{p}b^{p}$ in the American nomenclature. Recently a large insertion of approximately eight kb in intron 2 of the *TYRP1* gene (referred as the "b-locus" in other mammalian species) has been found in the *AP* phenotype segregating in a back cross in Pearl mink. This insertion results in the brown phenotype in this family. In the same study other brown phenotypes, were found to have the same insertion in homozygotic form. However, not all animals with brown phenotype are homozygous for the insertion allele; therefore, further investigation of other possible mutations located in the *TYRP1* gene is warranted.

Keywords: mink, genetics, mutations, b-locus, colour-gene

In American mink *(Neovison vison)*, a total of 12 phenotypes display a brown appearance in a wide range (Nes *et al.* 1988). However, the farming and commercial names of these colours are not based on a very clear phenotype description but rather on a specific grading. The latter is set at the fur centers where a certain phase is graded by experts and/or machines and allocated to a commercial name. All brown phenotypes are inherited as a simple recessive trait, thus only homozygous individuals display the phenotype. The various brown coat colors in American mink have a brownish appearance that can vary within the phenotype but also during the animal life. However, for commercial purposes the colours are recorded at the pelting time, which is when the fur colour has reached its "maturity". One important colour in the brown spectrum of mink is *American Palomino (AP)* symbolized as *kk* in the Scandinavian nomenclature and $b^{p}b^{p}$ in the American nomenclature (Nes *et al.* 1988). The *AP* mutant appeared first in a farm in Canada in the middle of the XXth century (King, 1951). The coat colour appears as a clear pale tan, and on a prime pelt it easily turns slightly red while the eyes are pink or red (Nes *et al.* 1988). Currently, only few pure *AP* mink are commercially produced as the colour is used particularly in combination with genes encoding grey colour such as the Aleutian colour gene $(b^{p}b^{pll})$ (Anistoroaei *et al.* 2012) and the Silverblue dilution gene $(b^{p}b^{p}dd)$ (Cirera *et al.* 2013). It is also segregating in back-crosses of the AP phenotype $(b^{p}pb^{p}p)$. Another brown phenotype, which has a darker appearance than the *AP*, is commercially called *Dawn* and the gene name assigned to this variant is $b^{D}b^{D}$.

In American mink, Shackelford (1980) described for the first time that different mutations of the *b* locus, alone or in combination with other loci bring different brown colour phases such as Green eyed pastel *(bgbg)*, Moyle buff *(bmbm)*, Ambergold *(abab)* and Socklot *(bsbs)*. In several species, brownish phenotypes have been found to be associated with the *b* locus *(TYRP1)*: in dog (Schmutz *et al.* 2012), pig (Ren *et al.* 2011), sheep (Gratten *et al.* 2007), cat (Schmidt-Küntzel *et al.* 2005 and others), cattle (Nonneman *et al.* 1996; and others), horse (Rieder *et al.* 2001), it has been reported that brown phe-

notypes are caused by specific mutations in the *TYRP1* gene. Moreover, a small number of mutations in the *TYRP1* gene have been found to cause oculocutaneous albinism type 3 in humans (Kenny *et al.* 2012).

Tyrosinase-related protein-l *(also known as 5,6-dihydroxyindole-2-carboxylic acid oxidase, DHICA, TYRP1)* was named as such due to its homology to tyrosinase (Jackson, 1988). It is one of the three tyrosinase related protein (TRP) family members, besides *Tyrosinase (TYR)* and *Dopachrome Tautomerase (Dct).* These three enzymes are type I membrane glycoproteins with significant structural homology, and they are all targeted to and located in the melanosomal membrane. They are part of a cascade reaction in the melanogenic pathway controlling melanin production in melanosomes (Hearing, 1999; & Hearing, 2005). TYRP1 is one of the most abundant glycoproteins in melanocytic cells (as reviewed by Kobayashi & Hearing, 2007), and it thus plays an intrinsic role in the formation and stabilization of melanogenic enzyme complexes. While TYR product abnormalities lead to oculocutaneos albinism, also described in mink (Anistoroaei et al. 2008; Benkel *et al.* 2009), TYRP1 has distinct catalytic functions in melanin synthesis downstream of TYR. In contrast to mutations in TYR, mutations in TYRP1 affect the quality of synthetized melanin rather than the quantity (Kobayashi & Hearing, 2007). In brown mouse, the melanin produced by a mutant TYRP1 gene is less polymerized compared to the melanin in wild type mice (Ozeki et al. 1995). TYRP1, in addition to having enzymatic functions, stabilizes TYR and co-expression of TYR with TYRP1 in melanocytes increases pigmentation (as reviewed by Kobayashi & Hearing, 2007). Moreover, TYRP1 determines whether eumelanic pigment will be black (when active) or brown (when inactive or absent) (Bennett *et al.* 1990; Zdarsky *et al.* 1990). In mink, brown types produce only slightly less eumelanin than wt mink. The *TYRP1* gene is represented by 7 exons with a full length coding sequence of 1.6 kb, yielding a protein of 537 amino acids, including the signal peptide (Sturm et al. 1995). The mature glycosylated protein has a molecular weight of about 75 kDa (Halaban & Moellmann, 1990; Hearing & Tsukamoto, 1991).

We have mapped the locus responsible for one of the mink brown phenotypes - *AP* – to be TYRP1 gene. Comparisons of the *TYRP1* genomic DNA sequences between *wt* and the *AP* mink, revealed a large insert at the beginning of intron 2. The insert was also detected in a *Dawn* phenotype by long range PCR. This is the first reported case of a b-locus mutation caused by an enlarged intronic insertion in the animal world. The findings of the present study enable the development of genetic tests for analyzing the color selection in American mink and supplement the catalogue of brown colours in mammal species involving the TYRP1 protein.

One individual from another brown mink phenotype commercially named Dawn was also investigated at the molecular level by long-range PCR and the same size insertion appears to be present. By this we suggest that certain modifiers of TYRP1 would induce different brown colour degradation, which results in at least two different phases of brown.

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Genetic markers associated with economically important traits in mink *(Neovision vison)* - preliminary results

Are there more alleles in the b-locus (TYRP1) in the American mink?

Part 4. Nutrition, Feeding & Management

Amino acid availability from protein meals of different quality to mink kits

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Abstract

Three protein ingredients with expected different quality; lamb meal (LM), poultry meal (PM) or fishmeal (FM) were used as main protein source in three extruded diets given to 12 mink males at age 8-11 weeks in a Latin square design. The diets had similar, but lower protein content than recommended (23-24 % of metabolizable energy) to ensure growth response. The LM diets with expected low quality revealed lower level of digestible essential AA than the PM and FM diets. Nitrogen retention differed significantly and was determined to 0.86, 1.04 and 1.30 g /$BW^{0.75}$/d, and the growth rate to 8.2, 26.8 and 35.3 g/d of the diets, respectively. Different essential AA content and digestibility was the main factors for the variation in growth response. Methionine is the most limiting AA in mink and the suggested requirement of methionine for mink kits at the same age as in the present study is 0.31g/MJ. The LM, PM and FM diets supplied 0.17, 0.26 and 0.33 g methionine /MJ, respectively, and was therefore probably the main reason for the observed difference in growth. The study demonstrated that information on AA availability of protein ingredients is crucial for obtaining adequate growth in mink kits.

Keywords: digestibility, protein efficiency ratio, nitrogen retention, growth

Introduction

Mink has a high dietary protein requirement and reliable information on protein quality of feed ingredients is necessary for correct diet formulations that will meet the requirement and give optimal animal performance (Lassen et al. 2012). Main factors in protein quality assessment are amino acid (AA) content, composition and digestibility. Amino acid availability is a term defined as the proportion absorbed in a form utilizable by the animal. Nitrogen balance studies with protein ingredients provide the most complete information on AA availability as digestibility, urinary nitrogen excretion, nitrogen retention is determined. In growing animals, protein efficiency ratio (PER) which is g growth/g protein indigested is a useful measure that sum up the AA availability factors and enable ranking of ingredients regarding protein quality and availability.

Due to high protein and AA requirement, mink is likely to show a pronounced growth response to differences in protein quality. The original objective of the present study was therefore to investigate if growing mink can be a model to evaluate protein and AA bioavailability in extruded dog foods applying protein ingredients with known different digestibility and AA content to demonstrate the limitations of using dietary content as a measure of nutritional adequacy. Parts of this study concerning growth parameters have been published earlier (Ahlstrøm *et al.* 2013), but not in relation to AA

digestibility and AA plasma concentration and comparisons to current knowledge and guidelines for dietary AA supply.

Material and methods

The selected protein sources were intended for the pet food market, and included lamb meal (LM), poultry meal (PM) and fish meal (FM). The extruded diets were produced at Centre for Feed Technology, Norwegian University of Life Sciences, Ås, Norway, and more details of the production is described elsewhere (Tjernsbekk et al., 2014). The diets were designed to have different protein digestibility and AA content, but similar CP content. Chemical composition of the diets is presented in Table 1. Digestibility of CP was measured in adult mink as part of a pre-experimental screening process, and was found to be 67.7 %, 80.9 % and 87.5 % in the LM, PM and FM, respectively. The experiment was performed at Copenhagen University, Fur animal laboratory, Rørrendegård, Taastrup, Denmark. More details in (Ahlstrøm et al. 2013).

Bioavailability of protein and AA was measured as apparent total tract digestibility (ATTD), N balance and PER, during three balance periods in 12 male kits as a 3 x 3 latin square design. The balance periods included a three days adaptation period and four days with quantitative collection of faeces and urine and accurate registrations of feed intake.

The extruded dog foods and faeces were analysed for dry matter (DM), gross energy (GE), ash, N, crude fat, starch and AA according to standard methods described in Tjernsbekk et al. (2014). Plasma AA were determined with method described in Larsson et al. (2012). Metabolizable energy (ME) was calculated based on digestibility data, using the following equation: ME (kJ) = g digestible protein * 18.42 kJ + g digestible fat * 39.76 kJ + g digestible carbohydrate * 17.58 kJ (Lassén et al., 2012). Data of N balance was calculated in relation to metabolic weight of the mink kits, so that comparisons could be made across periods. Digested N (DN, g/kg $BW^{0.75}$/day) was calculated as N intake – faecal N, whereas retained N (RN, g/kg $BW^{0.75}$/day) was calculated as N intake – (faecal N + urinary N). Protein efficiency ratio (PER) was calculated as: PER = weight gain (g/day)/protein intake (g/day). Apparent total tract digestibility of nutrients was calculated as: ATTD (%) = ((nutrient ingested (g) – nutrient in faeces (g))/nutrient ingested (g)) * 100.

Statistical analyses of data was performed with the SAS 9.3 computer software (SAS Institute Inc., Cary, NC, USA). Data from the mink experiment were analysed by use of the MIXED procedure, according to the following model: $Y_{ijkl} = \mu + \alpha_i + \varepsilon ijkl$, where μ = general mean, α_i = fixed effect of diet= α_i, ε_{ijkl} = random error component. The results are presented as least-square means.

Results

Mink kits fed the PM and FM diets had a greater ($P < 0.05$) feed intake than kits fed the LM diet, whereas DM intake was similar ($P > 0.05$) between diets (Table 2). Intake of ME differed ($P < 0.05$) between diets, and was lowest with the LM diet and highest with the FM diet. A similar tendency was observed for the N intake ($P = 0.08$). As expected, excretion of faecal N differed ($P < 0.05$) between diets, which was highest for the LM diet, intermediate for the PM diet and lowest for the FM diet. Excretion of urinary N was greater ($P < 0.05$) for the FM diet than the PM diet, with an intermediate excretion for the LM diet. N retained was found to be lower ($P < 0.05$) for the LM diet compared with the PM and FM diets. The utilization of digested N for retention (RNDN) was lower ($P < 0.05$) with the LM diet than with the PM or FM diets.

The FM diet resulted in a higher ($P < 0.05$) average body weight of the mink kits than the PM and LM diets (Table 2). Body weight of the animals increased ($P < 0.05$) from period 1 to 3. Since PER values are influenced by the weight gain of the animals, it was shown that also the PER values were higher ($P < 0.05$) for the PM and FM diets than the LM diet. Differences between diets in PER values during the three, separate experimental periods showed the same pattern as for the weight gain values (data not shown).

Digestibility of main nutrients and all AA was different ($P < 0.05$) between diets, except for Hyp (Table 3). Generally, digestibility was lowest in the LM diet, intermediate for the PM diet and highest in the FM diet. Differences in digestibility of main nutrients between diets affected the ME content, which was 13.7, 15.0 and 16.5 MJ/kg, for the LM, PM, and FM diet, respectively (Table 3). The ME contribution from protein was 23 % for all diets.

Plasma AA did not differ significantly between diets, but was numerically highest for the LM diet and lowest for the FM diet (Table 4). Essential AA concentration was significantly lower with the LM diet compared with the PM diet, while the FM diet showed intermediate values and did not differ from either of the other diets. For the plasma concentration of non-essential amino acids, the FM diet revealed lower values than the LM diet, but both similar to the PM diet. For the single AA, significant differences between diets were found for Arg, Lys, Met, and Thr, where concentration was lowest for the LM diet and intermediate for the FM diet, thus partly reflecting the difference in digestible intake. An exception to this was the concentration of Lys, which was numerically highest for the FM diet. Significant differences between diets were also found for Gly, Hyp, Pro and Ser, with highest values found for the LM diet that contained most of these AA.

Discussion

Different digestibility level of protein and AA between the diets were as expected reflected in the N balance study, with significant differences in amount of faecal N excreted when animals were fed the three diets. The amount of N retained was found to be similar when kits where fed the PM and FM diets, with an average of 1.06 and 1.18 g/day/$BW^{0.75}$. Several studies of N-balance in mink kits of the same age as in the present study have found a lowered level of N-retention for diets with a low protein content providing between 18 % and 30 % of ME, compared with diets having a higher protein level (Larsson et al., 2012; Matthiesen et al., 2012; Vesterdorf et al., 2014). The N-retention levels found for the PM and FM diets were comparable to the N-retention level of 1.09 g/kg $BW^{0.75}$/day found by Vesterdorf et al. (2014) when providing a diet with 24 % of ME as protein. Mink kits fed the PM and FM diets retained in average 48.6 % of the N digested. This was comparable to the RNDN levels found by others, where diets containing 18 % of ME and 24 % of ME resulted in RNDN levels of 49.3 % and 49.4 %, respectively (Matthiesen et al., 2012; Vesterdorf et al., 2014). The capacity for N-retention was, however, probably higher than the level observed in the present experiment, as N-retention levels close to 2.5 g/kg $BW^{0.75}$/day and an RNDN level of 55.7 % has been found in mink kits fed a diet with 32 % of ME as protein (Matthiesen *et al.*, 2012).

Based on the N-balance data and growth rates, it was apparent that the LM diet did not support the potential for N-retention and growth in young mink kits. Mink kits probably also have a higher capacity for N retention than the levels observed for the PM and FM diets. Still, the satisfactory level of RNDN and high growth rates observed for these diets despite the suboptimal dietary protein level, indicates a high availability of essential amino acids from these diets and that mink kits ranked the three diets according to the AA supply.

Table 1. *Analyzed chemical composition of experimental diets (g/kg).*

	Diet		
	Lamb meal	**Poultry meal**	**Fish meal**
Dry matter	943.3	914.1	922.0
Crude protein	255.1	248.7	251.3
Crude fat	202.8	186.1	187.7
Starch	257.9	268.8	269.1
Ash	119.6	72.3	70.3
Carbohydrates*	365.8	407.0	412.7
Metabolizable energy (ME), MJ/kg	13.7	15.0	16.5
Protein/fat/carbohydrate ratio, % of ME	23/40/37	23/38/39	23/40/37
Essential amino acids			
Arg	17.2	16.3	14.9
His	5.0	6.1	6.1
Ile	8.5	10.8	11.6
Leu	17.3	19.0	19.8
Lys	12.8	15.1	17.6
Met	3.6	5.0	6.2
Phe	9.5	10.6	10.5
Thr	8.8	9.7	10.2
Val	11.6	12.5	13.0
Total essential amino acids	94.3	105.1	109.9
Non-essential amino acids			
Ala	17.8	15.6	15.2
Asp	18.8	20.5	22.4
Cys	2.7	3.2	3.1
Glu	40.9	42.8	44.1
Gly	30.1	21.1	15.7
Hyp	11.1	5.5	1.9
Pro	21.6	17.0	12.6
Ser	11.5	11.2	11.3
Tyr	7.0	8.2	8.2
Total non-essential amino acids	161.5	145.1	134.5
Total amino acids	255.8	250.2	244.4

*Calculated by difference: carbohydrates = dry matter – (crude protein + crude fat + ash).

In addition, digestibility of the other main nutrients and GE was in disfavor the LM diet. This difference was especially prominent for crude fat digestibility and GE. This was possibly due to more saturated fat in the LM with lower digestibility than with the PM and FM. However, individual fat digestibility varied from 34.4 to 80.5 % with the LM diet, and from 6.3 to 91.4 % with the PM diet. Fat digestibility in the FM diet varied from 77.8 to 94.4 %. This remarkable variation could probably partly be due to individual differences in the ability of the animals to digest fat. Mink kits have a low, immature ability to digest fat, and a low level of fat digestibility in mink kits of the same age as in the present experiment has also been reported by others (Hellwing *et al.*, 2008, 2009).

Table 2. *Feed intake, ME intake, nitrogen balance, body weight, weight gain and PER (g/day/kg $BW^{0.75}$)*

	Diet			P-value	
	Lamb meal	**Poultry meal**	**Fish meal**	**SEM**	**Diet**
Feed intake	193.4[b]	222.6[a]	222.8[a]	7.1	<0.001
DM intake	63.8	67.2	68.0	2.9	0.121
ME intake	0.93[c]	1.10[b]	1.22[a]	0.04	<0.001
Nitrogen balance					
N intake	2.76	2.93	2.97	0.1	0.076
Faecal N	0.91[a]	0.76[b]	0.53[c]	0.03	<0.001
Digested N	1.85[c]	2.16[b]	2.44[a]	0.08	<0.001
Urinary N	1.19a[b]	1.11[b]	1.25[a]	0.05	0.006
Retained N	0.67[b]	1.06[a]	1.18[a]	0.05	<0.001
% RN of DN	35.23[b]	48.82[a]	48.46[a]	1.60	<0.001
Body weight (g)	1110[b]	1120[b]	1171[a]	54.2	0.001
Weight gain (g/d)	8.2[b]	26.8a	35.3[a]	2.90	<0.001
PER	0.38[b]	1.38a	1.71[a]	0.14	<0.001

In accordance with the lower proteolytic (Elnif, 1988) and pancreatic lipase activity (Hedemann *et al.*, 2011) found in young mink compared with adults, the ability of the mink kits to digest crude protein and crude fat in the present experiment was lower than digestibility observed in adult mink fed the same diets in an earlier experiment (Tjernsbekk et al., 2014). The difference was most pronounced for fat digestibility, which averaged to 14.8 % units, while the average difference in crude protein digestibility was 3.5 % units. The difference in crude protein digestibility between adult mink and mink kits was in line with the results reported from others (Skrede, 1978; Tauson, 1988). Both Skrede (1978) and Tauson (1988) found a numerically higher fat digestibility in adults than kits, but not to same extent as in the present experiment. This divergence may partly explained by differences regarding dietary factors like fat sources and ash content.

Reports regarding differences in amino acid digestibility between mink kits and adults is scarce. In the present experiment, such differences varied between the amino acids. Amino acid digestibility was in average 4.5 percentage units lower in the kits compared with the adults, although a higher digestibility of His, Thr and Ser was found in the kits. It is probable that differences in endogenous secretions between adult and young animals had affected the results, since they were apparent values. The difference in digestibility between kits and adults was most pronounced for Met with an average of 7.7 percentage units lower in the kits. The difference in Met digestibility was, however, dependent on diet, since it decreased from 11.6 percentage units with the LM diet, to 7.3 and 4.1 percentage units with the PM and the FM diets, respectively.

Concentration of amino acids in blood plasma has been found to increase with increased dietary protein intake in mink (Larsson *et al.*, 2012). In the present experiment, digestible amino acid intake varied significantly (Table 4). Still, amino acid intake seemed to be only modestly reflected in the concentration of amino acids in blood plasma. For most AA it was even observed that the PM diet had the numerically highest concentration in plasma, although the intake generally was significantly highest for the FM diet. The lowest total concentration of essential amino acids found for the LM diet did, however, correspond well with the low intake of digestible essential amino acids for this diet.

Table 3. *Digestibility of main nutrients and amino acids in growing mink (%)*

	Diet				P-values
	Lamb meal	**Poultry meal**	**Fish meal**	**SEM**	**Diet**
Dry matter	66.7^{c}	75.6^{b}	81.5^{a}	0.983	<0.001
Crude protein	66.8^{c}	74.0^{b}	82.1^{a}	0.547	<0.001
Crude fat	68.1^{b}	77.0^{ab}	88.5^{a}	5.981	0.001
Total CHO	79.5^{b}	82.9^{a}	83.9^{a}	0.433	<0.001
Energy	71.0^{c}	77.8^{b}	84.4^{a}	1.903	<0.001
Essential amino acids					
Arg	82.6^{c}	87.2^{b}	91.1^{a}	0.618	<0.001
His	72.8^{c}	80.6^{b}	86.2^{a}	1.174	<0.001
Ile	72.7^{c}	81.4^{b}	88.4^{a}	0.801	<0.001
Leu	76.7^{c}	83.4^{b}	89.6^{a}	0.691	<0.001
Lys	74.1^{c}	82.2^{b}	89.9^{a}	1.085	<0.001
Met	66.5^{c}	78.7^{b}	87.8^{a}	1.031	<0.001
Phe	79.4^{c}	83.7^{b}	87.8^{a}	0.676	<0.001
Thr	64.4^{c}	75.7^{b}	81.2^{a}	1.014	<0.001
Val	69.2^{c}	77.4^{b}	85.4^{a}	0.954	<0.001
Non-essential amino acids					
Ala	77.2^{c}	81.7^{b}	87.4^{a}	0.792	<0.001
Asp	42.7^{c}	58.7^{b}	75.6^{a}	1.984	<0.001
Cys	3.3^{b}	32.9^{a}	36.7^{a}	3.447	<0.001
Glu	77.3^{c}	84.2^{b}	90.1^{a}	0.792	<0.001
Gly	74.8^{c}	77.6^{b}	82.0^{a}	1.011	<0.001
Hyp	54.2	55.9	49.9	3.330	0.239
Pro	78.5^{c}	82.2^{b}	85.2^{a}	0.700	<0.001
Ser	71.1^{c}	80.4^{b}	84.5^{a}	1.095	<0.001
Tyr	72.1^{c}	79.3^{b}	84.9^{a}	0.964	<0.001

The lack of a more clear-cut difference in blood plasma amino acid concentration between diets in the present experiment could probably be due to the low and relatively similar protein concentration in the diets used. According to Fernández-Fígares *et al.* (1993), however, there are inconsistent reports of whether or not there exists a relationship between dietary intake of amino acids and the concentration of amino acids in plasma. Despite this, they observed that for the most limiting dietary amino acids, the rank order in dietary protein and plasma paralleled each other (Fernández-Fígares et al., 1993). This fits fairly well with the present findings, at least for Met which was the amino acid with lowest dietary intake.

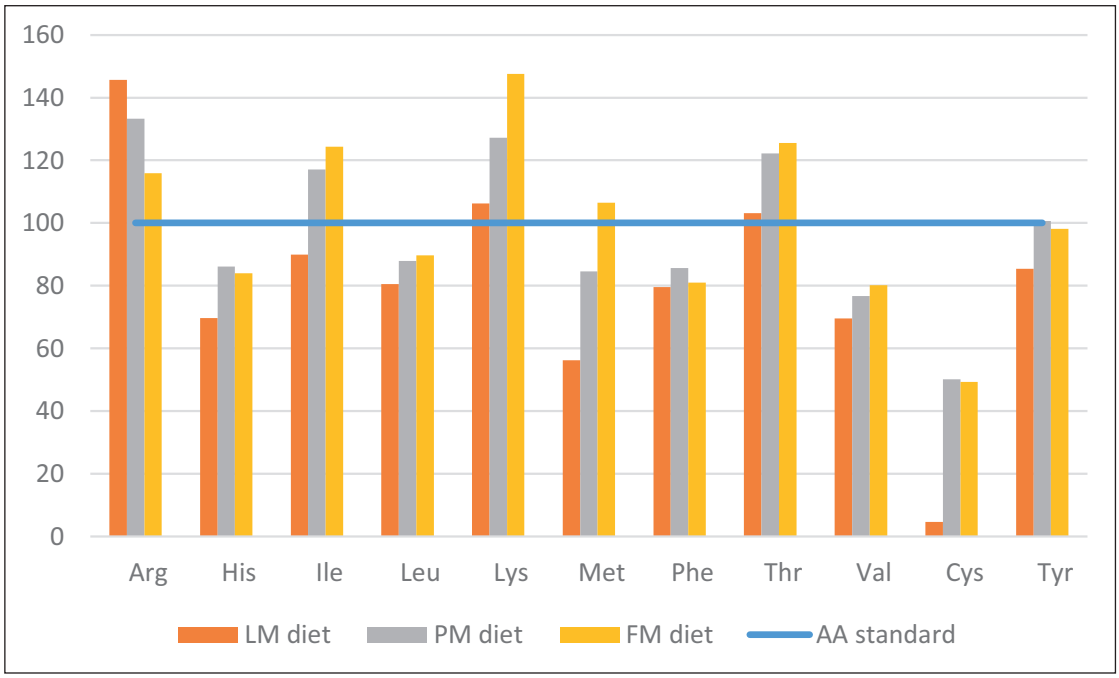
All diets used in the present experiment contained deficient amounts of His, Phe, Tyr, Leu and Val in relation to the current standard used for amino acid requirements in mink kits (Figure 1). Despite this, mink kits had a satisfactory level of N-retention and growth when they were fed the PM and FM diets, in which the average concentration of His, Phe, Tyr, Leu and Val was 0.33, 0.58, 0.43, 1.07, 0.66 g/MJ ME, respectively. These results implies that the true requirement of these amino acids in the early growth period really is lower than the levels set as the maximum requirement by Børsting

Table 4. *Amino acids in blood plasma, µmol/ml.*

	Diet				P-values
	Lamb meal	Poultry meal	Fish meal	SEM	
Essential amino acids					
Arg	0.099[b]	0.176[a]	0.126[b]	0.016	0.002
His	0.069	0.083	0.071	0.005	0.135
Ile	0.061	0.070	0.060	0.004	0.178
Leu	0.117	0.122	0.106	0.008	0.332
Lys	0.084[b]	0.143[a]	0.161[a]	0.012	<0.001
Met	0.042[b]	0.058[a]	0.053[ab]	0.003	0.007
Phe	0.087	0.093	0.084	0.005	0.479
Thr	0.125[b]	0.175[a]	0.168[ab]	0.013	0.020
Val	0.153	0.157	0.139	0.008	0.297
Tot EAA	0.836[b]	1.072[a]	0.969[ab]	0.065	0.054
Non-essential amino acids					
Ala	0.494	0.442	0.397	0.036	0.108
Asp	0.022	0.020	0.019	0.001	0.072
Asn	0.059	0.064	0.057	0.005	0.565
Cys	0.001	0.001	0.001	0.000	0.109
Glu	0.116	0.127	0.137	0.007	0.088
Gln	0.602	0.630	0.686	0.035	0.240
Gly	0.766a	0.614[b]	0.442[c]	0.033	<0.001
Hyp	0.232a	0.205[a]	0.120[b]	0.013	<0.001
Pro	0.201a	0.149[b]	0.110[c]	0.013	<0.001
Ser	0.274a	0.219[b]	0.151[c]	0.014	<0.001
Tyr	0.062	0.073	0.061	0.005	0.130
Tot NEAA	2.829[a]	2.544[ab]	2.180[b]	0.111	0.002
TotAA	3.665	3.620	3.149[b]	0.168	0.070

and Clausen (1995, 1996), although long-term effects of the lowered levels on growth, fur quality and health are unknown. Kits fed the LM diet had a low growth rate and a significantly higher amount of the digested N excreted as urinary N. This implies that one or more amino acids were supplied in deficient amounts to support the capacity of N retention and growth in mink kits. It is reasonable to think that the restricted growth rate was primarily related to the Met content of the diet, since the concentration of this amino acid was considerable lower in the LM diet compared with the other diets. In addition, the concentration of digestible Met in the LM diet of 0.17 g/MJ ME was below the concentration of 21 g/MJ ME found to reduce growth in mink by Børsting and Clausen (1996). For all diets, the concentration of Cys was far below the level set as recommended level. Both the PM and FM diets contained approximately only 50 % of the this level, with around 0.07 g digestible Cys/MJ ME. A correspondingly low level of 0.08 g Cys/MJ ME was used by Børsting and Clausen (1996) without negative effects on growth or fur quality. For the LM diet, however, it is probable that the extremely low level of digestible Cys contributed to the suppressed growth rate found when feeding the animals with this diet.

Figure 1. *Content of digestible AA in the Lamb meal, Poultry meal and Fish meal diet compared with the standard for AA requirements in mink kits (Sandbøl, 2012 in Lassen et al. 2012).*



Conclusion

The crucial importance of knowing AA composition in addition to CP and AA digestibility of protein sources was showed by the results in the present study, where differences in protein quality between diets of similar protein content demonstrably affected N-retention, weight gain and PER in mink kits. Differences in AA composition and digestibility between the protein ingredients were the main factors affecting protein quality.

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Differences between the kidneys of black and brown mink

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Abstract

Black mink are often considered more sensitive to changes in feed and environment than brown mink and the number of black mink dying during the growth period for various reasons is higher than the number of brown mink. To investigate if there could be any difference in kidney function we examined the water balance in groups of black and brown male mink in October, and at pelting the kidneys were examined histologically. The results showed differences in water balance and urine and histopatological changes of the kidneys.

Keywords: water balance, histopathology, testicles

Introduction

It is important that animals have free access to clean water of good quality and often black mink are considered more sensitive to changes in water supply than brown mink. Black mink are smaller than brown mink and there are significantly more black than brown mink dying with urinary tract problems ($p < 0.0001$) (own results, not publ.). Christensen (2014) found greater relative testicle weight in black males compared to brown males and there are more black than brown males with histological lesions in the testes and epididymis (Vangsgaard *et al*., 2014). The lesions found in the testes and epididymis in black male mink by Vangsgaard *et al*., 2014 were compatible to descriptions of autoimmune orchitis previously described to be a genetic disorder linked to black colortype in mink (Tung *et al* 1981, Tung *et al* 1984, Onderka, 1996).

To further investigate the difference between black and brown male mink we examined the water balance in groups of black and brown male mink in October and at pelting the kidneys, testicles and epididymis were sampled for histopathological examination.

Material and methods

To investigate the water balance of mink kits at the end of the growing season we used 20 brown and 20 black male mink. The water balance experiment lasted for 11 days starting October 27 and ending November 7. The test period was divided into 7 days of acclimatization followed by 4 days of collection. In the collection period daily records of feed consumption, water consumption, stool volume and urine volume was performed (Tinggaard, L., & Hvam, K., 2010) and the water balance was calculated. The animals used were born within a week around May 1 and from a litter size of 6 to 9. The kits were fed feed from the local Feed Kitchen with the energy distribution 27:58:15 (protein, fat, carbohydrates resp.) and an energy content of approximately 8.2 MJ / kg (196 kcal per 100 grams). The animals were fed ad libitum. Animals were weighed at the beginning and at the end of the experimental period. Blood samples were taken on October 30 after 12 hours of fasting. The blood was

analyzed for creatinine and urea. At pelting in November the animals were autopsied, kidneys and testicles were measured and weighed. Tissue samples for histopathologic analysis were fixed in 10% neutral buffered formalin for 3 days after which the tissues were processed routinely and embedded in paraffin wax. Sections of 4-5 µm were cut and stained with hematoxylin and eosin (HE). Masson trichrome stain was applied for visualization of collagen (Luna, 1968). For immunohistochemical staining a commercial mink Igg was applied (Rabbit anti-Mink IGg (HRP), Biorbyt, Cambridge, Cambridgeshire, UK).

The calculations were performed using the statistical program SAS (SAS Institute Inc., 1996). Feed intake, water consumption and urine analyzes were tested using PROC MIXED. The models included the main effects of the color type and day in the collection period as well as interactions between these main effects. Differences in weight and blood variables was tested with GLM. Differences between groups was considered significant at a probability level of less than 5%.

Results and discussion

Body Mass Index (BMI) at pelting in November was similar in black and brown mink but brown male mink are both heavier and longer than black male mink. During the investigation period black mink had a bigger weight loss than brown mink.

There was no difference in feed intake and amount of feces between brown and black mink, but the first day of collection the intake was lower than on the other days. However, there were differences in the uptake of drinking water ($p = 0.003$), black males drank more than brown males and on days where the feed consumption was highest the drinking water uptake and amount of urine was also highest. Black male mink have a higher water balance per 100 kcal, as compared to brown males (45.7 vs 33.6, $p = 0.04$), water in manure per 100 kcal was significantly higher, drinking water was higher (not significant), and urine quantity was lower (not significant). There was no difference between the days in the registration period. The total water intake by 100 kcal was 86.4 for black male mink and 77.1 for brown (NS). This corresponds to earlier investigations where a total water intake of 67-86 grams per 100 kcal in adult brown male mink was found (Clausen, 2012; Farrel & Wood, 1968; Neil, 1988; Tauson, 1999).

Blood samples showed no difference in hematocrit (hct) and creatinine between the two color types but there was a significant difference in the blood urea between the two types of mink, black had higher value than brown ($p < 0.0001$).

In black male mink the kidney weight in percent of body weight at pelting was higher than in brown male mink ($p < 0.0001$).

There was also a tendency towards greater relative testicle weights in black males compared to brown ($p = 0.07$) this was also found by Christensen (2014).

Histological examination of kidneys sampled at time of pelting showed a significant higher number of black mink with lesions in the kidneys ($p = 0.005$). There were also more black males with histological lesions in the testes ($p = 0.003$) but in epididymis the difference was not significant, this corresponds to investigations of Vangsgaard *et al.* (2014). Results of immunohistochemical investigations are not included in this abstract.

The greater relative kidney weight in black mink compared to brown and the higher blood urea suggests that the kidney function is not optimal in black mink. This may explain that black mink appear more sensitive to changes in water supply than brown mink. We also found a higher prevalence of lesions in the kidneys of black mink, compared to Brown mink. No infective agents (eg. bacteria were identified in or near the lesions in the kidneys). Further studies are necessary in order to determine if this apparently reduced kidney function and the kidney lesions may be linked to the autoimmune orchidis previously described in black mink (Tung *et al* 1981, Tung *et al* 1984).

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The effect of biochar-peat mixture on the gaseous emissions from fur animal manure

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Abstract

Gaseous emissions from fur manure cause local nuisance as well as leakage in the nutrient cycles of agriculture. Cover materials can be used during the storage of manure to reduce both the odour problem and to tighten the nutrient cycles. Peat is known as an effective cover material but its use as a non-renewable resource is questionable whereas biochar could have some additional benefits regarding e.g. the end-use of manure. The use of peat-biochar mixtures was tested in a large-scale laboratory study. Biochar-peat mixture was added on two different fur animal manures; first week as a covering material and during the two following weeks mixed with the manures. The results on the fluxes of ammonia and methane show that biochar-peat mixture reduced the emissions. The reductions increased with increasing proportion of biochar in the mixture. Right after mixing, the emissions increased temporarily but then gradually decreased. The reduction of ammonia emissions during the whole measurement period was 20-30% and those of methane 30-75% depending on the biochar–peat ratio. Biochar-peat mixture can be considered an effective cover reducing both ammonia and methane emissions but a consistent effect requires spreading new cover material approximately every ten days.

Keywords: biochar, fur animal manure, greenhouse gases, emission, ammonia

Introduction

Agriculture is the most significant source of ammonia that causes e.g. odour problems and acidification and is a large loss of nitrogen from agricultural systems. Ammonia is both a local problem as a nuisance to the neighbourhood and a challenge in the framework of the Convention on long-range transboundary air-pollution that sets limits to national emissions (UNECE 2016). The amount of greenhouse gases from fur animal production in Finland is estimated to be 120 Gg CO_2e that is emitted both as methane (CH_4) and nitrous oxide (N_2O) (Statistics Finland 2016). Gaseous emissions from fur manure cause local nuisance as well as leakage in the nutrient cycles of agriculture. This has set the business continuity in uncertainty. New emission reduction solutions are urgently needed.

Cover materials can be used during the animal housing and storage of manure to reduce both the odour problem and to tighten the nutrient cycles. Peat is known as an effective cover material but its use as a non-renewable resource is questionable whereas biochar could have some additional benefits regarding e.g. the end-use of manure. Biochar has the potential to bind nitrogen on its surfaces and slow the diffusion of gases from manure to the atmosphere (Taghizadeh-Toosi *et al.* 2014). As the manure is composted, the biochar also acts as an additive in composting and eventually ends up in agricultural soils where it has the potential to increase water holding capacity and carbon stocks of the soil as well as acting as a liming agent (Sun and Lu 2014). The use of biochar as a litter for fur animal

manure is a new innovation and one possibility to minimize the emissions from fur animal farms.

We estimated the potential and feasibility to use biochar for the odour control on fur farms. This study was the first experimental part of a project developing a novel way of recycling nutrients from fur farms.

Material and methods

In a laboratory test, biochar-peat mixture was added to two different fur animal manures, i.e. fox and mink manure. The properties of the raw materials are presented in Table 1. Three different amounts of biochar-peat mixtures (5%, 10% and 20% of the volume of fur animal manure) were used. All the tests were performed in three replicates.

Table 1. *The properties of the materials used in the laboratory test.*

Test materials	Total N g/kg	Ammonium_N g/kg	Total P g/kg	K g/kg	Dry matter %
Manure, mink	15,99	5,18	9,580	1,824	23,31
Manure,fox	15,99	5,11	10,815	1,585	22,33
Peat	5,94	0,05	0,182	0,077	60,01
Biochar	3,07	0,00	0,388	1,546	94,93

During the first week in a climate chamber the biochar-peat mixture was used as a covering material. In the climate chamber there were three different ambient temperatures used. For the first two days the temperature was set to 2 – 5 °C, for the next two days it was set to 12 – 15 °C and for the last two days it was set to 22 – 25 °C. The relative humidity was set between 60% and 65% for the whole period.

After one week the biochar-peat mixture was first mixed with the manures and then moved to a laboratory with normal room temperature (18-20 °C) and relative humidity (40 -50 %). Thereafter the test was continued for two additional weeks.

The amounts of ammonia and methane gases from the mixtures were measured using a photoacoustic gas analyser (Innova™ Multi-gas analyser). During the first week the measurements were performed every day, as for the last two weeks only two times a week.

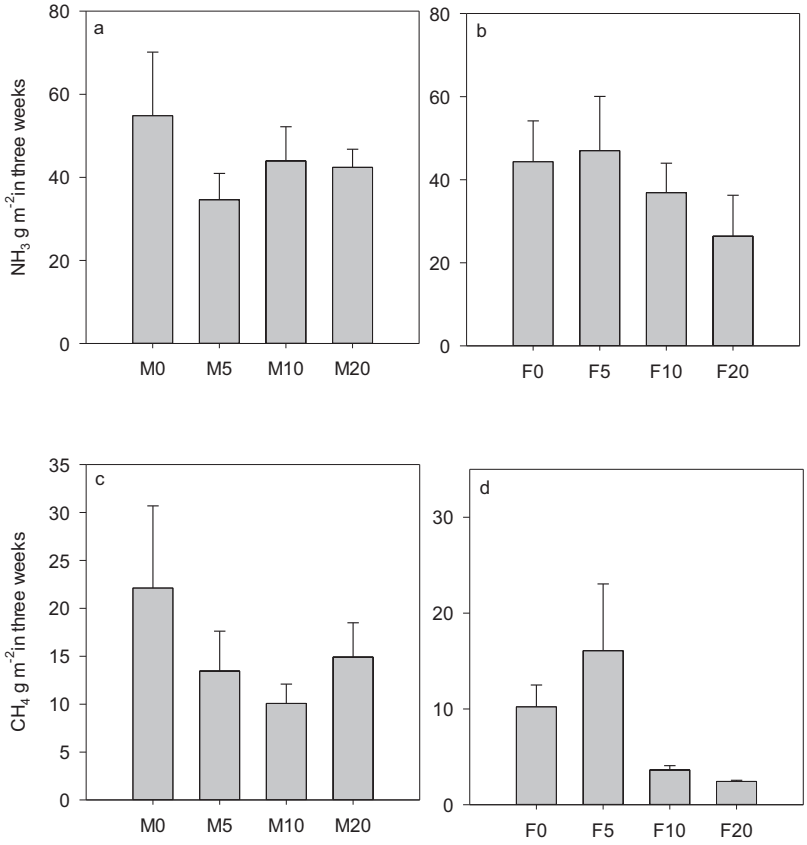
The data for ammonia and methane were analysed using the mixed model REML estimation method of SAS EG software (version 7.12). The values of ammonia were converted to square root and those of methane to logarithmic values to normalize their distribution. The degrees of freedom were calculated using the Kenward-Roger method. Percentage of biochar in the cover and animal type (mink/fox) as well as their interaction were included in the model as fixed effects.

Results

The results showed that biochar-peat mixture used as a covering material reduced the emissions from fur manure. Right after mixing the emissions temporarily increased but then gradually decreased (data not shown). The reduction of ammonia emissions in the cumulative flux in three weeks was 20-30 % and those of methane 30-75 % depending on the amount of biochar –peat mixture being used (Fig. 1).

The more there was biochar in the cover mixture the more ammonia (p=0.024) or methane (p=0.005) flux was reduced. The fluxes of ammonia or methane did not differ between mink and fox manure but there was an interaction between animal type and amount of biochar (p=0.026) in the case of methane indicating a higher mitigating effect for fox manu

Figure 1. *Effect of biochar-peat mixture on the cumulative fluxes (±one standard deviation of the mean) of ammonia from mink manure (a) and fox manure (b) and methane from mink manure (c) and fox manure (d). The number after M (=mink) or F (=fox) indicates the percentage of biochar in the mixture.*



Discussion

Using biochar cover on fur manure has the potential to reduce the gaseous emissions during animal housing and thus could be a solution to the local odour problems. The results indicated that the more biochar there was in the cover mixture, the better is the efficiency of methane and ammonia mitigation. Thus, the decision on biochar use clearly is a question of additional costs on fur farms. The biochar is easily saturated and new material should be added approximately every ten days to maintain the effect. If this could be feasible from the viewpoint of the fur producers, also other advantages in addition to odour control are foreseen.

Biochar is known to increase the water-holding capacity of soils which could be a benefit both in field use and especially if the manure is used as a nutrient source in e.g. growth media. If the manure is spread on soils, the added biochar has the potential to increase soil carbon stocks better than the more

labile carbon of untreated manure (Dias et al. 2010). This could be beneficial from the viewpoint of the climatic impact of agriculture especially if the biochar comes from a production process replacing fossil energy.

As positive effects of biochar cover were detected on farm, it may be expected that this amendment has benefits also in manure off-farm composting. Biochar used as compost amendment can improve the aeration of the process and thus decrease methane production and increase methane oxidation (Sonoki *et al.* 2012). Typically high percentage of ammonia is lost during composting (Tiguia and Tam 2000). As in our study, biochar can reduce these losses (Khan *et al.* 2014) thus increasing the N amount in the fertilizer product but too tight binding of N to the biochar may reduce the fertilizer value of the composted manure. However, there is evidence that the biochar-absorbed N can be well available to crops (Taghizadeh-Toosi *et al.* 2012). As fur manure is typically composted and not used as such, it can be estimated that the net effect of biochar on N availability to crops is positive due to higher retention of N.

It has to be kept in mind that the properties of biochar change in composting. Thus, all results derived from soil amendment with biochar are not valid in experiments with composted biochar. It is possible that the soil greenhouse gas emissions even increase when using composted biochar (Borchard *et al.* 2014). As always in studies with gaseous emissions from agriculture, the total effect considering emission swapping has to be estimated. If the ammonia reduction in composting is large enough a slight increase in nitrous oxide emissions from soil does not matter since some nitrous oxide emission is caused also by ammonia deposition.

There are known benefits of biochar in agricultural soils but its use is limited partly because it is not a pleasant material to handle. From the practical point of view, field application of biochar is simpler and less messy when mixed to manure. These considerations together with the reduced on-farm losses could motivate the use of biochar use in fur production.

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Protein requirement for gestation and retention in fetal tissue in mink *(Neovison vison)*

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Abstract

The mink has, similar to other strict carnivores, a high protein requirement. The requirement for gestation is incompletely known, although the importance of adequate gestational nutrition is well recognized. The objective was to determine the protein requirements before (BEF; 66 dams) respectively after (AFT; 40 dams) implantation. Protein provision made up 10, 15, 20, 25, 30, 35, 40 and 45% of ME (BEF) whereas the two lowest levels were excluded in AFT. Protein adequacy was determined using indicator amino acid oxidation (IAAO) and euthanasia for determination of implantation sites, number and survivability of fetuses. Fetal nutrient and energy retention at different time points after mating was investigated with 116 dams (FET). Implantation occurred at a normal rate even at the lowest protein provision, but embryo survival tended to be compromised when protein provided ≤ 20% of ME (BEF). All levels of protein provision AFT supported a similar rate of implantation, number and survival of fetuses. The IAAO results concurred with the euthanasia data, demonstrating the potential of the method. Late fetal growth is exponential, but only 0.6 g protein was retained in individual fetuses 50 days after mating (FET) confirming the low protein requirement for gestation.

Keywords: Delayed implantation, fetus, pregnancy, nutrition

Introduction

The knowledge regarding nutrient requirements of pregnant female mink is fairly limited but the importance of gestational nutrition, especially protein provision, has, however, long been recognized. Furthermore, it is well known that mink kits are born altricial, and that their body energy reserves are very limited at birth (Tauson, 1994), but still very little is known about the pattern of fetal growth and the quantitative accretion of nutrients and energy in the body. Because of the delayed implantation, stage in gestation can usually not be determined exactly and this hampers the possibilities to get accurate results. The objectives were to determine the protein requirement during gestation in mink before (BEF) and after (AFT) implantation and to monitor and measure protein and energy retained in fetal tissue during fetal development in pregnant mink dams at different time points after mating. Furthermore, an indicator amino oxidation (IAAO) technique was developed as a non-invasive tool for application in mink.

Material and Methods

The protein requirement in female mink was determined before (BEF) and after (AFT) implantation

and the studies were carried out from mating to close to parturition. The experiment was conducted using different levels of protein BEF (10, 15, 20, 25, 30, 35, 40 and 45 % of metabolizable energy (ME) from protein) and AFT (20, 25, 30, 35, 40 and 45 % of ME from protein) implantation. Furthermore, fetal nutrient and energy retention (FET) was studied at different time points from day zero to day 50 after mating.

Animals

A total of 222 dams of the standard brown color type was used in the BEF, AFT and FET studies. Sixty-six dams were used BEF, from mating until euthanization in Mid-April, and 40 dams were used AFT, from 4th of April from when implantation was anticipated to be completed until euthanization in late April just before parturition. The BEF and AFT dams were assigned to the experimental groups according to Table 1.

Table 1. *The experimental design and distribution of dams in before (BEF) and after (AFT) implantation*

Dietary treatment (% of ME from protein)	Before implantation	After implantation
10	4	-
15	8	-
20	11	8
25	11	8
30	11	8
35	7	8
40	7	4
45	7	4
Total number of dams	66	40

The remaining 116 females were used to study fetal nutrient and energy retention from day zero until day 50 after mating. Out of these, 6 dams were not mated and the remaining 110 dams were mated once in the period between 17 March and 25 March in order to avoid a long embryonic diapause, and to as large extent as possible to synchronise time of implantation. The non-mated dams were euthanized at the start of the experiment (0 days after mating), and the remaining dams were euthanized at days 9-12, 24-26, 32-35, 37-40, 41-45, 46-47 and more than 48 days after mating.

Diets

The experimental diets used BEF and AFT implantation were produced on site at the experimental farm, Rørrendegård, Tåstrup, Denmark, with 10, 15, 20, 25, 30, 35, 40 and 45 % of ME from protein, fat accounted for 50 % and carbohydrates accounted of the remaining part of ME. The diets consisted of industrial fish, poultry by-products, fish meal, blood product, corn gluten meal, corn oil, corn starch, potato mash powder, vitamins- minerals and water. The diets were fed BEF from mating to the 4th of April and from the 4th of April to euthanization in AFT. A conventional wet mink diet was used in the FET study (Sjællands Pelsdyrfoder A.m.b.a., Stårup, Denmark).

Experimental procedures

Protein adequacy BEF and AFT implantation was determined by the non-invasive Indicator amino acid oxidation (IAAO) method (Pencharz and Ball, 2003; Elango *et al.*, 2008) using breath tests

measurements with stable isotope technique simultaneously with indirect calorimetry to measure CO_2 production. The method was used and developed for application in mink using 1-^{13}C-L-leucine (99at%, Isotec, Miamisburg, USA) as substrate. The dams were measured once for a 10h period during the study BEF or AFT implantation and kept in balance cages during the measurements. A baseline of $^{13}C/^{12}C$ ratio was measured just before each 10h period followed by an administration of a single oral dose of 1-^{13}C-L-leucine (5mg/kg BW). Breath samples were measured every 10th minute for 10h by the Isotope Ratio Infrared Spectroscopy (IRIS, Wagner Analysen Technik GmbH, Bremen, Germany) and CO2 production by indirect calorimetry every 3rd minute. The cumulative recovery of the ^{13}C dose was calculated from the breath tests combined with the CO_2 measurements.

The dams were euthanized in Mid-April or late April in the BEF and AFT study, respectively. Anasthesia was achieved using intramuscular injection of 1.0 ml Ketaminol and 0.5 ml Narcoxyl per kg body weight (BW) (50 mg/ml and 20 mg/ml, respectively, InterVet International B.V. Boxmeer, The Netherlands). After loss of reflexes the animals were euthanized by excision of the heart. The uterus was removed and number of implantation sites and the survival rate of the fetuses were determined visually. In the FET study, the uteri were weighed and when implantation sites were present, the uteri were opened and fetuses retrieved and, when possible, their weight and length were recorded. On a subset of dams fetal livers were excised and weighed and the weight was scaled to the fetal body mass. From a second subset of dams fetuses were used for chemical analyses and for calculation of retention of energy and nutrients in fetal tissue.

Chemical analyses

In the FET study the uteri were analyzed for dry matter (DM) and nitrogen (N), and in the subset of fetuses for chemical analyses two individual fetuses and a pool of remaining fetuses per litter were analyzed for DM, ash, N, fat, gross energy (GE). The analytical procedures are described in details elsewhere (Matthiesen *et al.*, 2010)

Statistical analyses

The unbalanced data for implantation sites and fetal survival rates were analyzed with the MIXED procedure in SAS using protein supply as fixed effect in the BEF and AFT study. The GLM procedure in SAS was used in the FET study with a model analyzing the effect of number of days after mating on the dependent variables. Results are presented as LS-means and pairwise comparisons between LS-means were carried out using the PDIFF option. Differences between means were considered significant if $P<0.05$ and a tendency if $P<0.10$.

Results and discussion

All females except one had implantation sites at the time of euthanasia in the BEF and AFT studies. Number of implantation sites, number of live fetuses and survival rates were not affected by protein provision BEF or AFT. However, a tendency towards an effect of protein provision on fetal survival rates in the BEF study resulted in dams fed 15% of ME from protein having significantly lower fetal survival rates than dams fed 25-45% of ME from protein (Table 2). The preliminary IAAO results (data not shown) did not show any effects of protein provision in measurements carried out shortly after mating (from mating to Mid-March), indicating that the protein requirements of the BEF dams were fulfilled. This concurred with the euthanization data where implantation among BEF dams occurred even at very low levels of protein.

Table 2. *The number of implantation sites, live fetuses and fetal survival rates before (BEF) and after (AFT) implantation*

	Percent of metabolizable energy from protein								RR	P-value
	10	15	20	25	30	35	40	45		
BEF, *n=66*										
Implantation sites,	10	8.9	10.1	10.2	11.4	11.3	11.4	11.8	3.6	NS
Live fetuses	8.2	7.7	8.2	9.7	10.7	10.8	11.0	11.7	3.4	NS
Survival rate, %	84.6^{ab}	75.5^{a}	85.2^{ab}	96.8^{b}	94.2^{b}	96.5^{b}	97.1^{b}	99.1^{b}	18.2	0.1
AFT, n=40										
Implantation sites,			8.7	8.3	10.6	10.0	9.5	9.0	2.8	NS
Live fetuses			7.1	7.6	10.1	8.9	9.0	8.5	3.1	NS
Survival rate, %			81.3	91.4	95.7	89.1	95.4	93.1	19.6	NS

Values with different superscripts (a,b) in a row differ significantly ($P < 0.05$).

The IAAO results, from measurements performed closer to implantation, indicated that the protein requirement increased in early April around the time of implantation and that 10 to 15% of ME from protein was below the requirement. This was supported by the lower embryo survival rates among the BEF dams fed the lowest protein levels. No differences in IAAO data were found in the AFT study, similar to the euthanization results (Table 2). However, there was a numerically lower fetal survival rate among dams fed 20% of ME from protein which is in agreement with previous findings by Vesterdorf *et al* (2012) suggesting that the protein requirement after implantation is somewhat above 20% of ME from protein.

The first occasion when fetuses could be retrieved from the uteri in the FET study was 35 days after mating and sufficient mass for chemical analyses was retrieved 40 days after mating (Table 3). The proportion of total fetal mass to uterine mass increased from only 6.8% 35 days after mating to 45% 50 days after mating. Similar patterns of uterine growth and relation between fetal mass and uterine mass have been recorded in pigs (Noblet et al., 1985), showing that in early gestation the uterus with fluids and placenta make up the major part and then as fetuses grow the relative importance of fluids and placenta decline.

The decline in liver as % of BW from almost 12% at 40 days after mating to 6% at 50 days after mating could be compared with the even lower values of 3.5% found in neonatal mink kits (Tauson, 1994) and underline the vital importance of the liver in fetal metabolism. The DM content in the fetal bodies was very low and the main part of the DM was made up by protein. Both DM and protein increased with increasing fetal age but was still lower at 50 days after mating than values found in neonatal kits (Tauson, 1994). The fat content accounted for 0.6-0.7% of DM and did not increase as gestation progressed. The fat content of 1.4% in neonatal kits (Tauson, 1994) was higher than found in the fetuses but still very low and derived mainly from structural fat. The fetuses and neonatal kits do therefore not have any fat reserves for mobilization. The increase in GE with increasing fetal age reflected an increase in DM and protein content of the fetal bodies. The energy retained per fetal body up to 50 days after mating (19 kJ) is very low compared to the dams daily ME requirement for maintenance (527 kJ/ kg $BW^{0.75}$, Chwalibog et al., 1980), even when assuming a low utilization of ME for protein retention in fetal tissue. Energy retained in the fetal body is in fair agreement with the estimate of 31 kJ retained in neonatal kits with a BW of 11.1g (Tauson, 1994) indicating a very rapid accretion during the late part of fetal life.

Table 3. *Weight, liver weight, length of fetuses from day 35 to 50 after mating and chemical composition of fetuses from day 40 to 50 after mating in fetuses in different stages of fetal development*

	Days after mating					P-value
	35	40	45	47	50	
Weight and length of fetuses						
Weight of all fetuses, g	2.40[a]	10.3[b]	22.3[c]	43.4[d]	58.3[e]	<0.001
Fetal weight, % of uterus	6.8[c]	13.2[c]	21.0[bc]	40.1[a]	45.4[a]	<0.001
Weight of individual fetuses, g	0.164[a]	1.14[b]	2.43[c]	6.02[d]	6.37[d]	<0.001
Length of fetuses, mm	13.6[a]	24.9[b]	35.4[c]	53.6[d]	57.5[e]	<0.001
Liver data						
No. of observations	0	12	22	24	17	
Weight of liver, g	-	0.198[a]	0.229[a]	0.409[b]	0.407[b]	<0.001
Liver, % of body weight	-	11.8[a]	9.6[b]	6.7[c]	6.4[c]	<0.001
Chemical composition						
No of litters		2	3	6	7	0.1
Weight of fetuses		1.10	3.22	6.04	6.78	0.02
Dry matter, %		9.9[a]	11.0[a]	12.9[b]	13.1[b]	0.02
Ash, %		1.2	1.4	1.6	1.5	0.02
Crude protein, %		7.8[b]	7.6[bc]	9.0[ac]	9.1[a]	0.006
Fat, %		NA	0.64	0.66	0.68	NS
Gross energy, kJ/g		21.71[a]	23.97[a]	28.07[b]	27.98[b]	0.02
Retention in fetuses						
Retained protein, g		0.09	0.26	0.55	0.63	0.11
Retained fat, g		-	0.02	0.04	0.05	NS
Energy retained in protein, kJ		2	6	13	15	0.11
Energy retained in fat, kJ		-	1	2	2	NS
Energy retained, kJ		2	8	17	19	0.13

Values with different superscripts (a,b,c) in a row differ significantly ($P < 0.05$).

Conclusion

It can be concluded that implantation in the BEF study occurred even at the lowest levels of protein provision (10-15% of ME) whereas embryo survival rates tended to be compromised for protein levels below 20% of ME from protein. These findings indicate that the protein requirement is low in the first part of gestation and increasing as getting closer to the time of implantation as indicated by the IAAO results. The fetal survival rates were not affected by protein provision from 20 to 45% of ME in the AFT study. However, they were numerically lower in dams fed 20% of ME from protein, which suggests that the protein requirement after implantation in mink was fulfilled at protein levels slightly above 20% of ME. The low amount of energy and protein retained in fetal tissue up to 50 days after mating supports the low requirement for protein early in gestation and suggests that gestation cannot be considered as a particularly energy and protein demanding life stage in the production cycle of the female mink.

Acknowledgements

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Mineral and vitamin supplementation for mink

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Abstract

During the last years some feed kitchens in Denmark have started to use more organic minerals in mink feed because of higher bioavailability than the normally used inorganic forms. In a growth experiment we compared performance of organic minerals and inorganic minerals in different levels ranging from current recommendation to no extra supplementation on skin traits in brown mink. Results showed no significant difference in weight at pelting, skin length, skin quality, colour or purity, Moreover the study demonstrated sufficient of most minerals in Danish mink feed to fulfill minks needs with use of current raw materials. Finally we summarize recent studies on vitamin supplementation for mink in Denmark applying different levels ranging from current recommendation to no extra supplementation in multiple experiments that clearly demonstrate potential for future optimizing of both mineral and vitamin supplementation in Danish mink feed.

Keywords: *Neovison vison*, mink feed, Denmark, feed kitchens

Introduction

Mink feed is supplemented with minerals to prevent deficiencies and ensure optimal growth and fur development. During the last years some feed kitchens in Denmark have started to use organic minerals in mink feed because of higher bioavailability than the normally used inorganic forms. For many animals feed is supplemented with minerals to avoid deficiencies resulting in lower productive performance (Nollet *et al*. 2007). Zn deficiencies have been shown to result in reduced growth and hair changes in mink (Mejborn, 1986; Tauson *et al*., 1992). However the supposedly better performance of organic minerals also come at a higher price on the minerals and therefore it is important to evaluate if they offer a cost effective replacement to current practice and thereby to study if it is possible to reduce the dosage in mink feed. Therefore the aim was to evaluate the use of organic minerals in comparable and lower dosage compared to inorganic minerals in a growth experiment at Kopenhagen Farm. Similar experiments was conducted for vitamins E and B.

Materials & methods

In this experiment we included 7 groups of 135 brown mink pair, experimental setup is showed in table 1. There are several producers of organic minerals and in this experiment two different types were used, type A from Alltech (chelat based amioacid-hydrate; Bioplex Cu, Bioplex Zn og Sel-Plex 2300) and type B from Vilomix (chelat based on glycine). Mink kits were placed in the experimental groups in beginning of July and the experimental diet were fed from July 15th.

Mineral premixes were produced following recommendation for the Danish feed kitchens (15700 ppm Zn; 1280 ppm Cu; 100 ppm Se) (Lassén, 2014).

All male mink kits were weighted after they were placed in the experimental groups 8th July, 12th August, 22nd September an at pelting in November. After pelting all male skin were length measured and sorted by skin quality, silkiness, volume, color and purity.

The statistical calculations were performed using the statistical program SAS (SAS Institute Inc., 1996). GLM (ss4), LSMEANS/PDIFF procedures was used with a 5% significance level in calculations on weight and length. Appropriate covariates were included in the case they were significant. For skin quality, color and purity a non-parametric test (GENMOD) was used. For silkiness and volume PROBIT procedure was used.

Table 1. *Experimental setup for mineral trial growth period*

Group	Number of males	Minerals/ recommendation	Type / Product
36 Control	135	100 %	Inorganic minerals
29	135	50 %	Inorganic minerals
31	135	0 %	-
34	135	100 %	Organic minerals / A
30	135	50 %	Organic minerals / A
32	135	25 %	Organic minerals / A
33	135	50 %	Organic minerals / B

Results

Results from the growth experiment are shown in table 2, including weight of male mink 8/7, 12/8, 22/9 and at pelting. Results from table 2 show a significant difference in weight in the beginning of the experiment 8/7 and on the 22nd of September but not at pelting. Moreover table 3 shows no significant differences between any groups in the growth experiment in skin length, skin quality, silkiness, volume, color or purity of male skins.

Table 2. *Average weight of male mink kits 8/7, 12/8, 22/9 and at pelting.*

Group	Mineral	Weight 8/7 g	Weight 12/8 g	Weight 22/9 g	Weight pelting g
36	K100	1127 (166) ^{c}	2069 (185)	3025 (290) ^{c}	3632 (402)
29	K50	1141 (160) ^{bc}	2035 (204)	3051 (321) ^{bc}	3589 (435)
31	K0	1166 (171) ^{ab}	2081 (214)	3100 (330) ^{ab}	3655 (437)
34	A100	1158 (148) ^{ab}	2034 (187)	3014 (304) ^{c}	3566 (380)
30	A50	1148 (152) ^{abc}	2058 (222)	3055 (330) ^{bc}	3605 (421)
32	A25	1162 (164) ^{a}	2088 (220)	3155 (333) ^{a}	3660 (447)
33	B50	1165 (145) ^{a}	2071 (203)	3034 (299) ^{bc}	3652 (402)
		0,05	NS (0,08)	0,001	NS

NS indicates that there is no significant difference between groups; Different letters in a column indicates that there is difference between groups; Numbers in parentheses are the standard deviation

Table 3. *Average skin length and skin traits.*

Group	Mineral	Skin length cm	Quality *	Silkiness %	Volume Flat	Normal	Heavy
36	K100	95,7 (4,1)	6,2 (2,6)	15,4	19,2	71,5	9,3
29	K50	95,7 (4,0)	6,7 (2,4)	17,6	13,6	76,0	10,4
31	K0	95,6 (4,1)	6,3 (2,5)	15,3	9,2	77,8	13,0
34	A100	95,6 (4,0)	5,9 (2,5)	15,8	15,7	75,6	8,7
30	A50	95,5 (4,3)	6,5 (2,4)	18,3	9,2	76,3	14,5
32	A25	96,1 (4,5)	6,6 (2,7)	21,1	12,5	74,2	13,3
33	B50	96,1 (4,1)	6,3 (2,5)	14,2	13,4	74,8	11,8
		NS	NS	NS	NS		

* Scale from 1 – 12 and 12 the bested; NS indicates that there is no significant difference between groups; Different letters in a column indicates that there is difference between groups; Numbers in parentheses are the standard deviation.

Tabel 4. *Color and clarity*

Group	Mineral	Color	Clarity
36	K100	3,8 (0,9)	2,1 (0,7)
29	K50	4,0 (0,9)	2,1 (0,7)
31	K0	4,0 (0,8)	2,0 (0,7)
34	A100	4,1 (0,8)	2,1 (0,7)
30	A50	3,9 (0,7)	2,1 (0,8)
32	A25	3,8 (0,8)	2,0 (0,7)
33	B50	3,9 (0,9)	2,2 (0,7)
		NS	NS

NS indicates that there is no significant difference between groups; Different letters in a column indicates that there is difference between groups; Numbers in parentheses are the standard deviation. Color ranked by Kopenhagen Fur scale xpale = 1 and xxdark = 6; clarity ranked by Kopenhagen Fur scale 1 = most blue and 4 = most red.

Discussion

All results in this growth experiment show no significant effect of mineral supplementation on the skin trait measured. Studying the mineral content in the feed used in the experiment, based on common available raw materials for mink feed production, Figures 1-4, show that the mineral content in the mink feed is well over the established need for mink in the growth period for most experimental groups. The only group that is below the recommendations is group 31 for Zn, but this group had the same pelt quality as the group with the highest Zn addition (group 33). Similar studies have previously described that Zn deficiencies result in reduced growth and hair changes in mink (Mejborn, 1986; Tauson *et al.*, 1992), however we did not observe any reduction in growth. In a previous study supplementing mink feed with chelate minerals no positive effect was observed on skin length, skin quality and color (Clausen & Sandbøl, 2010; 2011). Therefore, as long as Danish feed kitchens have access to good raw materials

with sufficient minerals there is no need to add extra. If necessary to supplement mink feed with minerals this study show no positive effect of the organic forms compared to the commonly used inorganic forms, however it is possible to lower the supplementation using both organic and inorganic minerals as long the mink need is fulfilled. Therefore, we recommend that raw materials are more closely analyzed for mineral content and mink feed not supplemented on feed kitchens unless necessary.

Figure 1. *Zn content in minkfeed in the growth experiemnt (ppm dry feed)*

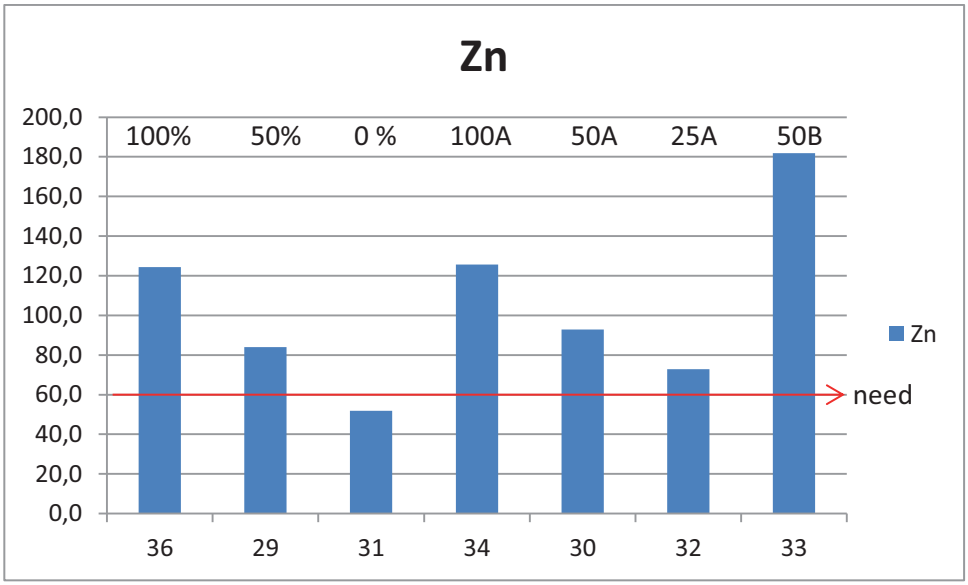


Figure 2. *Se content in minkfeed in the growth experiemnt (ppm dry feed)*

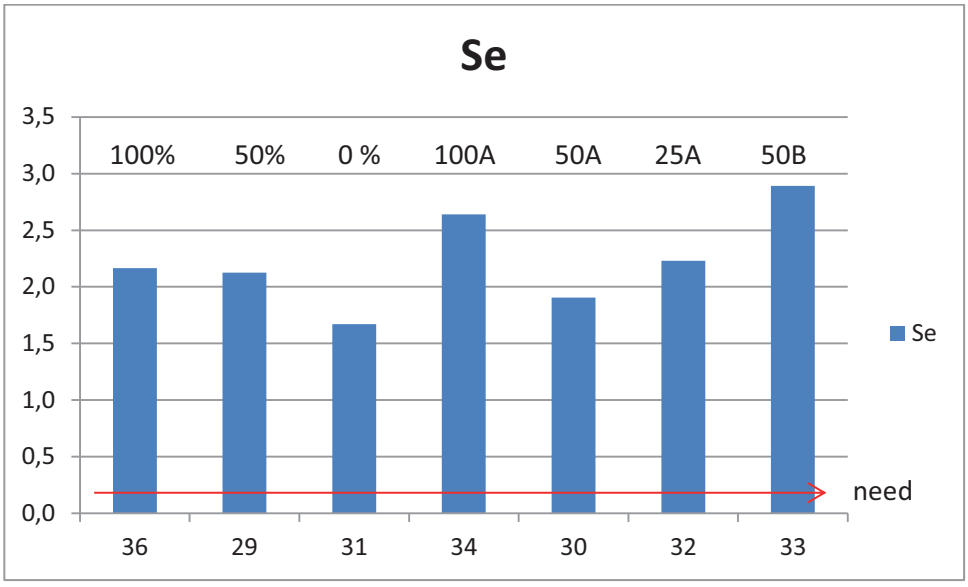


Figure 3. *Cu content in minkfeed in the growth experiemnt (ppm dry feed)*

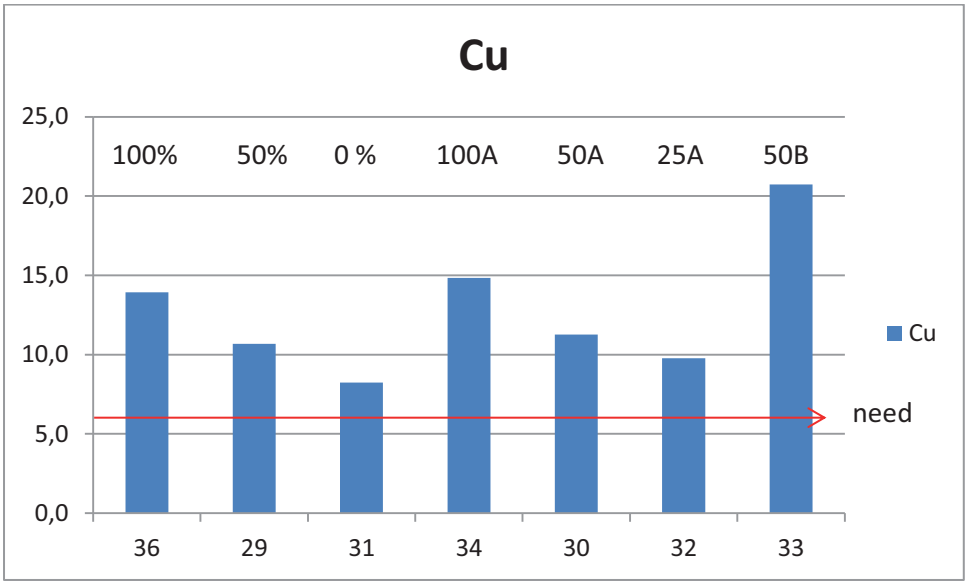
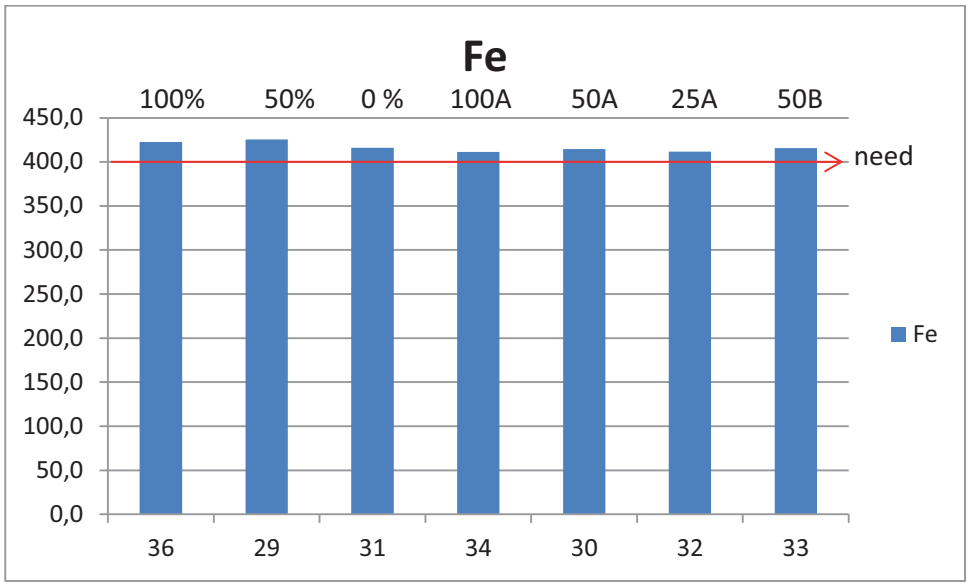


Figure 4. *Fe content in minkfeed in the growth experiemnt (ppm dry feed)*



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Trends in price and productivity in the fur sector

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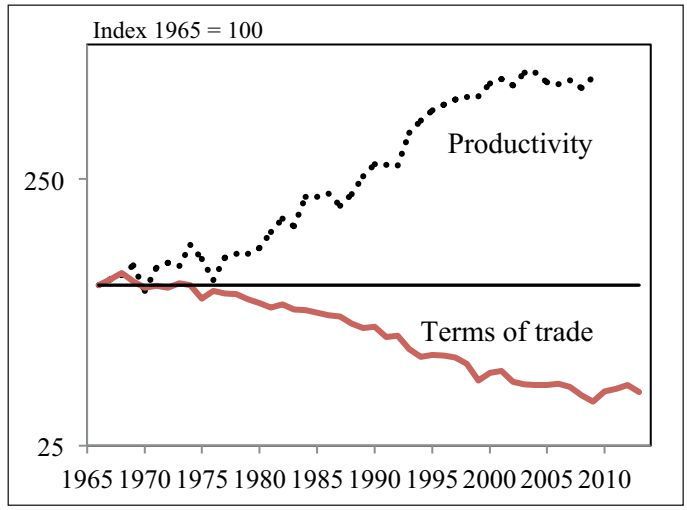
Abstract

Price and productivity are two important competitive factors for the fur skin production worldwide. In the paper it is demonstrated that there are significant long-term trends towards increasing productivity and decreasing price ratios. The trend follows the same pattern as for other agricultural products. The analysis is based on Danish conditions as a case.

By using different measurement methods it is demonstrated that productivity is increasing in several places in fur value chain. The fur skin prices, which are largely determined in international markets, are both very volatile and decreasing in the long term. The terms of trade - the ratio between output and input prices - is also unstable but with a clear negative long-term trend. The developments in prices and productivity cause high demands on management both on individual fur farms and further down the value chain.

Introduction

Prices and productivity play an important role for farmers around the world: Productivity - along with innovation and quality - is an important tool to improve farmers' competitiveness. Often farmers and fur farmers produce relatively homogeneous products for a free and open market with large and almost perfect competition, and this increases the importance of productivity as a competitive tool. Both farmers and fur farmers face a sustained real price decline and declining terms of trade. Figure 1 confirms empirically that for many years, productivity has been increasing, while the terms of trade have fallen - despite widely varying production conditions in the period.



Figur 1. *Development in productivity and terms of trade in Danish agriculture*

Note: Total factor productivity. Terms of trade is output prices / input prices.

Source: Own calculations based on Statistics Denmark (2016 and several issues)

Material and methods

In the paper price and productivity trends for the fur industry are identified, examined and quantified. The analysis is based on Danish conditions as a case - because data are available and well documented, because the Danish fur sector is of significant international importance, and because the trends are global and common for the fur sector in most countries. There will be comparisons with other countries and with agriculture in general.

Results

Productivity changes in Danish fur sector

There are no complete records of productivity growth in the Danish fur animal sector for many years, which can be directly compared with other sectors. However, it is clear that the structural change and scaling up of operations amongst fur animal producers of recent decades has resulted in significant productivity gains, where fewer and fewer employees have created a great increase in production.

Therefore, structural development can be considered a proxy for - or connected with - productivity. Through structural development economies of scale can be utilized, and this also creates improved productivity.

The structural changes almost follow identical global trends, indicating that global drivers push the structural development ahead. As figure 2 shows, the size of mink farms follows similar trends in so different countries as United States, Canada and Denmark.

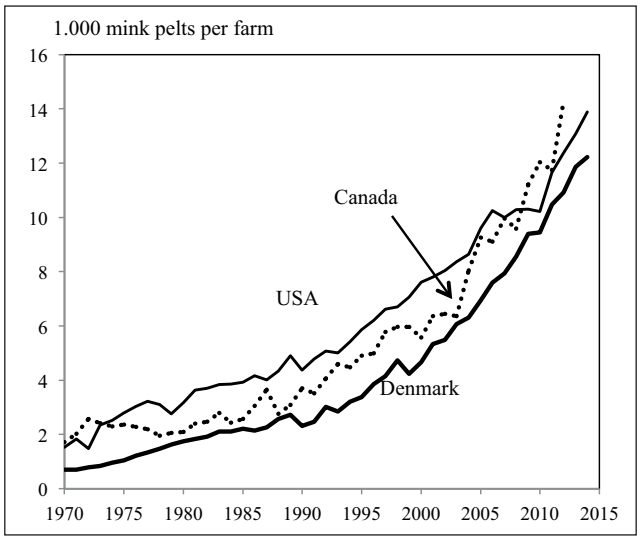


Figure 2. *Size of mink farms (number of produced mink pelts per farm)*

Source: Own calculations based on Statistics Denmark (2016), Kopenhagen Fur (2016a), USDA (several issues) and Statistics Canada (several issues)

Another estimate for the development of productivity can be seen by the fact, that the size of fur skins grow bigger year by year as a result of continuous and systematic breeding. The size of the fur skins can be used for a partial productivity measurement in terms of output (size of fur skin) per partial input (fur animal). As seen in Figure 3, the size (length) of fur skin is increasing year by year.

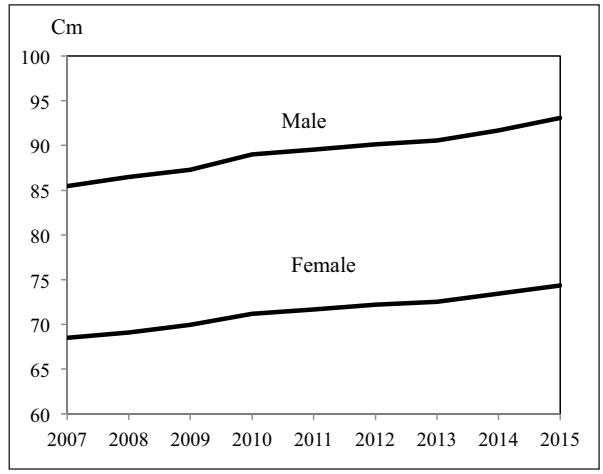


Figure 3. *Average size (length in cm) of Danish mink pelts sold by Kopenhagen Fur, 2007-2015*

Note: Data for the length of fur skins is split into size intervals, for example 83-89 cm, 89-101 cm. etc. The mink farmers will often adjust the length of the skins to fit the group divisions with the smallest possible margin to the lower limit. With a group, for example 83-89 cm, the average size is not 86 cm, but closer to 83. In this case it is assumed that the average size of the skins in each interval is the value for the lower interval + 25 per cent of the interval difference. In this case the average for the interval 83-89 cam is 84.5 cm.

Source: Own calculations based on Kopenhagen Fur (2016a)

The figure shows an almost parallel development of the size of skins for male and female mink animals. In both cases, the annual growth (increase in partial productivity) is one per cent per year.

Another way to illustrate increasing productivity is by measuring the litter size. The Danish data are reported by each fur farm, while data from other countries are calculated as the number of skins sold compared to the number of breeding females. The figures are not directly comparable from country to country, as factors such as survival rate, increasing or decreasing number of breeding females (whereby the number of sold skins changes) affect the results in some countries, while the Danish results are unaffected. However, the development over time may be used as an expression of a trend in partial productivity. Data for Denmark, USA and Canada are shown i Figure 4.

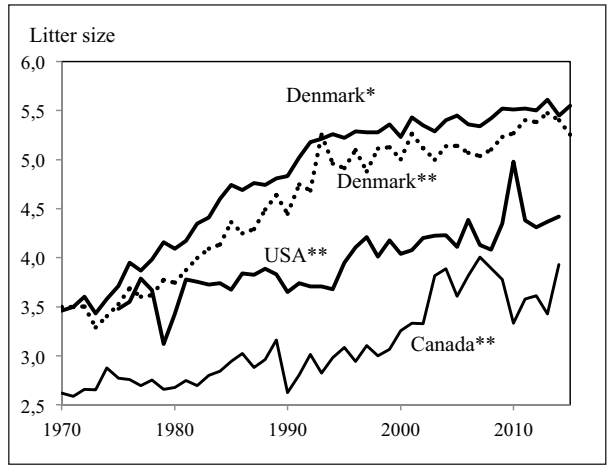


Figure 4. *Mink production: Litter size*

* Registered new born mink puppies per mink female
** Produced mink fur skin per mink female
Source: Own calculations based on Kopenhagen Fur (2016b), USDA (several issues) and Statistics Canada (several issues)

Figure 4 shows data for 3 different countries, and for Denmark litter size is measured both as new born mink puppies per mink female and produced mink fur skin per mink female. Data for Denmark illustrates an almost identical trend with an average annual increase of about 1 per cent. Data for USA Canada show a similar trend, although the annual increase is lower. However, litter size is increasing and it contributes to a higher productivity year by year.

With regards to processing, grading and sorting taking place at Kopenhagen Fur, productivity gains are ongoing, and a significant streamlining of their sorting system has been implemented. Ongoing investments in the development of new technology have made it possible to sort without increasing labor costs. Among other things, investments have been made in X-ray technology for sorting, and Kopenhagen Fur is working on a system to vacuum pack pelts so that they can be transported more efficiently.

Kopenhagen Fur has also achieved significant increases in productivity. Today, Kopenhagen Fur grades around 25-30 million mink pelts per season, but the number of employees in the sorting department has only increased modestly during the latest 10-12 years, when only approximately 12 million pelts were being sorted. Labor productivity, the number of sorted pelts per employee in the sorting department, thus rose significantly in the period (See Figure 5).

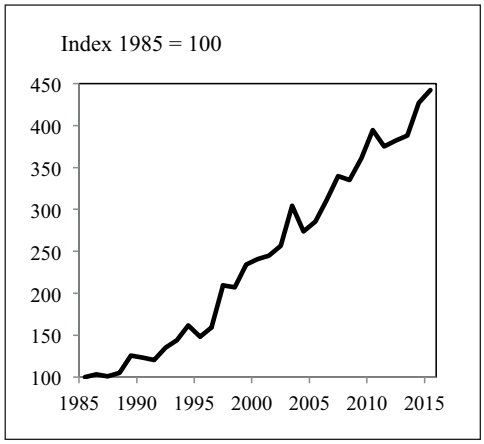


Figure 5. *Increase in labor productivity in the sorting department of Kopenhagen Fur, 1984/85-2014/15*

Source: Author's calculations based on data from Kopenhagen Fur (2016c).

As the figure shows, labor productivity increased from index 100 to index 450 in the period. In the period shown, there was an average annual increase in labor productivity of 5.1 percent.

The figure only shows part of the development in productivity at Kopenhagen Fur, and there are of course other inputs than labor. However, the development illustrates that significant rationalisation and productivity improvements can be made in key areas.

Price changes in Danish fur sector

The short-term price change for fur skin is, in many areas, noteworthy. There can be significant price fluctuations over time, and in many cases, the price depends on the size of the supply. The actual prices for mink fur at auction at Kopenhagen Fur in the period 1980-2016 are presented in Figure 6.

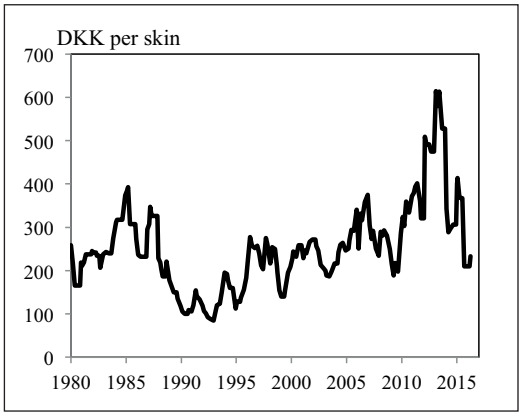


Figure 6. *Prices for mink fur skin in Denmark: Average price at auction, 1980-2016*

Note: the figure presents monthly prices based on, usually, 5 auctions per year. It is assumed that the price at a given auction continues until the next auction.

Source: Kopenhagen Fur (2016)

The long-term price change for fur skins follows the same development as other agricultural products: The prices increase at a very slow rate, which is often less than inflation.
If several costs are included, a more precise and comprehensive picture of the change in the terms of trade emerges. In the following, feed, capital and wage costs are combined and weighted according to their share of the total costs in the accounting result for Danish fur farmers. The change is presented in Figure 7.

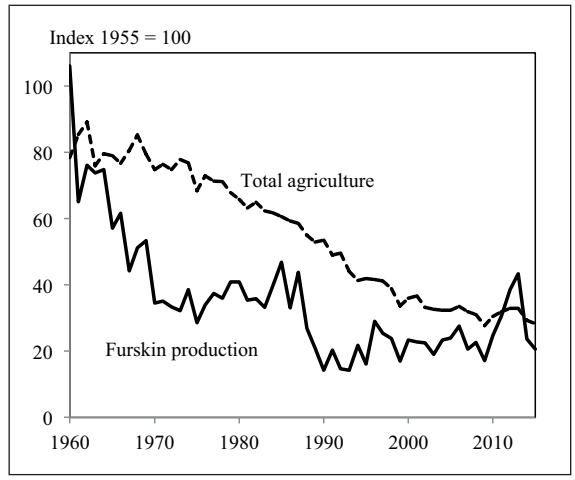


Figure 7. *Terms of trade for the Danish fur farmers and for agriculture in total, 1955-2015*

Source: Author's calculations based on Kopenhagen Fur (2015c) and Fodercentralen for Holstebro og Omegn (several years), Statistics Denmark (2016), L&F (2016) and NaturErhvervstyrelsen (several years).

Figure 7 shows the change in the terms of trade for the entire agricultural sector and the fur sector for the period 1960-2015. It is interesting to see that the trend over the whole period is almost identical: the terms of trade for agriculture as a whole and the fur sector fell from an index of 100 in 1995 to approximately 20-25 in 2015. In other words, the price of input factors increased 4-5 times more than the sales price.

Discussion

The empirical analysis and time series confirm that the fur sector is subject to considerable price and productivity pressures. It also appears that there is an ongoing and continuous trend of declining terms of trade and increasing productivity. There is no sign that the trends should change, and there is no prospect of significant changes in the basic conditions that create the productivity growth: Due to new technologies, new methods, R&D, breeding, improved management, advisory service, infra structure etc., fur farmers and the entire fur industry are able to increase production and productivity year by year. By that it is possible to compensate for the long term declining price trend.

It is also interesting that we see two coherent trends, as the declining price trend is both a result of and a cause for productivity increases. Product differentiation is a possible way to ease the price and productivity pressures, as it is possible to develop better qualities, which are not as price sensitive. However, product differentiation can not remove all the pressure, as innovation and differentiation relatively quickly can be copied and adopted by others in the market.

The fur industry operates on an open and free world market without significant trade barriers, so economic competitiveness is a major factor. The market forces will continue to stimulate productivity growth, and this will persistently lead to a demand for more basic research, innovation and development in the fur sector.

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Is vitamin B supplementation necessary for growing mink?

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Introduction

Vitamins of the vitamin B complex are essential for a large number of reactions in the body acting as coenzymes or precursors for coenzymes needed for many reactions in the metabolism. Fish, poultry and organ meats are rich sources of B vitamins, but some foods are especially good sources of just one B vitamin, while other foods contain several B vitamins. Hence, the natural content of B vitamins in mink feed may be high but the requirements of all may not be met. The requirement of vitamins for mink is largely determined on the basis of more than 40 years old research. Especially vitamins of the B complex comprise a challenge due to instability and analytical challenges for proper quantitative determination both in feed and animal (Lassén, 2000). A surplus of most B-vitamins is excreted via the urine and newly developed metabolomics methods, allowing the determination of different vitamin B metabolites, give possibilities to study the requirement of different B-vitamins in a new way.

Materials and Methods

An experiment was performed with four groups of brown mink (138 mink in each group) fed either 1) no vitamin B supplementation, 2) supplementation with 50 % of requirement, 3) supplementation according to requirement plus addition of 0.1 % Vitral B Mink Super and 4) a control group with supplementation according to requirement. The mink were fed the experimental diets from September to pelting in November. In September urine samples were collected from 10 mink per group. The samples were diluted and internal standards were added and the samples were analyzed using a LC-MS based non-targeted metabolomics approach. Chromatographic separation was performed on a Dionex UltiMate 3000 RSLC Binary UHPLC System (thermo Scientific Dionex, Sunnyvale, CA) equipped with an Acquity UHPLC HSS T3 column (Waters Corporation, Milford Massachusetts, USA) and the eluent was introduced into a Ultra-High Resolution Qq-Time-Of-Flight mass spectrometer (Impact HD, Bruker Daltonics GmbH, Bremen, Germany) by electrospray ionization. The acquired data were preprocessed in Compass DataAnalysis Version 4.2 (Bruker Daltonics GmbH) and data were explored by principal components analysis (PCA) using Latentix 2.10 (Latent5 Aps.). Loading plots were used to detect metabolite ions with the greatest influence on clustering. Compounds were identified based on queries in the METLIN (http://metlin.scripps.edu/) and Human Metabolome Database (http://www.hmdb.ca/) online databases for obtaining possible chemical structures using accurate mass and mass spectrometric fragmentation patterns (Nørskov *et al.* 2013).

Results and Discussion

PCA scores plots revealed that the metabolomics pattern for mink fed without vitamin B supplementation was different from the three other groups, which on the other hand were indistinguishable. The metabolites causing the separation between the non-supplemented group and the supplemented groups were predominantly metabolites of the vitamin B catabolism. The urinary excretion of N-methyl-2-pyridone-5-carboxamide and N-methylnicotinamide was significantly increased. These metab-

olites are products of the nicotinamide-adenine dinucleotide (NAD) degradation and they are closely related to the tryptophan metabolism which includes two vitamin B-6 dependent enzymes. Hence these metabolites may be potential markers of functional vitamin B-6 status. In support of this observation, the excretion of 4-pyridoxic acid, a catabolic product of vitamin B6 was increased in mink fed diets supplemented with vitamin B. Furthermore, the excretion of pantothenic acid and riboflavin was increased in mink fed the vitamin B supplemented diets. This excretion of unmetabolized vitamins suggests that they were available in surplus. The excretion of betaine, an important component of the one-carbon-metabolism also including choline, was increased in mink fed the vitamin B supplemented diets, suggesting that methylation pathways involving vitamin B2 and folic acid may were saturated.

The present study show that a metalbolomic approach to studying vitamin B metabolism is a very promising way to study the exact need for vitamin B supplementation and thereby secure a more precise supplementation in future mink feeding, though more research is needed in this field.

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International trade and specialisation between Europe and Asia: the case of Fur

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Abstract

The objective of this paper is to analyze, quantify and explain trade patterns and international specialisation within fur skin and fur garments focusing on Europa and Asia as two major trading partners. Data is provided from international trade statistics, national statistical institutions and organizations. The analyses combine international trade theories with trade statistics and in this way results and conclusions are presented.

The analyses show that international trade with fur skin products between Asia and Europe has increased remarkably during the recent decades. Europe accounts for a major share of world production and export of raw fur skin, and Asia accounts for the major part of the subsequent processing. This means that there is a significant export of raw fur skin from Europe to Asia, and a major export of fur garments from Asia to Europe including Russia. The conclusion is, that there is a major international trade of both fur skin and fur garments between Europe and Asia, and that the international specialisation in this sector is high.

Keywords: International fur trade, Fur garments, International specialization, Raw fur skin, Trade pattern

Introduction

International trade and specialization with agricultural raw materials and processed products is often rather limited due to trade barriers, logistic problems and food security. The production of raw fur skin - which is also considered an agricultural product - is quite different, as the export share and the orientation towards international markets is high. Most raw fur skin production takes place in the Western hemisphere, and to a high degree in Europe, while processing and production of fur garments now more and more takes place in Asia. This means, that a major part of the fur industry, i.e. the entire value chain from the farmer to the processor and finally to the consumer, is fairly internationalized. Europe and Asia are major players in this global value chain.

Up to now, the international fur industry has not been thoroughly described or analyzed. A major reason is the rather limited availability of national and international statistics about the fur sector. However, international trade and production statistics can provide important conclusions in relation to the interaction between Asia and Europe in this sector.

The role of the international trade in fur

International trade, imports and exports is very important for economic wealth, the business environment, employment, and consumers. International trade is important and beneficial for several reasons:

* International trade makes it possible to exploit the different strengths and comparative advantages of countries such as climate and factor endowments. Production is thus located in areas where it can be conducted most efficiently.

* International trade creates an international specialization, which reduces the level of costs, resulting in lower prices for consumers.

* International trade also increases competition, thereby reducing the risk of the emergence of monopolies. This increased competition also promotes innovation, the use of new technology and new knowledge, while it also creates further growth and development within society.
In general, international trade in agricultural and food products (raw fur skin is considered as an agricultural product) is relatively low compared to domestic trade and international trade in other products (Hansen, 2013). Therefore, there are apparent barriers to the growth of international trade in the agricultural and food sector.

The relatively low international trade in agricultural and food products is largely a result of the predominant agricultural and trade policies, although other factors also come into play. In general, the modest world trade in agricultural products is a result of transport barriers, self-sufficiency objectives, and fixed assets and low resource mobility in the sector.

When international trade increases, there is a stronger division of labor or specialization between countries. Each country specializes in the areas where they can optimally perform, and abandons the areas where they cannot compete internationally.

China is the only country that has both a significant production of both unprocessed mink fur, and a large fur processing industry.

In contrast to most other agricultural commodities, international trade and specialization play a very large role for fur production and for the fur industry. This is the case in most fur-producing countries, and international trade also has a great importance in countries which process fur.

Background and drivers

There are several reasons why international trade and specialization in fur business is big and well developed compared to other agricultural or agricultural-based businesses:

- Production is geographically limited to the specific climatic areas. The quality of fur pelts depend on the temperature when the animals grow up.

- Access to suitable fresh animal feed is an important competitive resource and factor, and this will limit the potential for production of fur skins in many areas.

- Significant knowledge and several competitive strengths are linked to existing fur clusters or value chains, and also cooperation between fur breeders, cooperative organisation, etc.
are important prerequisites. These comparative strengths cannot simply be transferred to other countries. The result is increasing international trade and specialisation.

- Opposite to most of agriculture in the Western world, the fur industry has never been subject to similar support schemes or market regulation under the agricultural policy. A more liberal and

free-trade-oriented sector with little or no trade barriers will - ceteris paribus - strengthen a trend towards more international trade and specialization.

The global fur industry

Production of fur animals is a part of the agribusiness sector. A major share of total fur production, about 85 per cent, comes from farmed fur which is raised and produced by farmers (Hansen, 2014).

The fur industry, and the entire fur cluster, is in many ways an interesting and instructive example from which many lessons can be learned:

Fur pelts are among the most internationally oriented agricultural products. In addition, a large and measurable effects of innovation, vertical integration and clear quality strategies have been identified. Fur clusters have been developed with direct connections with, e.g. the fashion and design industry, research, development and education. Forward integration in the value chain has also created a relationship with the international end users of fur.

Fur garments are mainly exported to high growth countries, and demand is rather income elastic compared to the demand for other agricultural products.

At the same time, fur markets are transforming and experiencing major changes. During recent decades, the fur processing industry has moved location from Western countries to being mostly located in Asia, especially in China. This international specialization and division of labor is extremely high within the fur sector.

Production and export of raw fur skin

In general, the production of fur pelts is concentrated in a few large countries, with China and Denmark together accounting for more than half of world production.

Furthermore, production almost exclusively takes place in the Northern Hemisphere, and almost all major fur producing countries, excluding China, are industrially developed high-income countries.

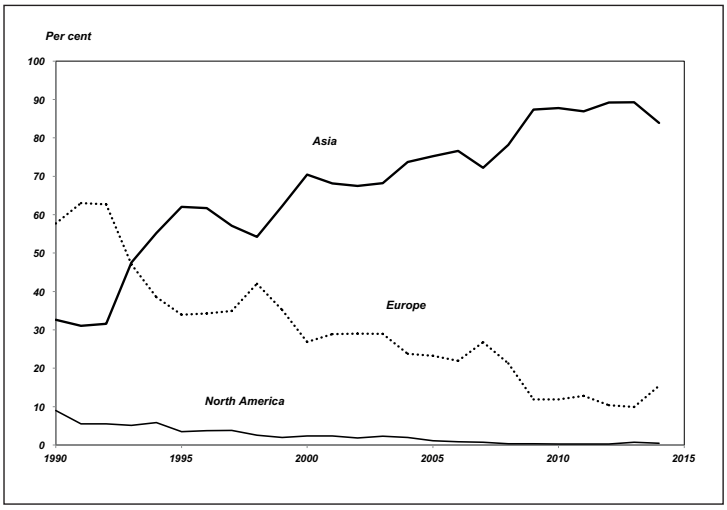
It is also noteworthy that a number of countries in Central and Eastern Europe in recent years have gained an increasing share of the global production of mink pelts. These countries now account for around 15 per cent of the total world production of mink pelts.

New world trade pattern and specialization

In the early 1960s, the U.K. and Germany were the dominant fur importing countries in the world. Together they accounted for 70 per cent of total imports. Subsequently, the pattern of trade has changed. A new international division of labor has taken place over the past 50 years or so. The world center for raw fur skin trade and demand has shifted from Western Europe and North America to Asia, particularly China. Countries like the UK, Germany, France, Belgium, USA and Canada were all major importing countries for decades, but their role has declined significantly and has been taken over by China in particular.

More recently, other Far Eastern countries have entered the field (especially South Korea), while Eastern European countries such as Poland, Estonia and Lithuania have also become important markets for the international marketing of fur. The shift from Europe to Asia is evident from figure 1.

Figure 1. *Europe's export markets for raw fur skin*

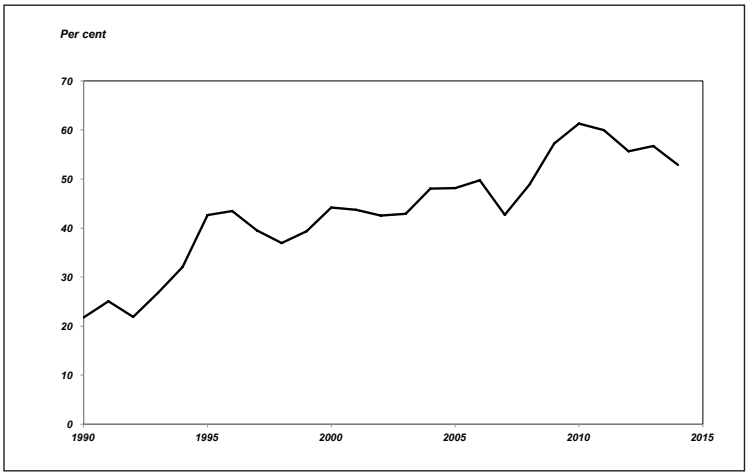


Note: Excluding re-export within Europe. It is assumed that export within Europe to Denmark and Finland is characterized as re-export aiming at fur auction sales in Copenhagen and Helsinki.

Source: Own calculations based on UN (2016)

The figure shows that an increasing share of exports of raw fur skin from European countries goes to Asia, while export to other countries within Europe is declining
Export of raw fur skin from European countries to Asia is now the most important international trade flow. About 50 per cent of all international trade of fur skin is export from Europe to Asia. See figure 2.

Figure 2. *Euro Asia trade: Share of total international trade of raw fur skin*



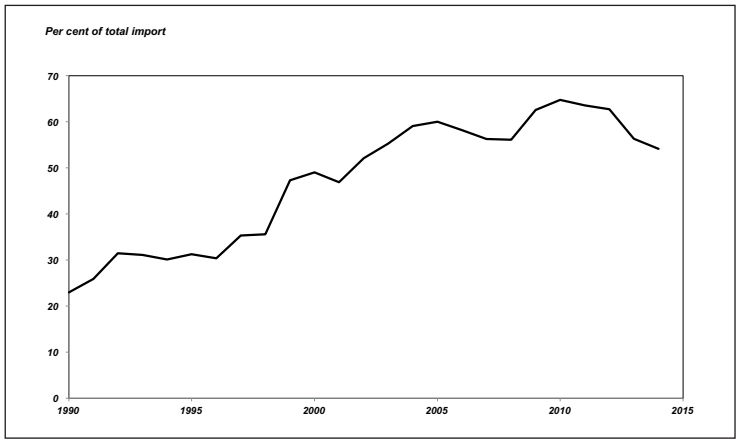
Note: Exclusively export from Europe to Asia

Source: Own calculations based on UN (2016)

Figure 2 shows that since 1990 Europe's exports of raw fur skin to Asia has represented an increasing share of total international trade of raw fur skin. However, during the recent years, the share has fallen. This is mainly due to increasing fur skin production in China, and increasing demand from fur processing industry within Europe, especially Italy and Greece. In general, when it comes to international trade flows of unprocessed fur skin, there is a clear west-east trade flow: Europe and North America produce raw fur skin, and a major part of the production is exported to Asian countries.

The Asian countries do not only import raw fur skin and process the raw skin into fur garments. The Asian countries also export fur garments back to Europe and North America. In the beginning of 1990s Asia did not play any major role as a supplier of fur garments to Europe. However, since then an increasing share of European import of fur garments now comes from Asia. Today, more than 50 per cent of imports to European countries comes from Asia, see figure 3.

Figure 3. *European countries' import of fur garments from Asia*



Note: Four most important importers of fur garments: Germany, France, Italy and Spain

Source: Own calculations based on UN (2016)

Figure 3 shows that international trade flows of products further down the value chain - here fur garments - are markedly different from the flows in figure 2. The international trade flow in fur garments is in reverse in that, to a great extent, it is an east-west trade flow.

Both figure 2 and 3 emphasize the growing interdependence that exists between Europe and Asia when it comes to international trade and specialisation within the fur industry. The trend has changed the recent years, which can be explained by mainly increasing production of fur garments in Europa.

Russia, which in this paper is regarded as a European country, plays a significant role on the international fur markets. Back in the early 1960s, the Soviet Union was the world's second largest exporter of raw fur skin with up to 20 percent of the world market. Subsequently, exports fell, so that, by the turn of the millennium, Russia's share of the world market only accounted for less than 2 percent. In contrast, Russia's imports of fur garments have increased significantly, and Russia is now the world's largest importer.

Conclusions

It can be concluded that international trade in furskin and fur garments is highly concentrated and specialised. This indicates that the international fur sector is relatively exposed to international competition, and that the comparative advantages between countries are significant and are very important for international trade and the division of labor. International trade and specialization in fur business between Europe and Asia has increased significantly during the recent decades.

Europe seems to be most competitive in production of raw fur skins, while Asian countries are the most competitive when it comes to the production of fur clothing and garments. International trade and specialisation enables both continents to exploit their comparative advantage in the fur sector for mutual benefit.

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A method for increasing the milkability of female mink

Russia

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Abstract

Research was carried out on increasing female milkability by including more organic selenium in their diet via the product Sel-Plex, which prevents anthropogenic-induced stress. Sel-Plex is obtained from growing bakery yeast in a selenium-rich environment. The main form of microelement (98%) is selenomethionine with a high absorption rate.

Research was carried out on standard mink females of a similar age and their pups at the populations of three fur farms. 210 females and 905 nursing pups were investigated. Doses tested were 100 and 50 mg/kg of body mass per day per head.

It was found that Sel-Plex has a positive effect on female milkability and the pups' physiological state. Criteria for judging female milkability was live weight of their 20-day old pups as this property is directly dependant on the dam characteristic. Females that were given Sel-Plex had larger 20-day old pups – their live weight was 13% higher than the control pups ($p<0,01$), male pups – by 14% ($p<0,05$). These pups show a higher-intensity growth rate and by 40 days (weaning) their average weight is higher than the control by 10% ($p<0,01$).

Adrenal weight in pups from Sel-Plex consuming mothers were sufficiently (25 %) smaller than in control animals.

Keywords: nursing pups, live mass, physiological state, Sel-Plex, selenomethionine

Introduction

In animal and poultry husbandry selenium-rich products are commonly used in order to support the high productivity of animals, prevent and treat a selection of diseases (Golubkina *et al.*, 2006; Kavtarashvili and Kolokolnikova, 2010).

Selenium plays a primary role in protecting the body from oxidation stress, destruction of hydroxides and peroxides. As a result products of lipid oxidation, which can damage membranes and lead to a fall in the functional properties of cell, are not built up (Boriaev *et al.*, 1996).

In animal husbandry products of inorganic selenium are used to increase animal productivity (Pereldik, 2000). However, the products of inorganic selenium (selenites, selenates), due to their high valency of the microelement, are highly toxic, which restricts their application. Synthetic organic-based sele-

nium preparations (selekor, DAFS-25) are less toxic and more cumulative (Evrienov, 2001; Pereldik *et al.*, 2010).

The general trend in the last several years, both in Russia and abroad, has been the use of natural derivatives of the microelement, primarily – selenomethionine, as this form of selenium has the highest absorption rate and significant biological effect.

Growing bakery yeast in a selenium-rich environment allows for the production of the preparation Sel-Plex, where the main form of the microelement (98%) is selenomethionine. The producer of the preparation is LLC "Alltech", Moscow. Sel-Plex has a high level of availability and low toxicity. It increases resistance to mycotoxins and oxidised fats, when they are present in feed. It increases the antioxidant status and support resistance to various forms of stress.

In the conditions of growing anthropogenic-caused stress of animals, in order to increase the milkability of females and growth power of kits, as well as tightly controlled feeding regimes, it is also important to use supplements that prevent stress.

The relevance of the current study is attributed to the demand for new methods of aiding the minks body in adapting to changing environmental conditions and a fuller exhibition of their genetic properties. The novelty of the study is in it being the first test of the influence of an organic selenium (from yeast) on the productivity of mink.

The purpose of the study was to increase mink productivity by using organic selenium (obtained from yeast) – the supplement Sel-Plex.

The influence of varying doses of Sel-Plex on the milkability of female minks and the growth power of nursing kits was tested. A method was developed for using organic selenium (obtained from yeast) in the form of the supplement Sel-Plex in mink breeding.

Materials and method

Research was carried out on female minks of varying ages of the colouring dark brown and their kits. In order to assess the milkability of the females, a standard method was used – assessing the live mass of kits at 20 days of age. When the groups of kits were formed, their birth date, litter number and sex were taken into consideration.

Test groups of females, in order to assess the growth power of their kits, were formed according to their fatness and analogous age.

Nursing kits were weighted every decade with an accuracy of 1 gram. A total of 210 females and 905 nursing kits were investigated. Doses of Sel-Plex tested were 50 and 100 mg/kg of body mass per day per head. Feeding and living conditions for all groups were identical.

Sel-Plex was fed during the lactating period with the views of increasing milkability. It was administered in 5 – 15 day courses (according to investigation year) into the feed and thoroughly mixed in.

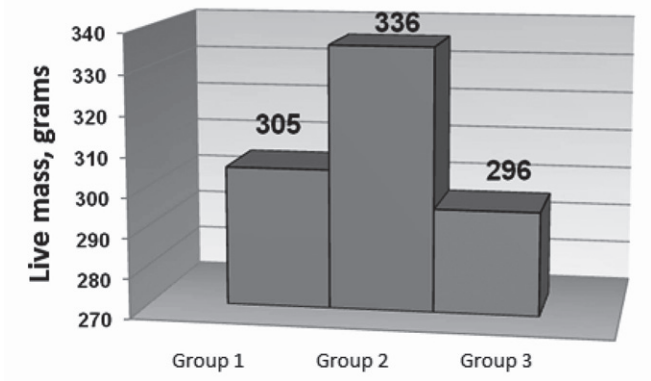
Criteria for assessing the supplement were the following properties: growth power of kits before the age of 20 days and before weaning from the mother (relative and average daily weight gain).

Results

Calculations of Se content in the native feed mix showed that in the years of the investigation there was 0.014 to 0.016 mg in 100 kcal. The average body mass of kits in the groups was 9.8 g. The investigation shows that the feeding of Sel-Plex has a positive influence on the milkability of females, when administered in two courses: during the first 15 days after whelping and 10 days before kits are weaned. From the two doses tested, the supplement performed best when administered in the dose 50 mg/kg of body mass per day, in which case the difference in weight at 20 days old in the kits of the test and control groups became statistically significant both in females (110.0±4.2 g as opposed to 97.5±3.5 g, $p<0.01$), and in male (130.7±5.6 g as opposed to 114.5±3.8 g, $p<0.05$), while in the group with the dose of supplement administered as 100 mg/kg of body mass per day – only in females (118.0±4.9 opposed to 97.5±3.5 g, $p<0.01$).

Recording of the behaviour of nursing kits showed that all properties, characteristic of its intensity (total, average daily and relative weight gain), were the highest at all periods of recording, all the way to weaning, in kits, the mothers of which were given Sel-Plex during lactation in the dose 50 mg. By the age of 40 days they had an average live mass of 336±7.91 g as opposed to 305±8.47 g for the control, $p<0.01$ (Figure 1).

Figure 1. *The live mass of nursing mink kits when weaned.*



Group 1 – control;
Group 2 – Test, 50 mg;
Group 3 – Test, 100 mg.

The livability of all nursing kits remained normal, which supports the assumption that testing varying doses of Sel-Plex is not toxic for the mink. It was found that the weight of the suprarenal glands of kits from mothers that were given Sel-Plex was sufficiently (25%) smaller than in the control. It is assumed that using a seleniumrich supplement increases the antioxidant qualities of the body and supports the resistance to various types of stress (Table 1).

A larger, that in animals from the test group, weight of suprarenal glands of the control group can be an indirect result of undergone stress and that Sel-Plex (organic form of selenium) can protect from the consequences of stress.

Table 1. *Suprarenal gland weight in mink kits (n=15)*

Group	Sel-Plex dose, mg	Suprarenal glands Mg,x ±Sx	To the control, %
1- Control	-	90±8.6	100
2- Test	50	76±10.0	75
3- Test	100	62±6.4	61

The achieved results comply with scientific evidence, that demonstrate the potential relationship between the size of the suprarenal glands and the corticosteroids that they produce, and the function of the suprarenal glands increases under stressful conditions (Osadchuk, 2001). Recording the feed consumption showed that Sel-Plex does not worsen the taste of feed as it was readily eaten by the animals.

The information about the perpetual changes in a minks body, obtained through this experiment, provide a clear understanding of the influence of Sel-Plex on the physiological state and productivity of mink. Everything stated shows that viability of feeding Sel-Plex to lactating female mink with the view of increasing their milkability, which provides for an advantageous start for their nursing kits.

Discussion

Introducing the selenium-rich supplement Sel-Plex into the diet of female mink during the whelping and nursing period in two courses: from the 1st to the 15th May and from the 27th May to the 5th June in the dose 50 mg/kg of body mass (0.05 mg of chemically pure selenium) ensures:

- An increase in their milkability – by the age of 20 days the live mass of the kits in the test group was significantly higher than in control, both in female kits (13% higher, $p<0.01$) and in male kits (14% higher, $p<0.05$);
- An increase in the growth power of the kits until weaning – by the age of 40 days nursing kits have a higher body mass than the control by 10% ($p<0.01$).

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Using the product Bio-Mo™ to increase mink *(Neovison vison)* life span and productivity

Russia

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Abstract

Research was carried out on the use of the product Bio-Mos™ with the intention of supporting the physiological state, supporting the productivity potential and increasing the economical effectiveness of mink pelt production. On the application of Bio-Mos™ to feed mixes, livability of nursing young with symptoms of digestive upset increased by 5-11%, nitrogen retention increased by 26%, an improvement was noticed in the state of the digestive organs: no pathological changes in the internal organs, an increase in intestine's area of nutritive absorption by 20-30%.

The positive influence of Bio-Mos™ on the physiological state and protein metabolism leads to increase in pup growth intensity (by 2%), and, as a result, an increase in end live weight (by 3%). Practical application of Bio-Mos™ achieves an increase in selling price by 10% from one pelt as a result of a 2% increase in area and 5% in quality.

Keywords: mink young, nitrogen balance, livability, live weight, pelt quality

Introduction

The postnatal development period in mink is characterised by a high intensity of growth and development. At this time the young are susceptible to gastrointestinal diseases. Gastrointestinal issues in animals, cause by bacterial agents, are widespread in all areas of animal husbandry and lead to a fall in productivity (Buriakov *et al*, 2008, Koslovskiy *et al*, 013, Musin and Gadiev, 2009, Tinaev, 2006). Research was carried out on the use of Bio-Mos™ (Alltech) with the intention of supporting mink physiological state, productivity potential and increasing the economical effectiveness of pelt production. This preparation is based on a complex of manna-oligosaccharides (MOS) extracted from the outside wall of yeast cells *Saccharomyces cerevisae*, which are not affected by digestive enzymes. Bio-Mos™ supports the build-up of bacteria that produce lactic acid, such as *Bifidobacterium* and *Lactobacillus* by decreasing the colonisation of the intestine with pathogenic microorganisms.

Materials and methods

Research was carried out for three years on two fur farms and laboratories of FSBSI NIIPZK on 1002 mink of the colours scanblack, scanbrown and sapphire.

Four doses were tested: 80, 150, 300 and 600 mg/kg of body mass.

Male minks were kept in sheds. Groups were formed by the analogous method according to age, sex and body mass.

Bio-Mos™ was given daily with combination feed between the months of June and October. The amount of preparation was adjusted monthly according to body mass. The first group (n=50) received a basic diet, the second group (n=52) received a basic diet and Bio-Mos™ at a dose of 300 mg/kg of body mass.

Digestion trial, zootechnical analysis of samples, histological investigations of the organs were carried out according to methodological guidelines (Petukhova *et al*, 1989, Kladovshikov and Samkov, 1975, Merkulov, 1969).

Protein content in serum was calculated using refractometric analysis (refractometer RDU 88-595). The state of gastrointestinal organs (liver, stomach, intestine) were analysed after slaughter.

Livability testing was carried out on lactating female minks (n=20) and their kits (n=104) with symptoms of digestive upset. Analogous groups were formed according to whelping time, female fertility and litter size at start of experiment.

In the first group (the control) kits from lactating females were injected with colibacillosis serum and the antibiotic ceftriaxone. Females from the second group (experimental) were given Bio-Mos™ with their feed in a dose of 600 mg/kg of body mass during the lactating period.

To assess growth intensity, kits were weighed in the morning before feeding with an accuracy of 0.5 g. The data from weighing was then used to calculated the overall, relative and average daily gain (Matseevskiy and Zemba, 1988).

At the end of the experiment the animals were measured: length – from the end of the nose to the tailhead and chest girth behind the shoulder blades (fleshing). An assessment of the pelt quality was carried out with an expert panel (Manual, 2003).

The obtained digital data was treated with variation statistical methods (Plokhinskiy, 1969, Microsoft Excel).

Results

As a result it was found that the best results were obtained when Bio-Mos™ was added to the combination feed in a concentration of 300 mg/kg of body mass.

An analysis of the properties that are characteristic of nutritive digestibility (the digestibility coefficient) did not reveal any significant difference between the control and experimental groups. It was shown, however, that animals that were given Bio-Mos™ with their compound feed had a lower percentage of nitrogen excreted with urine, than the control: 1.87±0.11g as opposed to 2.73±0.34g ($p<0.05$) and an increase in its retention with the fixed nitrogen - by 17.9% and digested nitrogen – by 26.4% accordingly.

After the histological assessment of the intestines an increase in the surface area of nutrition absorption was found. The length of the villi in animals that received Bio-Mos™ with their diet was 0.3-0.5 mm in the duodenum, 0.7-1.2 mm in the small intestine, as opposed to 0.2-0.4 mm and 0.5-1.0 mm for the control.

An assessment of the internal organs showed that 40% of the control animals displayed a pathology, while the condition of the organs in all the animals of the experimental group were found to be normal. The concentration of total protein in blood serum in the experimental group, when compared to the control, increased by 2.5%, while variability (Cv) decreased by 5 times. Livability of kits from females that received Bio-Mos™ during the lactating period was found to be 5-11% higher than kits injected with serum and an antibiotic.

By the end of the monitoring period the body mass of kits from the experimental group was higher than in the control group by 49 g (2436±39 g as opposed to 2387±38 g). Kits from the experimental group had a higher growing power: overall gain - 1968±35 g; relative gain - 136±1.3%; average daily gain – 14.4±0.3g as opposed to 1924±35g, 135±1.7% and 14.0±0.3g in the control. Results of the morphometric assessment of the animals were in accordance with growth performance and are shown in Table 1.

Table 1. *Body size data in young mink at slaughter*

Group	n	Data $\bar{x} \pm S_{\bar{x}}$		
		Body mass, g	Body length, cm	Chest girth behind shoulder blades, cm
Control	50	2506±28	50.7±0.4	24.9±0.2
Experimental	52	2573±43	51.5±0.4	26.0±0.2***
% to control			+2.7	+1.6 +4.4

***p<0.001

It is noticeable that the kits in the experimental group are larger than in the control, and the difference in fleshing have reached a statistical significance and are 26.0±0.2 cm as opposed to 24.9±0.2 cv (p<0.01) respectively.

An analysis of the sorting results (Table 2) shows that using Bio-Mos™during the growth period of mink (July – October) has increased (compared to control) the amount of defectless pelts by 23%, larger pelts- by 2%, which increases the pass rate according to quality (115±4.1, opposite 127±1.8, p<0.001), and increased the incremental revenue from one male mink pelt by 10.4%.

Table 2. *Results of the expert panel assessment of mink pelt*

Group	n	Data $\bar{x} \pm S_{\bar{x}}$		
		Pass rate by size, %	Pass rate by quality, %	Defectless pelts, %
Control	39	131±1.5	115±4.1	41
Experimental	25	133±1.1	127±1.8**	64
% to control		+2	+12	+23

**p<0.01

Discussion

As a result of the investigation it has been found that Bio-Mos™ has a positive influence on mink physiological state. The addition of Bio-Mos™ to the diet resulted in livability of suckling kits with symptoms of digestive upset increased by 5-11%, nitrogen retention in the body increased by 26%, the state of digestive organs improved: no pathological changes in the internal organs were found, the surface area of nutrient absorption increased by 27-33%.

The positive influence of Bio-Mos™ on the physiological state and protein exchange explains the increase in growth power of kits (by 2%) and, as a result: increased finite mass (by 3%) and morphometric performance (by 4%) at slaughter. The practical use of Bio-Mos™ resulted in the increase selling price by 10% from one pelt due to a 2% increase in its size and a 12% increase in quality.

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Bioactive Effect of the Preparation "Biostyl" on the Different Genotypes of American Mink (Neovison vison)

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Abstract

The different role of coat color mutations in the American mink on the per os effect of the biologically active preparation "Biostyl" was shown. The number of kits per female was the same in all control genotypes, including *Standard (+/+ +/+), sapphire (a/a p/p)*, and *lavender (a/a m/m)*: 4.4 ± 0.4, 4.4 ± 0.5, and 4.3 ± 0.5, respectively. Experimental groups of these genotypes have shown a great contrast among each other: stimulation of the reproductive function was 5.2 ± 0.3 in *Standard* minks, while suppression of the reproductive function was 3.8 ± 0.6, and 2.3 ± 0.5 in the double recessive mutants *sapphire* and *lavender*, respectively. The differentiation in body mass between experimental and control newborn *Standard* kits was not revealed. A significant decrease in the body mass of newborn experimental *sapphire* kits as compared to control group in a sex-specific manner was registered.

Keywords: coat color mutations, Biostyl, reproductivity, viability

Introduction

To date, 35 mutations responsible for modifications in coat color have been registered as a result of the historical domestication of the American mink *Neovison vison*. Twenty two of them are recessive, while 13 are dominant or semidominant (Ness, et al., 1988; Trapezov, 1997 a, b).

The study of color mutation phenogenetics revealed that they possess a wide spectrum of pleiotropic action. They affect both the form and spatial organization of pigment granules in the hair (Prasolova and Trapezov, 2007); they affect various brain regions modifying monoamine oxidaseactivity on the metabolism of biogenic amines (serotonin and dopamine) and, hence, the degree of the manifestion of domestication behavior (Popova *et al*., 1994, 1996; Voitenko and Trapezov, 2001; Trapezov *et al*., 2008). Coat color mutations influence the activity of digestive enzymes (Trapezov *et al*., 2009). Moreover, coat color mutations are responsible for cell immunity via the morphological and functional organization of leukocytes (Uzenbaeva *et al*., 2011; Alexandersen *et al*., 1994) and a decrease in both the total viability and resistance to viral and bacterial infections.

Environmental stress caused by extreme feed conditions results in glucocorticoid function impairments differing in their degree: animals homozygous by coat color mutations demonstrate a higher degree compared to the standard (Trapezov and Markel, 1989).

As a result of the cage breeding of American minks, it was detected that coat color mutations were responsible for suppression of the reproductive function (Kharlamova and Trapezov, 1999). Despite the development of specific technologies regulating reproductivity (Hamadani *et al*., 2013), many

modulating effects of mutations caused by environmental factors remain unknown. Our goal was to study the effect of coat color mutations caused by bioactive preparation "Biostyl" on the reproductive function and on kits' viability in American mink.

Material and methods

The material for the study included cage bred American minks *Neovison vison*, including females and males of three genotypes: *Standard (+/+ +/+)*; double recessive *sapphire (a/a p/p)*, which possess characteristics of single homozygotes *aleutian (a/a)* and *platinum (p/p)*; and double recessive *lavender (a/a m/m)*, which represent the combination of two mutant alleles, *aleutian (a/a)* and *mojle (m/m)*.

Biologically active "Biostyl" was applied to the animals *per os* 6 days prior to mating for 5 days and during mating for 3 days at 0.05 mL/kg of body weight. "Biostyl" represents the aqueous solution of biologically active, synthesized (trekresan) and natural (antiseptic Dorogov's stimulator ASD F2) substances (Rasputina, 2007).

A widely used mating system for minks was used for the mating of both experimental and control animals. To assess the results in the control and experimental groups of various genotypes, the fertility value and newborn kit weights were analyzed.

Results and discussion

According to Table 1, the integral fertility value (the number of 10 day old kits per female) in the control groups was the same for all animals. At the same time, treatment with "Biostyl" *per os* in the same dosedemonstrated a differentiated effect. An increase in the integral value of fertility for 0.8 kits was registered in the experimental *Standard (+/+ +/+)* animals, while its suppression was revealed in *sapphire (a/a p/p)* and *lavender (a/a m/m)* minks for 0.6 and 2 kits ($p < 0.01$).

The increase in the integral fertility value in the experimental *Standard (+/+ +/+)* genotype and the opposite decrease in the experimental double recessives *sapphire (a/a p/p)* and *lavender (a/a m/m)* might be explained by the effect of the similar dose, which resulted in a decrease in the number of females lacking newborn kits to 6.2% in the one case *(Standard)* and in its increase to 28.6% in *sapphire* and 31.6% in *lavender*.

A question arises regarding the observed opposing effects. According to published data, mutations affecting mink coat color decrease the total immunity resistance of the organism via its disorganizing effect. Since the genotype of *Standard (+/+ +/+)* minks represents wild-type alleles, the selected dosage (0.05 mL/kg of body weight) demonstrated a stimulating effect as compared to control, while the same dosage revealed a suppressed action on the mutant *sapphire (a/a p/p)* and *lavender (a/a m/m)* genotypes. Moreover, a dose of 0.05 mL/kg of body weight significantly increased both prenatal and neonatal mortality in *lavender (a/a m/m)* minks: as shown in Table 1, the stillborn kit rate was higher by an order (10%) as compared to control, while the number of kits that died within the first 10 days was the highest (20.6%).

The differential effect of Biostyl also affected the embryonic development of the offspring. According to data in Table 2, no differences in body weight (in both males and females) were detected between the experimental and control newborn kits with the *Standard (+/+ +/+)* genotype, while a trend toward a decrease in body weight was observed in experimental group.

Table 1. *Fertility indicators in minks Standard (+/+ +/+), sapphire (a/a p/p), and lavender (a/a m/m) under the action of the same dose of "Biostyl" (0.05 mL/kg of body weight*

Genotype	Group	Mated females	Kits born					Kits died in the first 10 days of life, %	Number of 10 day old kits		Females without offspring, %
			live		Born dead, %	live + dead					
			per successfully whelped female	per female in the group		per successfully whelped female	per female in the group		per successfully whelped female	per female in the group	
Standard (+/+ +/+)	Experiment	32	5.8 ± 0.3	5.5 ± 0.4	5.9	6.2 ± 0.3	5.8 ± 0.4	4	5.6 ± 0.3	5.2 ± 0.3	6.2*
	Control	32	5.8 ± 0.4	4.7 ± 0.5	2.6	6,0 ± 0.4	4.8 ± 0.5	6.6	5.4 ± 0.3	4.4 ± 0.4	18.7*
sapphire (*a/a p/p*)	Experiment	28	6.4 ± 0.4	4.6 ± 0.6	2.3	6.5 ± 0.4	4.7 ± 0.6	16.4**	5.3 ± 0.5	3.8 ± 0.6	28.6
	Control	27	5.5 ± 0.5	4.0 ± 0.5	6.1	5.7 ± 0.5	4.9 ± 0.6	3.2**	5.2 ± 0.4	4.4 ± 0.5	14.8
lavender (*a/a m/m*)	Experiment	19	4.8 ± 0.4	3.3 ± 0.6*	10**	5.4 ± 0.5	3.7 ± 0.7	20.6	3.3 ± 0.5	2.3 ± 0.5**	31.6**
	Control	17	5.2 ± 0.3	4.9 ± 0.4*	1.2**	5.3 ± 0.3	5.0 ± 0.4	14.3	4.6 ± 0.5	4.3 ± 0.5**	5.9**

* $p<0.05$
** $p<0.01$

Table 2. *Body weights of newborn minks of genotypes Standard (+/+ +/+), sapphire (a/a p/p) and lavender (a/a m/m) whose mothers were exposed to biologically active drug "Biostyl" during their pregnancy.*

Genotype	Group	Number	Birth weight, g	Average birth weight, g	Difference in birth weight, g	*P*
♂♂						
Standard (+/+ +/+)	Experiment	26	6.0-12.4	10.1 ± 0.3	– 0.2	0.6
	Control	28	6.4-13.8	10.3 ± 0.3		
sapphire (*a/a p/p*)	Experiment	57	5.9-12.3	9.4 ± 0.2	– 0.8	0.002
	Control	45	7.6-15.4	10.4 ± 0.2		
lavender (*a/a m/m*)	Experiment	29	6.7-12.6	10.0 ± 0.3	– 0.6	0.2
	Control	45	7.4-14.4	10.6 ± 0.3		
♀♀						
Standard (+/+ +/+)	Experiment	36	6.4-11.9	9.3 ± 0.2	– 0.4	0.3
	Control	33	6.8-12.2	9.7 ± 0.2		
sapphire (*a/a p/p*)	Experiment	35	4.9-11.6	8.2 ± 0.2	– 1.3	0.0005
	Control	42	7.3-12.9	9.5 ± 0.2		
lavender (*a/a m/m*)	Experiment	19	5.1-11.6	9.1 ± 0.3	– 0.6	0.2
	Control	27	4.1-12.1	9.7 ± 0.3		

The effect of "Biostyl" on the embryonic development of double recessive mutants depends on the combination of the *aleutian (a/a)* allele. The combination of the *aleutian (a/a)* allele with the *mojle (m/m)* allele in *lavender (a/a m/m)* minks results in a suppressing effect on body weight; however, this does not significantly differ between experimental and control animals. At the same time, the combination of the *aleutian (a/a)* allele with the *platinum (p/p)* allele in *sapphire (a/a p/p)* minks resulted in a significant decrease in body weight of newborn kits as a result of "Biostyl" application (in the same dose of 0.05 mL/kg of body weight) in a sex specific manner: females appeared to be more sensitive than males.

Therefore, the same dosage of a biologically active substance might result in either a stimulating or inhibiting effect, depending on the genotype that received it.

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Heterozygosity and stress resistance of the adrenal glands cortex structure in American mink *(Neovison Vison)*

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Abstract

The reaction of the homozygote of coat color mutations *Standard (+/+)*, *hedlund-white (h/h)*, *aleutian (a/a)*, and heterozygote *hedlund-white (h/+)* and *aleutian (a/+)* to the stress starvation was studied. Homozygous *aleutian (a/a)* animals characterized with the lowest body mass in comparison with another genotypes as in experiment as in control ($p < 0.05$). In control homozygous animals as on the wild alleles *Standard (+/+)*, as on the mutant alleles *hedlund-white (h/h)* and *aleutian (a/a)*, characterized with the tendency of more highest zona fasciculata and zona reticularis in comparison with experiment. At the same time heterozugous animals *hedlund-white (h/+)* and *aleutian (a/+)* in the experiment shown tendency to increasing of zona fasciculata and zona reticularis.

Keywords: coat color mutations, mutation *hedlund-white*, mutation *aleutian*, starvation

Introduction

Stress resistance of living organisms of different genotypes under optimum conditions appears to be quite different from that under stress conditions. Resulting from the selective advantage of heterozygous over homozygous individuals, the polymorphism of heterozygotes exists in populations, which is maintained by the pressure of natural selection. But for the enhanced adaptability of heterozygotes (so called superdominance), the population pays by the segregation of the genetic burden, homozygotes with decreased viability, which are hidden under the standard phenotype appearance (Trapezov and Markel, 1989; Trapezov, 1997 a, b; Trapezov *et al*., 2008, 2009; Voitenko and Trapezov, 2001; Uzenbaeva *et al*., 2011).

The current work presents some fragments of the project concerned with studying the effects of heterozygosity for mutations affecting the pelage pigmentation on the response to environmental stress caused by extreme feeding conditions, in order to understand the mechanisms of genetic adaptation of the American mink to various ecological niches.

Materials and methods

The study was carried out using cage bred mink males with the following genotypes and fur color: *Standard dark brown (+/+)* with dark brown fur color, *hedlund white (h/h)* monorecessive homozygotes with white fur color, and *aleutian (a/a)* homozygotes with the dark iron gray fur color and *hedlund white (h/+)* and *aleutian (a/+)* heterozygotes with the dark brown fur color.

As a chronic stressing factor, we decreased by certain quantities the forage portions fed to the animals during their growth period (Fig. 1). The monthly average of the forage amount fed to the experimental

animals was as follows (percent of the control amount): 50% in July, 34% in August, 55% in September, 55% in October, and 60% in November. In other words, in our experiment, we imitated the fodder shortage conditions to which the natural populations of the American mink are normally exposed. The control animals were fed *ad lib*.

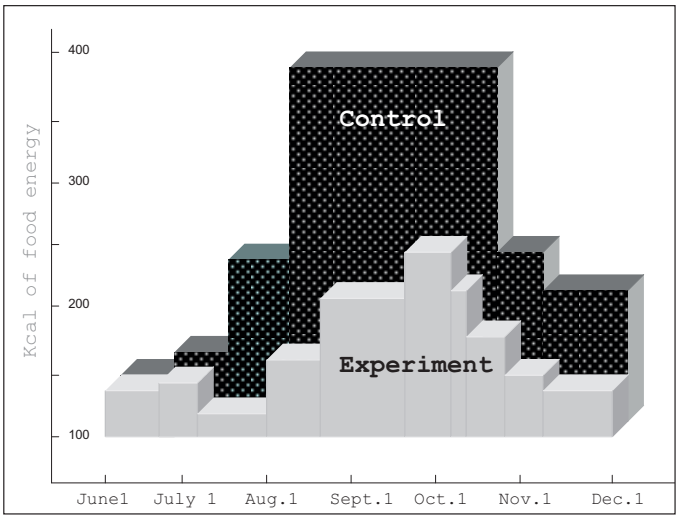

Figure 1. *Forage consumption per day by the experimental and the control animals.*

Results and discussion

As can be seen in Fig. 2, in the experimental groups, the minks of all genotypes tested showed a statistically lower body weight compared to the control animals as a result of the long-term feed restriction during their period of growth. At the same time, homozygosity for the *Aleutian mutation (a/a*) exerted a marked recessive effect, the animals with this genotype demonstrating lower growth rates than other genotypes both in the experiment and in the control.

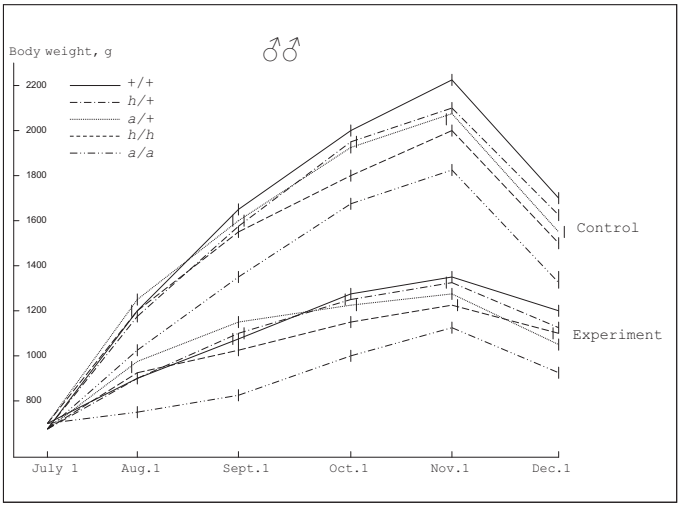

Figure 2. *The effects of mutations affecting coat pigmentation in the American mink on the body weight dynamics in the control experiment and under the stress of starvation. The animals homozygous for the Aleutian mutation (a/a) showed a statistically slower growth rate than animals of other genotypes both in the experiment and in the control ($p < 0.05$).*

The data provided in the table indicate quite paradoxical shifts at first sight; namely, under the control conditions, the animals homozygous for both the wild type *standard allele (+/+)* and the mutant *hedlund white (h/h)* and *aleutian (a/a)* alleles show the evident tendency for broadening of the zona fasciculata and zona reticularis of the adrenal cortex, producing glucocorticoid hormones, compared to the experiment. At the same time, in the animals heterozygous for the *hedlund white (h/+)* and the *aleutian (a/+)* mutations, we observed the clear tendency for increasing thickness of the zona fasciculata and zona reticularis.

When interpreting this fact, it should be taken into account that cage breeding of growing mink youngsters in November and December, when night temperatures in Siberia fall to about –30°C, places high demands on the animal's organism. As a result of this, adaptive mechanisms may be activated, including the stress systems. It is worth noting that, in the control minks homozygous for both the wild type *standard dark brown allele (+/+)* and the mutant *hedlund white (h/h)* and *aleutian (a/a)* alleles, the stress level was higher than in the heterozygous *hedlund white (h/+)* and *aleutian (a/+)* animals. This was directly confirmed by the differences in the width of the cortical zona fasciculata and zona reticularis, these being wider in the homozygous minks compared to the heterozygous ones (table 1). The fall of temperature in winter leading to stress in animals is indirectly indicated by the loss of their body weight, which is apparently caused by the enhanced consumption of the energy stored in fat.

The five months during which the winter cold stress was enhanced by the chronic stress associated with the feed restriction led to genotype dependent histology cal changes in the adrenal cortex. The comparison of the data obtained in the two experiments showed that the decreased thickness of the zona fasciculata and zona reticularis observed under the stress of starvation in the animals homozygous for both the wild type *standard dark brown (+/+)* allele and the mutant *hedlund white (h/h)* and *aleutian (a/a)* alleles was not caused by the insensitivity of the animals to the stress factors, but was rather associated with the development of the third stage of stress, which is the lack of physiological compensation and exhaustion of the organism's adaptive resources. It is worth mentioning that, for the young animals with the corresponding genotypes, the very winter temperature fall under the normally used cage breeding and feeding regimen is quite a stressing factor compared to the *hedlund white (h/+)* and *aleutian (a/+)* heterozygous animals. Two unfavorable factors, the feed restriction and the temperature fall in winter, acting together lead to adaptation breakdown accompanied by the destructive processes in the adrenal cortex of the homozygous animals.

Table 1. *Width of the adrenal cortex and the adrenal cortex zones under chronic starvation stress conditions in minks homozygous and heterozygous for the mutations affecting coat pigmentation*

Genotype	Group	Number of individuals	Width of adrenal cortex and adrenal cortex zones, μm		
			zona glomerulosa	zona fasciculata and zona reticularis	cortex
Standard (+/+)	Experiment	9	116.0 ± 9.4	383.9 ± 21.0	499.3 ± 30.4
	Control	9	88.6 ± 4.7	429.8 ± 26.8	518.4 ± 31.5
Homozygotes *hedlund white* (*h/h*)	Experiment	10	89.3 ± 5.2	397.9 ± 20.8	487.2 ± 6.0
	Control	11	87.4 ± 6.1	413.6 ± 2.0	501.0 ± 28.1
Homozygotes *aleutian* (*a/a*)	Experiment	8	90.2 ± 7.5	418.3 ± 20.0	508.5 ± 27.5
	Control	8	87.9 ± 6.1	431.6 ± 23.6	519.5 ± 9.7
Heterozygotes *aleutian* (*a/+*)	Experiment	8	83.0 ± 5.2	444.1 ± 25.3	527.1 ± 30.5
	Control	8	85.5 ± 5.6	420.4 ± 3.6	505.9 ± 9.2
Heterozygotes *hedlund white* (*h/+*)	Experiment	10	82.9 ± 8.5	445.8 ± 3.1	528.7 ±31.6
	Control	9	98.0 ± 8.3	409.8 ± 2.1	507.8 ± 0.4

Control – zona fasciculata and zona reticularis in the homozygotes is wider than zona fasciculata in the heterozygotes (l = 0.5), which indicates a tendency. Experiment – zona fasciculata and zona reticularis in the heterozygotes is wider than the corresponding zones in the homozygotes (l = 1.3), $p < 0.2$, which is also a tendency.

Starvation stress had its most dramatic effects in the case of minks homozygous for the *Aleutian (a/a)* mutation. In the cortical zona fasciculata and zona reticularis, not only single dead cells but also entire fields of cytolysis were observed; blood vessels in the zona fasciculata were enlarged with hemorrhages at the border with the zona reticularis; zona fasciculata lost its typical columnar structure and showed a somewhat honeycomb structure with the constant presence of cells with pyknotic nuclei (Fig. 3, 4).

We may suppose that one of the important genetic physiology mechanisms of this type of heterosis is the involvement of neuroendocrine systems, the adrenocortical system among them, which provide the adaptation of the organism to unfavorable factors, in the organism's systems on which functioning of the genes under study exert their pleiotropic effects. The advantage of the minks heterozygous for the mutations affecting coat pigmentations expressed in the form of their higher stress resistance may be one of the causes of the maintenance of polymorphism for the discussed loci in mink populations.

Acknowledgements

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Figure 3. *Histological pattern of the adrenal cortex zones in the minks homozygous for the Aleutian (a/a) mutation under chronic starvation stress. In zona fasciculata and zona reticularis, there exist not only single dead cells but also entire fields of cytolysis. Blood vessels in the zona fasciculata are enlarged with hemorrhages at the border with zona reticularis.*

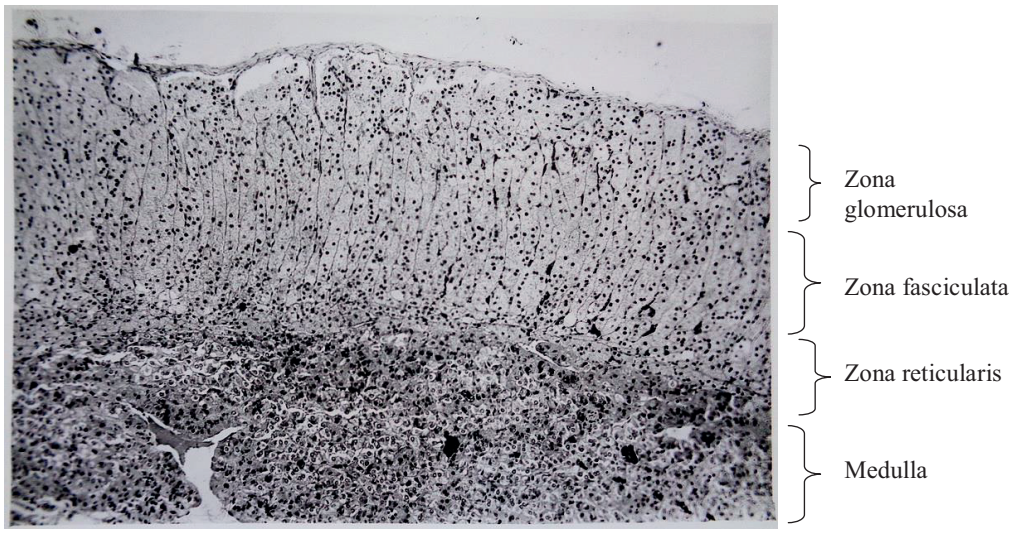
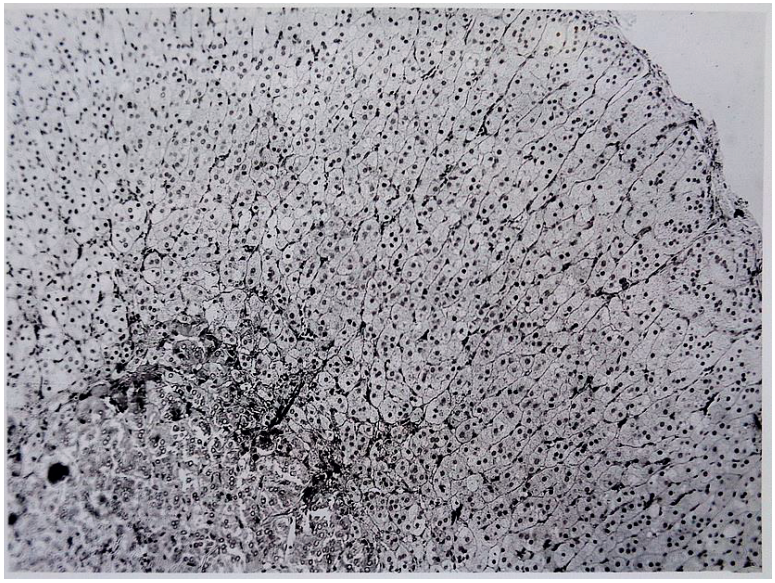


Figure 4. *Histological pattern of the adrenal cortex zones in the minks homozygous for the Aleutian (a/a) mutation in the control. Adrenal gland is of normal shape. The histological structure of all zones is preserved.*



Use of Biochar to reduce emissions from fur farms and to enhance the value of fur animal manure

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Abstract

Fur farms are currently sources of nutrient and greenhouse gas emissions and odor annoyance. This has set the business continuity in uncertainty. Therefore new emission reduction solutions are urgently needed. The use of biochar as a litter for fur animal manure is a new innovation. The project aims to find solutions for processing of fur manure in order to reduce the environmental load and also to create a new business model by making the most of the properties of biochar.

Productization of fur animal manure is a huge chance for the fur production sector, and it will contribute to the continuity of the business as a whole. The acceptability of the sector is improved when the disadvantages of the production are reduced and the benefits of sustainable use of fur manure are communicated to the public.

Keywords: greenhouse gases, ammonia, manure processing, productization

Introduction

Agriculture is the most significant source of ammonia emissions that cause e.g. odour problems and acidification. Ammonia emissions also cause large losses of nitrogen from agricultural systems. Ammonia is both a local problem as a nuisance to the neighbourhood and a challenge in the framework of the Convention on long-range transboundary air-pollution that sets limits to national emissions. The amount of greenhouse gases from fur animal production in Finland is estimated to be 120 Gg CO_2e that is emitted both as methane (CH_4) and nitrous oxide (N_2O). Gaseous emissions from fur manure cause local nuisance as well as leakage from the nutrient cycles of agriculture. This has diminished the acceptability of the business among citizens. New emission reduction solutions are urgently needed.

Peat is known as an effective cover material to reduce emissions from manure but its use as a non-renewable resource is questionable whereas biochar could have some additional benefits regarding e.g. the end-use of manure. The use of biochar as a litter for fur animal manure is a new innovation and one possibility to minimize the emissions from fur animal farms. Biochar has the potential to bind nitrogen on its surfaces and slow the diffusion of gases from manure to the atmosphere. This beneficial character of biochar can also reduce the emissions from processing of fur animal manure and save the nutrients in a growth media processed from the manure. The aim of the project is to find out economical mixtures of biochar and peat to be used both on fur farms and on composting plants to reduce the environmental load. The project also aims to find functional solutions for the production chain to be able to produce a growth media product with high marketing potential in Finland and abroad.

Material and methods

In laboratory different biochar -peat mixture were tested as covering material for fur animal manure. The amounts of ammonia and methane gases from the mixtures were measured using a gas analyser. According to the results of the laboratory test the most suitable biochar-peat litter will be chosen to be tested on four fur farms. A predefined amount of the mixture will be spread on the top of the fur manure under the animal houses according to a preset schedule. The amount of litter used for one fur animal will be calculated and the working time needed for spreading is to be measured. Also the amounts of ammonia and methane gases from the manure are measured using a gas analyser.

Processing of fur manure and biochar-peat mixture to a growing media is carried out in a composting plant. Ammonia and methane emissions from the process are measured. The properties of the final product are tested both in laboratory and on field trials. Surveys of the productization and marketing of fur animal manure are carried out.

Results

The results of the laboratory test showed that biochar-peat mixture used as a covering material reduced the emissions from fur manure. These results are discussed more detailed in Hellstedt & Regina (2016). The tests on fur farms are running at the moment. On the market there are implements suitable for spreading the mixture. No results from the emission measurements are yet available. The composting process is in progress and preliminary results are ready by the end of this year. First results of the Productization and marketing surveys show that there is a clear demand for growth media with balanced nutrient concentration and increased water-holding capacity.

Discussion

Using biochar cover on fur manure has the potential to reduce the gaseous emissions during animal housing and thus could be a solution to the local problems. If the manure is spread on soils, the added biochar has the potential to increase soil carbon stocks better that the more labile carbon of manure. This could be beneficial from the viewpoint of the climatic impact of agriculture especially if the biochar production process produces energy replacing fossil energy. Also, from the practical point of view, field application of biochar is simpler and less messy when mixed to manure. Biochar is known to increase the water-holding capacity of soils which could be a benefit both in field use and especially if the manure is used as a nutrient source in e.g. growth media. These considerations together with the reduced on-farm losses could motivate the use of biochar use in fur production.

Acknowledgements

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Hellstedt, M. and Regina, K., 2016. The effect of mixture of biochar and peat on the gaseous emissions from fur animal manure. Proceedings of the IFASA 2016 conference.

Nutrition, Feeding & Managements

Oral presentations

Amino acid availability from protein meals of different quality to mink kits

Differences between the kidneys of black and brown mink

The effect of biochar-peat mixture on the gaseous emissions from fur animal manure

Protein requirement for gestation and retention in fetal tissue in mink *(Neovison vison)*

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Using the product Bio-Mo™ to increase mink *(Neovison vison)* life span and productivity

Bioactive Effect of the Preparation "Biostyl" on the Different Genotypes of American Mink *(Neovison vison)*

Heterozygosity and stress resistance of the adrenal glands cortex structure in American mink *(Neovison Vison)*

Use of Biochar to reduce emissions from fur farms and to enhance the value of fur animal manure

Part 5. Behavior & Welfare

Development of environmental enrichment in blue foxes *(Vulpes lagopus)*: a review

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Abstract

Environmental enrichment aims to provide for animals stimuli that improve their welfare. The present paper provides a review on almost 60 studies carried out during the past three decades to develop environmental enrichment for farmed blue foxes *(Vulpes lagopus)*. These attempts have included changes in the basic cage structure, furnishing of the cages and activity or gnawing objects, as well as social enrichment. A wide variety of behavioural, physiological and health and production related measurements have been utilized to assess the foxes' welfare. The extent of the use of various enrichment objects and structures, as well as behavioural opportunities induced by group housing, indicate that enabling certain species-specific behaviour patterns is beneficial for the welfare of blue foxes. However, the welfare effects of several enrichments are either controversial or not clarified yet. There is a need for further studies to support not only legislative needs but also to support the refinement of the WelFur on-farm welfare assessment protocol. These include in particular housing systems combining different enrichment and social environments in innovative ways to 'multi-enriched environments' that are applicable on commercial farms. Also new methods for assessing the welfare effects, such as positive indicators of animal welfare, should be used.

Keywords: animal behaviour; species-specific behaviour; animal welfare

Introduction

Environmental enrichment (EE) can be defined as the addition of environmental features that will enhance the complexity of the animal's housing environment, resulting in beneficial effects on behaviour and other aspects of biological functioning (Newberry, 1994). EE can help animals to cope with stressors in their surroundings, reduce frustration, increase the fulfillment of behavioral needs, promote more positive affective states etc. (Mandel *et al.*, 2016). Methods can vary from the provision of a single enrichment item into various features in a re-constructed environment.

WelFur (2015, p 48) welfare assessment protocol for foxes defines EE as follows: '…an object or material inside the cage, or with regard to straw or such material also outside the cage so that it is available for the animal which allows species-specific manipulation and/or interaction with it, e.g. gnawing, carrying or digging with it. Enrichment can be wooden block, bone, pile of straw, rope, ball, year-round nest-box, digging substrate (e.g. sand), scratching plate or some other objects or material that are not harmful for the animals'.

In early 1990's, the European animal welfare recommendations (European Convention, 1991) led to intensive efforts to improve the cage conditions and/or to seek alternative housing environments for foxes. Since then, intensive research on various EE solutions and their potential welfare implications have been carried out (e.g. Mononen, 1996; Hovland and Bakken, 2000; Nimon and Broom, 2001; Ahola, 2007; Koistinen, 2009). Also the WelFur fox protocol (WelFur, 2015) is based on this knowledge. However, the scientific knowledge on the welfare effects of EE is still undoubtedly insufficient and in many cases also controversial. The objectives of the present paper are i) to give an overview of the studies on various forms of EE in blue foxes *(Vulpes lagopus)*, ii) to summarize their main welfare effects and iii) to provide future research perspectives.

Environmental enrichment and welfare of blue foxes

Table 1 summarizes the ways of providing EE to blue foxes, the welfare indicators utilised in EE studies and the welfare effects of EE. The table, and our paper, includes peer reviewed studies. Studies include changes in basic cage structures (pens, enclosures, complex housing, multi-level set ups; note that here also the size of the cages has been regarded as EE), furnishing of the cages (observation or resting platforms, year-round and seasonal nest boxes, concealment screens) and activity or gnawing objects (wooden block, straw, bone, pet toys, rope, hockey buck, stone), as well as social enrichment (group housing, flexible and loose-housing systems). In these studies a wide variety of behavioural, physiological and health and production related variables have been employed. Most common methods used for evaluating enrichment value of commodities have been the time spent with and behaviour directed to EE ('Use' in Table 1). Both simple preference tests ('Prefer') but also more sophisticated preference methods, such as resource motivation ('RM') have been employed. Also normal and abnormal behaviour ('Behav') and various behavioural tests ('BT'), physiology ('Physiol') and production variables ('Product') have been used. Also some health ('He') variables have been used.

Cage furniture

In experimental conditions various kinds of platforms have been provided for blue foxes. The foxes spend a marked proportion of their daily time on resting on or observing their environment from the platforms. Foxes prefer platforms that enable possibility to survey surroundings. Actual influences of platforms on animal welfare and production performance have been minor (Table 1). One study showed that wearing of fur and hair in foxes with platforms was twice as high as that in foxes without platforms (Korhonen and Niemelä, 1993).

Foxes seem to avoid cage sites where they cannot properly see around (Mononen *et al*., 1998). Visual barrier may affect temperament by increasing fearfulness (Pedersen *et al*., 2002). However, growing foxes with concealment screen were less stressed in September than those without (Mononen et al. 2001). Opaque walls have no effect on production result. Effects of platforms, nest boxes and visual barrier on organ size and health indices are open (Table 1).

Floor and top nest boxes have been studied during growing and breeding seasons as well as year-around. Only the study by Mononen et al. (1999) have shown that nest box type essentially affect reproduction, i.e. there were more barren vixens in the floor box than in the top box group. Year-round access to a nest box increases fearfulness and declines fur quality and growth (Korhonen *et al*., 2006). Harri *et al*., (1998) showed that access to a nest box decline fur quality and increased fearfulness during growing season. The nest box may serve the function as alternative option to withdrawal in challenging situation. In operant cost methods male foxes value access to nest box as well as a sand floor more than to a platform and wooden block during breeding season (Koistinen *et al*., 2009b).

Occupational objects

5Foxes use wooden blocks for carrying, chewing, poking and sniffing (Korhonen *et al.*, 2002). Wooden blocks affect positively on reproduction by increasing whelping success (Korhonen and Niemelä, 2000). Foxes have been found to favor wooden blocks to straw as enrichment (Korhonen *et al.*, 2002). Welfare and production effects of straw are open. Both, wooden blocks and bones are long-lasting objects, which enable various occupational activities, and may prevent development of stereotypic behaviour and dental plaque (Koistinen *et al.*, 2009c, Ahola *et al.*, 2010). Pet toys seem to have positive influence by increasing exploravity and reducing or delaying the occurrence and severity of dental plaque and gum inflammation (Pedersen, 2004).

Cage and housing structures

Foxes do not prefer to stay on the ground/sand floor over wire-mesh floor. Actually the situation most often seems to be opposite. Foxes do not choose only one floor material, yet they use each one that is available (e.g. Korhonen and Niemelä, 1997; Koistinen *et al.*, 2007; Koistinen, 2009). This is because different floor and resources serve different behavior and needs. In standard wire-mesh cages, foxes are able to perform the most basic behaviours and they do not seem to avoid walking on the wire mesh floor (e.g. Korhonen *et al.*, 2001a,b).

Research has been unable to show welfare implications of having access to ground flooring and larger cage (Table 1). For example, comprehensive study on housing in three different-sized cages equipped with wire-mesh flooring and in one enlarged pen combined with wire-mesh and earthen flooring did not reveal essential benefits of ground floor and large cage on physiological and behavioural parameters (Korhonen et al., 2001d). The wire-mesh section was distinctly preferred to the earthen floor section for most behaviours. Locomotor stereotypy tended to increase with increasing cage size. Floor material had minor effects on activity and stereotypies. Sand floor is beneficial for claws (Ahola *et al.*, 2009). Access to ground contact provides foxes opportunity for species-specific behavior like digging, however. Any superior welfare impacts of digging behaviour have been not yet proved.

Effects of floor material on production result have been slight (Table 1). Ahola *et al.* (2009) showed that sand floor may positively influence fur development in juveniles. Korhonen *et al.* (2001d), on the other hand, found indication for deteriorated fur quality (dirty furs) with earthen floor and large cage size. Dirty fur coat may be problematic because it declines insulation capacity of pelage and, thus, exposes animal to cold. Access to ground floor increases also occurrence of parasites (Korhonen *et al.*, 2001d). Blue foxes can be housed in hall housing conditions but with poorer reproduction performance (Table 1).

Social enrichment

Keeping several animals together in the same housing system could be expected to provide opportunities for both positive (e.g. playing, grooming, feeling safe) and agonistic (e.g. aggression) interaction. Potential benefits of social enrichment obviously vary with age, sex and season and depend on social relationships, available resources and enrichments, and how they are distributed in the cage area. Effects of social enrichment on production performance (fur properties, reproduction) have been typically negative (Table 1). Ahola *et al.* (2005) showed that group housing caused females more bite scars and led to higher serum cortisol levels than in males.

Complex housing conditions

Flexible/loose multi-tone housing systems can be assumed to provide a variable environment, which can stimulate physiological, behavioural and psychological needs. Flexible environment provides foxes with more choice, more control of the environment and a possibility to various horizontal and vertical

activity. Negative effects on production variables (reproduction, fur properties) and temperament were found in foxes kept in a very complicated, multi-enriched grouping set-up (Korhonen and Niemelä, 1996). A current multi-enriched study (Korhonen and Eskeli, 2013) is an example how the modern housing could look like. Cages with platform and wooden block were furnished with additional enrichments like a bone, a scratching plate, a hockey buck, a ceiling rope, a wall rope and straw. The basic idea was to provide the animals with opportunities to fulfil various behavioural motivations, and hence stimulate the animals both physically and mentally. For most enrichments both the time spent in contact and the frequency of contacts with the enrichments declined with time. This decline was not so evident for the platform, top nest box and wall rope. Blue foxes used readily the opportunities for more diverse behaviour.

The enrichment value of certain commodity seems not directly to be related to the amount of its daily use or preferences. More important is that enrichment is available whenever animal is willing to use it. Since the use of objects varies within and between individuals, it may reflect differences in the motivation to explore due to properties of the object or animal itself.

Future perspectives on research and implementation

To enrich housing environment is to give animals something to do, .i.e. something that maintains/enhances their well-being. "That something" should be one way or another linked to animal's species-specific behavior and needs. Further ideas and studies are needed in particular on housing systems combining different enrichment in innovative ways to a 'multi-enriched environment' that is applicable on commercial farms. Also new methods for assessing the putative welfare effects, such as positive indicators of animal welfare, should be clarified.

Enrichments may require a different housing system, but some types of enrichment may give the animals benefits with relatively minor changes. Temporary vs. regular changing schedules of enrichment objects should be considered. Seasonal opening of partition walls between cages would be a potential way to occasionally increase available area, cage enrichments and social activity. Methods for modified nest boxes and individual withdrawal need to be investigated, too. The floor space requirements have been frequently discussed. Additional space may not improve welfare per se, but it enables installations of various environmental enrichments. It also may provide possibilities to develop and conduct natural behaviour, including exploration and appropriate stimulation through play and activity, species-specific behaviour and social relations. Effects of space requirements on production results require further evaluation.

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Table 1. *Enrichments and methods employed in the studies from 1989 to 2016. Use= the amount of enrichment use, either as percentage or min per observed time. Prefer = simple preference testing. RM=measurement of resource motivation and/or operant cost. Behav= evaluation of single behavior (normal, abnormal) or wider ethogram. BT=behavioural in-cage and/or out-cage testing. Physiol= physiology, including measurements of hormonal, clinical-chemical and/or physical indices, and organ scaling. HE=health, including diseases, wounds, biting marks, haematological and clinical-chemical status. Product=measurements of production results. Welfare effects were roughly classified to positive, negative and non-effective. Positive welfare effects are marked in bold, negative ones underlined.*

Enrichment	Use	Prefer	RM	Behav	BT	Physiol	HE	Product
Platform	1, 3, 4, 7, 8, 9, 11, 12, 16, 27, 30, 53	5, 6, 46	55	4, 7, 8, 9, 11, 16, 30, 32, 45, 53, 54	5, 9, 27, 30, 32, 54	27, 29, 30		4, 7, 27, 30
Opaque wall	30			19, 30, 32	30, 32	**30**, 32		30
Nest box	2, 3, 11, 12, 15, 20, 41	5, 6, 13, 45, 53	**47**, 55	20, 29, 31, 38, 45, 47, 53, 54	15, 35, 38	31, 32, 38, 54		15, **20**, 24, 37, 38
Wooden block	23, 31, 41, 53	45, 53	55	23, **31**, 45	23, 31, 55	29, 31	**31**	**23**, 31
Bone	48			**48**		50	**50**	
Pet toys				35	**35**	35	**35**	
Straw	26, 54			26, 54	26, 54	26		26
Hockey buck				54	54			
Stone				2, 28				
Rope				54	54			
Digging plate	34, 47, 50	34, 47, 52		34, 47, 54	52, 54	52, 54		47, 54
Sandbox	33, 34, 53	45, 52	55	33, 34, 45, 47, 53	**33**, 34, 47, 52	33, 34, 44, 47, 52	34	33, 44, 47, 52
Floor	14, 21, 22, 28, 27, 29	6, 14, 18, 22, 22, 37, 49	40, 42, 43, 46, 47, 49, 55	14, 21, 22, 26, 27, 28, 40, 42, **43**, 44, 46, 47, 49	25, 2, 36, 39, 42, 45, 46	24, 29, 44	24, 29, **44**	24, 29, 37, **44**
Cage size	21, 27, 28, 29, 52	17, 21, 24, 26, 29, 46	46, 47	17, 21, 25, 26, 27, 28, 36, 46, 47, 51, 52	52, 53	24, 25, 26, 29, 36, 46, 51, 52	25, 29, 50, 51	24, 25, 29, 36, 51, 52
Complex housing	10			10	10			10
Hall				39, 41	39, 41	39, 41	39, 41	37, 39, 41
Social	2, 10			2, 10, 25, 36	10	25, 36		10, 25, 36, 37

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Juvenile silver foxes' preference for various environmental enrichment objects

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Abstract

Providing farmed silver foxes with suitable enrichment objects is important to support their needs for e.g. gnawing, exploration and play. Here, we assessed 30 juvenile, singly housed silver foxes' short-term preference for five different, but familiar, enrichment objects based on the immediate use of the objects when the foxes were given simultaneous access after one week of deprivation. Also, wear and tear was assessed as a measure of use and to estimate the robustness and durability of various objects. A meat bone (cattle femur) was clearly the first ranked object (P≤0.05), followed by a rawhide bone, a mutual pulling device mounted between neighbouring cages, and straw and a plastic cube as the least used objects. All of the objects were, to a various degree, gnawed by the foxes. After one week of use, about 30% of the cattle bone mass was reduced, compared to about 88% of the rawhide bone mass. Although the plastic cubes were almost indestructible and therefore durable objects their low ranking do not necessarily make them good enrichment objects for foxes. The preferred meat bones are relevant enrichment objects for foxes that elicit oral manipulation and play, activities that potentially support foxes' welfare.

Keywords: enrichment, gnawing objects, meat bones, fox farming, behaviour, welfare

Introduction

Farmed silver foxes *(Vulpes vulpes)* tend to nibble and gnaw available objects (e.g. wooden sticks) or the cage interior, like e.g. their nest boxes, shelves or food trays (Mononen *et al.*, 1998; pers. obs.). The propensity for exploration and oral manipulation are linked to foxes' biology as opportunistic hunters and scavengers (Fox, 1975) and likely based on combined, intrinsic motivations for exploration and foraging (hunting). Also, motivations for play may be present, especially in young animals (Bekoff and Byers, 1998). Consequently, access to appropriate types of enrichment, especially such that stimulate exploratory and predatory motivations, is assumed to be important as means to support foxes' welfare. Knowledge about the type of oral enrichment silver foxes would prefer is based on experience from commercial situations and from research on related species such as farmed blue foxes, dogs and other captive carnivores (e.g. Koistinen and Korhonen, 2013; Pullen *et al.*, 2010; Cloutier and Packard, 2014). These studies show that e.g. meat bones, wooden blocks, rawhide, straw, sand and dog toys are alternative enrichment objects for carnivores. Previous studies on wooden blocks, commonly used as gnawing objects for farmed foxes, have shown that splinters may cause irritation and bleeding in the stomach (Korhonen *et al.*, 2002). Examining foxes' preference for alternative objects that are suitable for oral manipulation is therefore important. As a first step towards exploring alternative oral enrichment objects for silver foxes, their immediate preference for objects that differed in sensory qualities and level of which they were chewable was measured in a multiple-choice set-up. These objects were a cattle bone, a rawhide bone, straw, a reciprocal pulling device, and a plastic toy cube. We hypothesized

that the cattle bone and the rawhide bone would be the most preferred objects. Furthermore, wear and tear of the objects was assessed as an additional estimate of use and durability.

Material and methods

Thirty juvenile silver foxes, 15 females and 15 males from a Norwegian commercial line participated in the study. From the age of about 12 weeks onwards they were housed in male-female pairs in a wire mesh cage (length x width x height: 117 x 107 x 76 cm) with a resting platform (25 x 106 cm), top nest box (98 × 55 × 48 cm) and wooden sticks. At the age of 5-6 months, the foxes were individually housed in a similar cage that also acted as the experimental cage in where the various enrichment objects were tested. The animals were fed a food paste for fur animals once a day and had free access to drinking water. The enrichment objects were: a) Mutual pulling device consisting of a metal wire and two hard plastic tubes mounted between two neighbouring cages; b) Straw - a metal hayrack mounted outside the cage and filled with 1 litre of straw: c) Pressed rawhide dog bone, 21 cm long, average weight 157 ± 12 g; d) Cattle femur bone – the upper (knuckle) part of the bone, heated and dried, average weight 695 ± 140 g e) Buster® soft cube, 10 cm in diameter, hard (ABS) plastic (380 ± 2.5 g) (for details see Hovland *et al*., *in press*). The animals were housed singly for one week to adjust to individual housing. Then, the foxes had continuous access to all the objects simultaneously for 10 days (habituation period). The foxes were then deprived of the objects for one week but with access to a wooden gnawing stick (deprivation period). After this, the wooden stick was removed and new objects of identical type were put in the cage yet again with continuous access for 7 days (reintroduction period). The foxes' activity and interaction with the objects (e.g. sniffing, pawing, gnawing, manipulating, playing) was video recorded continuously the 1st hour after admission in the Reintroduction period, and then for one hour on the 2nd and 4th day after access. Variables recorded during the 1st hour were 1) latency to first contact, 2) latency to first use and 3) total time interacting with each object. On the 2nd and 4th day, total time interacting with the objects (for 1 h each day) was recorded. Preference for the objects was determined based on latency to first contact and use and total interaction time. The objects were weighed at the start and end of both access periods. Deterioration of the cubes was assessed based on a subjective, visual grading from photos on a scale from 1 to 5 with 5 as the most worn. GLM models (JMP 12) were used to test the effect of object type on the various time variables. Mean values are given ± SE. The study lasted from 27th of September to 28th of October 2013 and was completed at the NMBU research farm. The university's local Animal Research Authority approved the study.

Results

Latency to contact and use the cattle bone was significantly shorter compared to all other objects (contact: $F_{4,116}$=13.66, $P < 0.0001$; use: $F_{4,116}$=27.15, $P < 0.0001$; Figure 1). Total interaction time ranked the cattle bone as the most preferred object (1751.4 ± 128.52 s), followed by rawhide (313.6 ± 76.60 s), the pulling device as the 3rd ranked item (62.4 ± 8.19 s) and straw (31.4 ± 6.15 s) and cube (24.7 ± 3.56 s) as the least preferred objects ($F_{4,116}$=150.27, $P < 0.0001$).

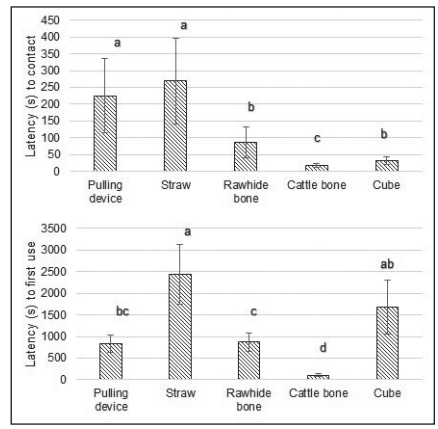

Figure 1. *Latency (s, mean ± SE) to first contact with and first use of the various objects during the first hour after reintroduction. Different letters denote significant differences between the objects.*

There was a significant effect of object type on total time spent interacting with the objects both Day 2 ($F_{4,116}$=8.04, P<0.0001) and Day 4 ($F_{4,116}$=4.51, P = 0.002). Time spent with rawhide and cattle bones was higher compared to the other objects on Day 2 ($P \leq 0.01$). On Day 4 time spent with the cattle bone was higher than with the other objects ($P \leq 0.01$) except for the pulling device (P > 0.05). The foxes used the pulling device mostly alone ($F_{1,28}$=70.9, $P \leq 0.0001$). The first hour after reintroduction the cattle bone was used mainly as a gnawing object, which was also the case on Day 2 and Day 4. The percentage change in weight of the objects, excepting the cube that was 100% intact, is given for both periods in Table 2. The rawhide bones were worn the most whereas there was only a slight decrease in the weight of the pulling device (Table 1). The mean values from the visual grading of the cubes was 2.65 ± 0.23 during Habituation and 3.15 ± 0.23 during the Reintroduction, which was significantly different (Paired t-test, t=5.06, $P \leq 0.0001$). These data show that the foxes gnawed the cubes in both periods.

Table 1. *The percentage reduction (± SE) in mass of the objects measured as weight (g) and/or volume (dl), for the cattle bone, rawhide bone, straw and pulling device at the end of both periods.*

Period	Cattle bone (%)	Rawhide bone (%)	Straw (%)*	Pulling device (%)
Habituation	28.7 ± 1.53	93.4 ± 3.18	40.0 ± 4.94	8.4 ± 1.96
Reintroduction	29.2 ± 1.98	88.0 ± 4.75	12.7 ± 1.91	2.1 ± 0.58

* most of the used straw fell through the cage floor and was located under the foxes cages

Discussion

Juvenile silver foxes preferred the cattle bone over all other objects as this was the object they sniffed and interacted with first, and spent almost half their time with (49%) during the first hour of access following deprivation. Gnawing was the predominant activity with the cattle bone. Based on time spent in interaction with the object the second most preferred object was the rawhide bone. The pulling device was rated as the third most preferred based on total interaction time (2%). Finally, straw and cube were used the least during the first hour of access and latency to start using these objects was longer. During the 1 h observation period on Day 2, foxes spent more time with the cattle bone and the rawhide bone than with the other objects. On Day 4, time spent with objects were shifted towards the pulling device. In a study with young laboratory housed beagles, Hubrecht (1993) found that out of the three available chewing objects rawhide, gum bone and plastic hose pipe, the rawhide and gum bone was gnawed the most. Our results showed that both the cattle bone and the rawhide bone served as gnawing objects but also elicited play and other activities. The data further revealed that foxes had used all objects during both periods and that the rawhide bone was the object of least endurance. On average, only about 10% of the rawhide bone was left as more than half the foxes had consumed all or most of their rawhide the fifth day after reintroduction to the objects. Further, our results showed that wearing of the cattle bones, in terms of weight loss, was about 30%. In a study examining long-term use of raw cattle femur bones in blue foxes Ahola et al. (2010) assessed the weight reduction after 6 months to average about 32%. The bones used in that study were heavier and thus, probably more solid compared to the bones in our study. When constructing the pulling device our intention was to develop an object that would elicit positive interactions related to play and hunting. However, as the pulling device was used mostly alone, and seemed to trigger defensive responses, additional 24h recordings would be needed for a more reliable evaluation of its relevance as an enrichment object. Also Hubrecht (1993) tried a kind of pulley system with rawhide bones between opposite pens 'however, he found the system not to be of 'any practical advantage' for kennel dogs. Foxes used straw for chewing, nibbling and play but use was considerably reduced from the habituation period to the reintroduction period, which may have been related to a habituation effect or to dislike, as straw may cause irritation to the mucous membrane of the stomach

and intestine (Korhonen *et al*., 2002). One of the least preferred objects, the cube, was the most endurable and indestructible item, although our recordings showed that it was also used for gnawing. Seen from a fox farmer's perspective, economic considerations would likely affect their choice of enrichment. Based on durability the cube would probably be long lasting, and therefore economically defendable in a long-term perspective. However, as the foxes ranked the cube as one of the least preferred objects the enrichment value of this object is uncertain. The first ranked cattle bone was 70% intact after one week, which could defend the initial cost. However, this type of bones, initially produced for the family dog market, would probably be too costly. Alternatively, raw cattle femur bones or raw elk or deer femur bones, regularly available for a low cost during the hunting season, would likely be good substitutes.

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WelFur welfare assessment results from a sample of Finnish fox farms in 2012-2014

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Abstract

The welfare of all farm animals needs to be improved, and 'a perfect fox farm' in terms of animal welfare does not exist. The ultimate aim of the WelFur scheme is to encourage the fur farmers to improve animal welfare. In the Fur Farm 2020 project in 2012-2014, a full three-period set of WelFur welfare assessment data was collected from 57 Finnish fox farms. The aims were to test the WelFur fox scheme in practise, and collect data for the further development of the protocol. We report here the overall assessment results, as well as the results from the four welfare principles and 12 welfare criteria. The percentages of the farms in the four overall welfare classification categories were: 'Best current practice' 11 % (6 farms), 'Good current practice' 82 % (47 farms), 'Acceptable current practice' 7 % (4 farms) and 'Unacceptable current practice' 0 % (0 farms). We conclude that WelFur fox protocol can serve as an important tool in improving the welfare of farmed foxes.

Keywords: animal welfare, feeding, housing, health, behaviour

Introduction

The development of WelFur welfare assessment protocols for farmed foxes and mink started in 2009 (Mononen et al., 2012). The protocols were finalised and published in 2015 (WelFur, 2015a,b).

The WelFur welfare assessment protocols are based on four welfare principles that comprise of altogether 12 welfare criteria (WelFur, 2015a; see also Table 1 below) under which the animals' welfare is assessed by one or more measurements. The total number of measurements in the fox protocol is 25, but all measurements are not carried out in all three production periods (P1 = winter period, P2 = summer period, P3 = autumn period). The measurement results are first calculated to criterion scores (from 0 to 100), then the criterion scores are combined to principle scores (from 0 to 100), and finally the principle scores are combined into an overall assessment by using specified rules. The overall assessment assigns each farm to one of the four welfare categories: 'Best current practice', 'Good current practice', 'Acceptable current practice' or 'Unacceptable current practice'.

The WelFur fox protocol was tested in practise during the development phase. The largest testing was carried out in 2012-2014 in the Fur Farm 2020 project in Finland (Ahola et al., 2014). Ahola *et al.* presented, however, the results only at the level of the original measurements, because the complicated formulas for calculating the criterion and principle scores (see WelFur, 2015a) were still being checked. The objective of the present paper is to report and discuss the final criterion and principle level scores

and overall classification results for those 57 Finnish fox farms from which complete assessment data was collected in the Fur Farm 2020 project.

Material and methods

WelFur fox welfare assessment data was collected from 57 voluntary Finnish fox farms according to the WelFur fox protocol (WelFur 2015a; N.B: minor changes were made to the protocol after the visits). Four of the assessors were experienced researchers or research assistants who had participated to the development of the fox protocol, and eight assessors were specially trained to do the assessment visits. The former carried out 15 % and the latter 85 % of the 171 assessment visits. The number of visits per assessor ranged from 1 to 32 (median 11). For practical reasons, there was variation between the farms in terms of whether all the three visits were carried out by one and same assessor (51 % of farms), or two (30 %) or three (19 %) different assessors.

On the farms the data was collected on recording sheets and calculated afterwards to 'percentages of various situations', and then to criterion scores, principle scores, and overall assessment (WelFur, 2015a). While synthesising the four principle-scores into an overall assessment "an indifference threshold equal to 5 is applied: for instance, 50 is not considered significantly lower than 55". The overall results presented here include applying this rule, but the principle scores are presented and interpreted as the 'original scores'.

The results are presented descriptively, i.e. the number and percentage of farms in each of the four overall welfare categories together with minimum, maximum, mean, standard deviation and median for each criterion and principle.

Results

The percentages of the farms in the four overall welfare classification categories were: 'Best current practice' 11 % (6 farms), 'Good current practice' 82 % (47 farms), 'Acceptable current practice' 7 % (4 farms) and 'Unacceptable current practice' 0 % (0 farms).

In the case of the *Good feeding* principle the average of the farms exceeded (median but not mean) slightly the lower bound (score 80) of the highest category ('Best current practice'), whereas the average for the *Appropriate behaviour* principle was just below the upper bound (score 55) of the second lowest category ('Acceptable current practice') (Table 1; see WelFur, 2015a, pp. 19-25, for the interpretation of the scores). The average scores for the principles *Good housing* and *Good health* were clearly in the second highest 'Good current practice' category. None of the individual farms was classified as 'Unacceptable current practice', i.e. the lowest category, for any of the principle scores. The maximum principle scores were either near or at the lower bound ('Good housing' and 'Appropriate behaviour') or the upper bound ('Good feeding' and 'Good health') of the highest category. All the minimum values were near the lower bound of the second lowest category.

Averages for the criterions scores were at the 'Best current practice' level for six, at the 'Good current practice' level for four and at the 'Acceptable current practice' level for two criteria. The maximum reached the highest category for ten out of the 12 criteria, and in the case of the two remaining criteria the best farms were clearly in the 'Good current practice' category. The worst farm, in turn, was in the 'Unacceptable current practice' for seven criteria, and for the remaining five criteria the category of the worst farm was 'Acceptable current practice'.

Table 1. *Descriptive statistics of the principle and criterion scores of the WelFur welfare assessments of 57 Finnish fox farms in 2012-14. P1 = winter, P2 = summer, P3 = autumn: periods in which each measurement is carried out.*

PRINCIPLES & Criteria (and measurements within each criterion)	Minimum - Maximum	Mean ± SD	Median
GOOD FEEDING	**30 - 100**	**78 ± 20**	**82**
Absence of prolonged hunger (Body condition score, P1-3)	19 - 100	78 ± 24	82
Absence of prolonged thirst (Continuous water availability, P1-3)	35 - 100	87 ± 18	100
GOOD HOUSING	**26 - 78**	**62 ± 10**	**65**
Comfort around resting (Cleanliness of the fur, P1-3; Availability of a platform, P1-3)	13 - 100	83 ± 25	100
Thermal comfort (Protection from exceptional weather conditions, P1-3)	38 - 100	65 ± 12	67
Ease of movement (Floor area, P1-3; Cage height, P1-3)	41 - 77	64 ± 9	67
GOOD HEALTH	**28 - 99**	**63 ± 16**	**66**
Absence of injuries (Difficulties in moving, P3; Skin lesions and/or other observed injuries to the body, P3)	14 - 100	72 ± 23	80
Absence of disease (Bent feet, P3; Ocular inflammation, P3; Impaired mouth and teeth health, P3; Diarrhoea, P3; Urinary tract infection, P1; Obviously sick fox P3; Mortality, P1-3)	28 - 100	61 ± 16	60
Absence of pain induced by management procedures (Killing method, P1-3)	43 - 99	96 ± 13	99
APPROPRIATE BEHAVIOUR	**23 - 78**	**49 ± 13**	**52**
Expression of social behaviours (Social housing, P3)	9 - 100	83 ± 25	100
Expression of other behaviours (Opportunity to use enrichment, P1-3; Opportunity to observe surroundings, P1-3; Stereotypic behaviour, P1-3; Fur chewing, P1-2)	12 - 70	49 ± 12	49
Good human-animal relationship (Feeding test, P1)	3 - 94	46 ± 20	44
Positive emotional state (Temperament test, P1; Transport of live foxes, P1-3)	4 - 83	58 ± 21	66

Discussion

The welfare of all farm animals needs to be improved, and 'a perfect fox farm' in terms of animal welfare does not exist. The ultimate aim of the WelFur scheme is to encourage the fur farmers to improve animal welfare (e.g. WelFur, 2015a), and it can serve this purpose in several ways. Benchmarking is important to the farmers (Lepistö *et al.*, 2014), and the overall category and principle scores will be useful for this and for certification purposes, whereas criterion level data will probably serve more advisory purposes.

A majority of the fox farms in our study was classified into the category 'Good current practice', and many of them have realistic opportunities to rise to the highest category. None of the six farms in the 'Best current practice' category scored to this category (at least 80) in all principles, i.e. also these farms can do some progress. Three out of the four farms in the 'Acceptable current practice' category can reach the 'Good current practice' with rather minor improvements in animal welfare. Accordingly, at the overall assessment level the WelFur fox scheme works well, and there is no need for major modifications in the rules how the farms are assigned to various categories (WelFur, 2015a, p. 25).

Information collected during the WelFur assessment visit is communicated to the farmer who can then focus on those areas of animal welfare that need the most immediate attention (Lepistö et al., 2014), and/or to create an animal welfare improvement strategy (Manteca and Jones, 2013). The variation between the farms was higher at the criterion than principle level, and the range was extended predominantly to the lower scores. This suggests that the criterion level data, in particular together with the even more detailed measurement level data (Ahola *et al.*, 2014), have plenty of potential for advisory purposes. We conclude that WelFur fox protocol can serve as an important tool in improving the welfare of farmed foxes.

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On the way towards on-farm welfare assessment protocol: what do we know about the welfare of Finnraccoons

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Abstract

In this paper I review the literature concerning the welfare of the Finnraccoon *(Nyctereutes procyonoides ussuriensis)* in the light of the development of an on-farm welfare assessment protocol. The four principles of animal welfare originally recognized in the Welfare Quality® will be covered, in terms of *Good feeding, Good Housing, Good Health* and *Appropriate Behaviour*. The Finnraccoon follows an annual fluctuation in appetite and general activity, including intermittent superficial hibernation in winter. The endocrinology of the energy metabolism is well understood. *N. procyonoides*, as a species, is vulnerable to numerous diseases and parasites, but on fur farms the general health status is good. The information concerning housing conditions, including cage size, environmental enrichment and management practices, is sporadic in small scale studies.

Keywords: behaviour, feeding, fur farming, health, housing

Introduction

After developing WelFur on-farm welfare assessment protocols for foxes (*Vulpes lagopus* and *V. vulpes*) and mink *(Neovison vison)*, next step is to look at the less numerous fur animal species raised in Europe (see e.g. Fur Europe 2016), like the Finnraccoon *(Nyctereutes procyonoides ussuriensis)*. The Finnraccoon is the farmed type of the wild European raccoon dog (Kasperek *et al.* 2015), one of the six subspecies of a fox-like canid originating from Asia (Ward and Wurster-Hill 1990). Finnraccoons have been raised in the same fur farms with other fur animal species from early 1970s (Mäkelä 1978). Typically, Finnraccoons are housed and managed like foxes *(Vulpes lagopus, V. vulpes)*, although their behaviour differs from that of fox species.

In the on-farm welfare assessment protocol, attention must be paid to the key points of the welfare of the species (Welfare quality® 2009a, b, c). In this paper I review the knowledge concerning the welfare principles of *Good Feeding, Good Housing, Good Health* and *Appropriate Behaviour* recognised in the Welfare Quality® and employed in the on-farm welfare assessment protocols for cattle, pig, poultry (Welfare quality® 2009a, b, c), foxes and mink (WelFur 2015a, b). The aim of the work is to bring the information visible for the development of the on-farm welfare assessment protocol. The use of references is limited to the main publications of each topic.

Good Feeding

The principle *Good feeding* aims at assessing the prolonged hunger and prolonged thirst (Welfare quality® 2009a, b, c).

The annual fluctuation of the appetite and following fluctuation of the body condition are well-known

phenomena in the Finnraccoon (e.g. Asikainen 2013, Korhonen 1987a). The voluntary feed intake exceeds the maintenance in summer/autumn and reduces below maintenance, even ceases in winter (Korhonen 1988a, b, Mustonen *et al.* 2004a). Finnraccoons tolerate periods of food scarcity and may fall in periodical, superficial hibernation in winter without deleterious effects in their physiology, health and reproduction (Asikainen 2013, Asikainen *et al.* 2002, 2003, 2005, Kinnunen *et al.* 2015, 2016, Koistinen *et al.* 2015, Łapiński *et al.* 2008, Mustonen *et al.* 2001, 2004a, b, 2007a, Nieminen *et al.* 2001, 2002, 2004, 2005, 2011). Weight regulation peptides regulated by photoperiod, with melatonin as a signal, are involved in the fluctuation of the activity level and appetite, as well as retention and mobilisation of fat reserves. The feeding on farms can be usually managed according to this natural fluctuation of the appetite in Finnraccoon.

In the Finnraccoon, water consumption takes typically a few minutes in a day, occurring more frequently in summer than in winter (Bielański et al. 1996, Korhonen 1988c, Korhonen *et al.* 1991a). Drinking may be suppressed in winter because of the down regulation of the metabolism (Asikainen *et al.* 2005). The type and functioning of the watering system affect the availability of the water and thus also welfare of the animals. Continuous water availability is usually secured on farms by providing water through an automatic frost-protected watering system.

Good Housing

The principle *Good Housing* aims at measuring the comfort around resting, thermal comfort and ease of movement (Welfare quality® 2009a, b, c).

Finnraccoons seem not to favour elevated platform (Korhonen *et al.* 1997, Mononen and Harri 1989) or a wooden plate on the cage floor as a resting site (Korhonen 1987b). Instead, nest box is used for resting, and it may invite superficial hibernation in winter (Asikainen 2013). In social housing, Finnraccoons prefer to allohuddle (Korhonen 1988c, Ahola *et al.* 2007).

Thermoregulation of the Finnraccoon has gained much attention. Past decades it was measured that the lower critical temperature would be around ambient temperature (T_a) of 15 °C and 10 °C in the summer and winter fur, respectively (Korhonen *et al.* 1983, 1985, Korhonen and Harri 1984). However, the size of the animal has increased, and the fur insulation has changed from those times so much that the results may not be applicable today farm population. More recent research indicate that the fur, white adipose tissue (adults) and shivering thermogenesis maintain the body temperature (T_b) (Niiranen *et al.* 2015), which follow oscillation of T_a (Mustonen *et al.* 2012), as well as diurnal oscillation including periods of shallow hypothermia in winter (Nieminen *et al.* 2005, Mustonen *et al.* 2007b, 2012). Shivering have been observed on T_a:s -6 - -8 °C in fasted animals (Asikainen *et al.* 2003). The heat loss can be decreased by providing a nest box (Korhonen and Harri 1986), in which the temperature remains at least 1-2 °C higher than the Ta as long as the animal stays in (Harri *et al.* 1989). The Finnraccoon pelage is favourable for capturing warmth from the solar load; Finnraccoons can warm themselves by basking in the sun (Harri and Korhonen 1988). The new born cubs with undeveloped thermoregulation are dependent on the external heat source, but they can tolerate hypothermia well by recovering from T_bs as low as 25 °C (Harri *et al.* 1991a, b).

The sporadic knowledge regarding the ease of movement indicate that relatively small changes in the available area and cage structure (typically combined with variation in group size) do not affect systematically any of the used welfare and production indicators (Ahola *et al.* 2004, 2007, Korhonen and Harri 1988a, b, Korhonen et al. 1986, Mohaibes et al. 2008). Cage-housed Finnraccoons use an outdoor run for locomotion but do not avoid staying in the mesh cage (Korhonen and Alasuutari 1993). A

larger enclosure is not used randomly, but constant path ways are established (Korhonen *et al.* 1991a).

Good Health

The principle *Good Health* was designed to evaluate the absence of injuries, diseases and pain induced by management procedures (Welfare quality® 2009a, b, c).

Injuries are rare in Finnraccoons. Skin lesions are observed in 0.5-1% of Finnraccoons, and moving difficulties in 1-2% of the Finnraccoons on Finnish farms (Koistinen *et al.* 2013, 2014). Although the wild, *N. p. ussuriensis* is recognised as a vector of several infectious diseases and parasite, of which many are zoonosis (e.g. Laurimaa *et al.* 2016, Sutor *et al.* 2014), the health status of the Finnraccoon is good on farms (Koistinen *et al.* 2013, 2014, Korhonen and Harri 1983). The diseases encountered on farm are described in the book by Kangas (2000). The research on the health of Finnraccoons is continuous (e.g. Zhao *et al.* 2015); recently, a necrotic pyoderma causing abscesses between the toes (Nordgren *et al.* 2014) and bent feet (Koistinen *et al.* 2013) has been described in Finnraccoon.

Since Finnraccoons are not typically subjected to any mutilations to their body, systematic transportation is rare, mainly natural mating is used and handling of the animals occurs seldom, the risks for pain caused by management practices is relatively low. There is no information of these slight handling procedures on the welfare of the Finnraccoon. The breeding success of Finnraccoons is relatively good (Profur 2016), and mortality low. Electrocution has been proved effective killing method in blue foxes (Korhonen *et al.* 2009), and there is reason to suspect that this method is effective also in the Finnraccoon.

Appropriate Behaviour

The principle *Appropriate behaviour* aims at evaluating the expression of social behaviours, expression of other behaviours, good human-animal relationship and positive emotional state (Welfare quality® 2009a, b, c).

Group housed juvenile Finnraccoons are more active but stereotype less than pair housed juveniles (Ahola *et al.* 2007, Hänninen *et al.* 2002). Other physiological and production-related measurements do not show consistent and systematic difference in the welfare between various group compositions (Ahola *et al.* 2004, Hänninen *et al.* 2002, Kasanen *et al.* 2000, Korhonen 1984, Korhonen and Harri 1988a, Korhonen et al. 1986, Piórkowska et al. 2002). Finnraccoons tend to synchronize their behaviour and allohuddle (Korhonen 1988c, Ahola *et al.* 2007). Olfactory signals, vocalisation, body gestures and behaviour are used for social communication. If possible, elimination occurs in a specific area, forming a pile dung called latrine, or in sites where the droppings do not easily fall through the perforated floor (Korhonen 1988c, Korhonen et al. 1991b). Elimination is somewhat ritualised. Communal defecation including anal sniffing is observed (Korhonen *et al.* 1991a, b). Behavioural communication include greeting, allogrooming and play (Korhonen et al. 1991a). Vocalisation of the Finnraccoon is poorly understood; only vocalisation of small cubs has gained some attention (Chaadaeva 2002).

The expression of other behaviours is linked to normal and natural behaviour of the species (Welfare quality® 2009a, b, c). In the wild, raccoon dog roam in dense vegetation, and avoid moving on open areas (e.g. Drygala 2008, Kauhala 1992). They forage for food, catch small prey and manipulate the food items. In winter, they remain mainly lethargic, periodically hibernating in a den. Adults live in pairs or in family groups. It can be assumed that opportunity for exploration, object manipulation, social communication and wintering are important features of the housing conditions of the Finn-

raccoon. With the exception of wintering and social communication (see above), the information of these features of the housing is sporadic in small-scale studies (e.g. Korhonen 1988c, Korhonen and Alasuutari 1993, Korhonen *et al.* 1991a, b). Stereotypic behaviour, occurring 0-3% of time (Ahola *et al.* 2007) and being more frequently in autumn than in other seasons (Koistinen *et al.* 2014), is probably linked to the circadian and annual activity rhythm, and feeding regime (Ahola *et al.* 2007, Korhonen 1988c). Also fur chewing is observed, up to 27% of the animals (Barabasz *et al.* 2011, Korhonen and Harri 1988b, Piórkowska *et al.* 2000), but the aetiology has not gained scientific attention.

Various tests for human-animal relationship and/or temperament have been used in the Finnraccoon. Feeding test is a validated test of human-animal relationship in foxes (Rekilä 1999), but may not been optimal test for Finnraccoon (Fortuńska and Barabasz 2003). Mere subjective evaluation of the temperament shows constant results throughout the growing season (Korhonen and Harri 1988b). In the empathy test (described by Gacek 1999) and capture test, the blood cortisol level is lower in Finnraccoons scored confident than in those scored aggressive or fearful (Fortuńska and Barabasz 2003), suggesting validity of these test in relation to stress hormone levels. In the empathy test, 14-17% of Finnraccoons were scored aggressive, 50-74% confident and 13-33% fearful (Barabasz *et al.* 2011, Łapiński *et al.* 2013).

Conclusions

As a conclusion, in the literature concerning Finnraccoon, the principle *Good feeding* of the criterion *Absence of prolonged hunger* is well covered, whereas the criterion *Absence of prolonged thirst* has not gained that much scientific attention. Knowledge is available of the *Good Housing*, especially on the thermoregulation, but some of this information may need to be updated. Furthermore, more research on various housing conditions is needed. The principle Good Health is relatively well covered in the literature and much basic information can be derived from the wild conspecifics and other species e.g. foxes. In the case of the principle *Approprate behaviour*, research is needed in the use and welfare effects of various enrichments and cage structures. The ongoing research project in Luke will concentrate on these topics and also to the questions related to resting preferences and ease of movement in the criterion *Good Housing*.

Any welfare scheme has a great potential in improving the welfare of the animals on commercial farms. Therefore, the development of the on-farm welfare assessment protocol also for the Finnraccoon is important.

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Insignificant effect of human activity and access between cages on group housed juvenile mink

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Abstract

Housing more than one juvenile mink of each sex in the same cage (group housing) has been shown to increase aggression, and bite marks in the leather side of mink pelts has been shown to be an ideal indicator of this aggression. In addition to high heritability, profound environmental factors are found to affect the number of bite-marks in both a selection and control line. Two hypothesises for such an environmental factor in group housing was tested: 1. The level of human activity around the mink. 2. Placing the access hole between the lower and the upper part in the back of the cage. We scored the number of bite-marks in a group selection line against bite-marks and an unselected control line of brown, group-housed mink over five successive years. Based on the bite mark score from the leather side of the skins at pelting, the two environmental factors tested had little and insignificant effect.

Keywords: group selection, bite-marks

Introduction

A three years selection experiment "Genetic adaptation of mink to group housing" has demonstrated a genetic variation in bite-mark score in group-housed mink (Berg & Møller, 2010; Alemu *et al*, 2012). Therefore, there is a potential for group selection against bite-marks on the skin side of the pelt. The total heritable variation of both direct and indirect genetic effects was more than two-fold greater than the heritability of a model with direct effects only (Alemu *et al*, 2014). Expressed as proportion of phenotypic variance, the direct and indirect genetic effects accounted for more than half the variation, while the rest was due to management and other environmental effects. In general, the bite-mark score varied significantly between years despite the genetic trend, indicating a need for investigation of the effect of non-genetic factors on bite marks.

In order to describe the reasons for this variation a number of factors has been investigated, including early or late separation of the kits into group housing (Møller & Hansen, 2013) and wire netting or solid plastic walls between the two-stores climbing cages (Møller & Hansen, 2014). Although early separation into group hosing could reduce the level of bite marks compared to late separation, this effect was not constant across farms and years (Møller & Hansen, 2013) and there were no difference between solid and wire netting walls between cages (Møller & Hansen, 2014).

Some farmers claim that the level of activity in the sheds should be limited to a minimum in order to reduce aggression among group-housed mink. It is known that the activity level of mink is low during daytime unless disturbed by human activity such as feeding (Hansen & Møller, 2008). Activity induced by human activity might be a potential onset of interaction between group-housed mink, including aggressive interactions. We therefore wanted to test the effect of high or low human activity

on the aggression level of group-housed mink.

When group housed mink are fed on top of the cage, some of them will access the feed standing on their hind legs at the bottom of the cage. Others will access the feed from the upper floor, through the access hole, if this is placed in front of the top cage. This might be an opportunity for aggression due to feed competition, and might lead to bite marks in the front of the body. We therefore wanted to test the effect of placing the access hole between cages in the front or the back of the top cage for group housed mink.

This investigation set out to test two hypothesises for bite-marks in the skin side of the pelt: 1. It is possible to reduce the bite-mark score in group housed mink by reducing the level of human activity. 2. It is possible to reduce the bite-mark score in group housed mink in climbing cages by placing the access hole between the lower and the upper part in the back of the cage rather than in the front.

Material and methods

A group selection experiment against bite-marks in brown, juvenile mink and an unselected control line, all group-housed 2 males + 2 females, was established at the Aarhus University research farm in Foulum in 2009. The results until 2011 were used for estimation of total heritable variation of both direct and indirect genetic effects (Alemu *et al*, 2012; 2014). The selection experiment was prolonged in order to investigate the effect of environmental factors in 2012 and 2013 (Møller & Hansen, 2013; 2014). In 2014, the effects of human activity and of connecting the cages in the back or the front of the cage were tested. Sixteen sections of two stores climbing cage with six cages each was distributed in two multi-row sheds. Eight cage sections were placed close to the entrance to the farm where all visitors to the farm will enter. The other eight cage sections were placed in the shed most far from the entrance to the farm where few, if any visitors to the farm will enter. In every second cage section, the top was turned around so that the access hole between the lower and the upper part was placed in the back of the cage rather than in the front. Two males and two females were placed in each cage, alternating between juveniles from the selection line and controls that were naïve to group housing.

A total of 384 juvenile mink was included in the experiment and distributed as described in table 1. Due to 6 fatalities, and loss of ID marks or pelts during the pelting process the number of bite-marks was scored on 345 identifiable mink pelts from the experiment in which half of each selection line was separated into a shed with high or low activity and connection between climbing cages in the back or the front of the cage, as described in Table 1.

Bite-marks were scored from 0 to 9 in the Neck, Body, and Tail region on the skin side of the pelt during the pelting procedure, just after fleshing, according to the procedure described by Berg & Møller (2010).

The model used to analyse the bite-mark score included the selection line (selection, control), level of activity (high, low), access hole between climbing cages (front, back) and interactions between these fixed effects. A probability level (P) of 0.05 was chosen as the limit for statistical significance in all tests.

Results

There was no significant difference between bite mark scores in sheds with high and low levels of activity or between cages with the opening connecting the lower and upper part of the cages placed in the back or the front of the cage (Table 2).

Table 1. *Number of mink in the experiment and number of pelts inspected for bite-marks (in brackets) in each combination of selection line, high or low human activity and access hole between climbing cages in the back or the front of the cage in 2014.*

No. of mink and (no. of pelts inspected)	Activity level	
Access hole, Selection line	High	Low
Front, selection	48 (43)	48 (46)
Front, control	48 (41)	48 (43)
Back, selection	48 (45)	48 (43)
Back, control	48 (39)	48 (42)

Table 2. *Sum of neck, body and tail bite-mark scores (Least Squares Means) of juvenile mink according to selection line (selection, control), level of activity (high, low) and connection between climbing cages (front, back).*

Selection line	Human activity level	Cage connection	Bite-mark score
Selection	High	Front	3,7
Selection	High	Back	2,8
Selection	Low	Front	2,9
Selection	Low	Back	3,1
Control	High	Front	8,5
Control	High	Back	10,3
Control	Low	Front	8,9
Control	Low	Back	10,2

The bite-mark score in the line selected against bite-marks was one third of the score in the unselected control line ($P < 0.0001$). (Table 2).

Discussion

The difference in human activity between the two groups had no influence on the bite-mark score. This indicates that more frequent activity by visitors and farm personnel during daytime, when the mink would otherwise be inactive, does not trigger aggressive interactions between the animals leading to bite-marks. We therefore reject our 1st hypothesis that it is possible to reduce the bite-mark score in group housed mink by reducing the level of human activity.

The access hole between the lower and upper floor of climbing cages in either the front or the back had no influence on the bite-mark score. This indicates that feed competition between mink feeding while standing on their hind legs at the bottom of the cage and mink feeding from the upper floor through the opening in front of the top cage, does not increase aggression and bite-marks. We therefore reject our 2nd hypothesis that it is possible to reduce the bite-mark score in group housed mink in climbing cages by connecting the cages in the back of the cage rather than in the front.

The large difference between selection and control lines indicate a continuous effect of selection against bite marks. This is also to be expected based on the high values of total heritable variation and especially the large indirect genetic effects that are included in group selection (Alemu *et al*, 2014).

The search for management or environmental factors affecting the level of bite-marks in group housing

should be continued in order to minimise the level down towards pair-wise housing of juvenile mink.

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It is possible to take a representative sample of animals based on the number of cages in use in each mink shed

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Abstract

Present study evaluates a new sampling method that has been developed for the welfare assessment protocol WelFur-Mink. The method is based only on information on the number of cages in use in each shed on the farm. In short, the number of cages in use is summarised shed by shed, and whenever the sample unit, i.e. the total number of cages in use divided by the number of cage sections in the sample, is reached, a section of cages is randomly selected in the respective shed. The new sampling method has proven feasible in practice and independent of the assessor. Based on a simulation study, we found that the accuracy and precision of the method were acceptable for the use in WelFur-Mink. Error ranged from -12.3 to 15.5 percentage points and Mean absolute deviation from the median ranged from 0.0 to 9.8 percentage points. The order the sheds were summarized in did not affect the representativeness of the samples. In conclusion, the new method is a systematic procedure for taking a representative sample of mink, which can be used in WelFur-Mink.

Keywords: animal welfare, sampling, stratification, welfare assessment, WelFur

Introduction

The WelFur Welfare assessment protocol for mink (WelFur-Mink) is based on a sample of 20 sections of 6 cages (120 cages) in the lactation and winter period and 15 sections of 6 cages (90 cages) in the growth period (Møller *et al.*, 2015). The animals are selected in sections of six cages for practical reasons, i.e. to simplify the assessment of the animal based measurements. To ensure a representative sample, the sample is stratified (divided into subgroups before sampling) according to a number of factors, which may affect the welfare of the animals. The factors include the animal's characteristics and housing conditions (Møller *et al.*, 2015). This means that a lot of information about the animals and housing systems on the farm is needed in order to do the stratification. This is time consuming, and in some situations is it not possible to obtain all of this information. Even with all the information at hand, it may be challenging to distribute the sample sections on the farm, so that the resulting sample is representative according to all the factors. Finally, there is a risk of an unknowingly bias by the assessor, who is responsible for selecting the sections on the farm. We developed a new sampling method, based on the systematic structure of mink farms, in order to overcome these problems. Mink farms are often built and expanded in stages, where the housing is more uniform in sheds built in the same stage. Hence, many farms consist of several units. The mink are also often gathered in the sheds according to e.g. colour type, sex and social housing. In the new sampling method, the sections in the sample are distributed between the sheds according to the number of cages in use in each shed relative to the total number of cages in use on the farm. By doing this, we aimed to ensure that the samples were stratified according to both the physical characteristics of the farm and the animals' characteristics. We also wanted to ensure that the samples were not based on animals in one single shed, as could

have been the case with a completely random selection. The European test of WelFur on 27 mink farms in January and February 2016 included a practical test of the new sampling method. We had developed an Excel sheet for this purpose; when entering the number of cages in use in each shed it was calculated how many sections to select in each shed. We also included a function that randomly selected the sections within the respective sheds. Based on the practical test, the new sampling method was found feasible in practice. The selection of the sections on the farm was independent of the assessor due to the randomising function; hence, the method was repeatable. The aim of the present study was to evaluate the representativeness of samples selected with the new sampling method. Also, as the number of cages in use is summarized shed by shed, it is possible that the order of the sheds, may affect the distribution of the sampled sections and thereby the representativeness of the samples. The effect of the shed order was, therefore, also evaluated. It was hypothesised that samples selected with the new sampling method would be representative for the farm, and that the order of the sheds would affect the distribution of the sampled sections but not the representativeness of the samples.

Material and methods

The new sampling method is based only on information on the number of cages in use in each shed. The starting point is the relation between the number of cages in use in each shed and the sample unit, i.e. the total number of cages in use on the farm divided by the number of cage sections that we want to sample. In order to take the number of animals in the previous sheds into account, all the cages in use are summarised shed by shed, and the ratio between the number of cages in use and the sample unit is calculated for the addition of each shed. This means, that first the ratio between the number of cages in use in shed 1 and the sample unit is calculated, then the ratio between the number of cages in use in shed 1 and 2 and the sample unit is calculated, etc. until the number of cages in use in all sheds have been summarised. The ratio for each shed is rounded to an integer, and this number, minus the number of cage sections that have been selected in previous sheds, is the number of cage sections that should be randomly selected within the respective sheds.

The accuracy and precision of the method was evaluated based on the simulation of the sampling of 300 samples on a model farm. The model farm was based on a private Danish mink farm to ensure a realistic layout and distribution of animals. The farm consisted of 23 sheds and about 42,000 cages, and was chosen as a model due to its complexity, i.e. the many different combinations of social housing, colour type, and housing conditions. The farm was visited in October 2015 and all cages were described section wise according to a range of factors (number of animals per cage, sex, age, colour type, social housing, cage type, cage wall, nest box insulation, nest box position, and rows per shed). The model farm was based on this description.

The selection of 100 samples on the model farm using the new sampling method was simulated for three different shed orders. Each sample consisted of 90 cages (15 sections of 6 cages) as in the growth period in WelFur-Mink (Møller *et al*., 2015). Order 1 followed the existing numbering of sheds on the farm while orders 2 and 3 were shed orders that would be natural to use if the sheds had been unnumbered. For each of the three shed orders, it was calculated how many sections to select in each shed using the new sampling method. A program that made a random selection of the 15 sections within the respective sheds was developed. The distribution of the animals in relation to the factors included in the stratification in WelFur-Mink (i.e. sex, age, social housing, colour type, cage type, cage wall, nest box position, nest box insulation, and shed type) was calculated for the samples and the model farm.

The accuracy and precision of the method was assessed individually for each combination of factor and shed order. The accuracy was assessed as the difference between the actual distribution on the model farm and the median of the 100 samples for each shed order (Error), and the precision was assessed as the mean absolute distance between the value of each sample and the median of the 100 samples for each shed order (MADM). Distributions of the calculated Error and MADM were evaluated by visualisation and the Shapiro-Wilks normality test. As data did not meet the assumptions of parametric testing only nonparametric tests were used. The effect of shed order on the accuracy and precision of the resulting samples was analysed using the Friedman test of differences with Error or MADM as response variable, shed order (1, 2 or 3) as explanatory variable and factor as block. The Wilcoxon rank sum test was used to analyse if the accuracy and precision differed between the animal related factors (i.e. age, sex, social housing, and colour type) and housing related factors (i.e. cage type, cage wall, nest box position, nest box insulation, and shed type) with Error or MADM as response variable and the type of factor (animal- or housing related) as explanatory variable. Excel was used for the calculations of how many sections to select in each shed. The remaining programming and statistics was made in R (R Core Team, 2015).

Results

Based on the three simulated shed orders (order 1, 2 and 3), sections were selected in 12, 13, or 14 different sheds. The resulting samples were based on a random selection of sections within almost the same sheds, despite the differences in shed orders. Shed order 1 and 2 only differed by the selection of one section, shed order 1 and 3 by the selection of two sections, and shed order 2 and 3 by the selection of three sections. For most of the animal and housing related factors, the majority of the samples were centred around the sample medians with fewer samples further away from the median. Error ranged from -12.3 to 15.5 percentage points and MADM ranged from 0.0 to 9.8 percentage points with some differences between the factors. Error and MADM did not differ between shed orders (Error: Chi Square value =0.17, p =0.92; MADM: Chi Square value= 0.25, p =0.88). Every section in the sample represents 6.7% of the total sample; hence, an Error of ±3.35 percentage points is as exact as the method can be. Including a ±2.5 percentage point margin, 72% of the Errors were within this range. Similarly, a sample range (min. to max.) of 6.7 percentage points is as exact as the method can be. Including a 5 percentage point margin, 22% of the sample ranges were within this range. MADM differed between animal and housing related factors (W=1185, p = 0.00016) with higher values for the animal related factors (median [25;75%-quartile], Animal: 5.1[4.0;6.9] vs. Housing: 1.8[0.6;4.8]), while Error did not differ (W=736.5, p = 0.60).

Discussion

The presented sampling method is a systematic procedure for taking a representative sample of mink at farm level. The accuracy of the method was high for most of the included factors, but for a few factors ('Percent adults housed individually', 'Percent housed in single/pair cages' and 'Percent housed in group cages'), the Error was more than ±10 percentage point. The calculated Error is affected by the initial distribution of the sections between the sheds. If the selected sheds include, for example, proportionate more group housed animals than on the farm, then the probability of selecting a cage section with group housed mink is increased compared to the actual probability on the farm. The model farm had more variation between sheds than most farms have. We, therefore, expect that the accuracy will be equal or higher on farms with less variation between sheds. Also, the precision differed between the factors, and was better for the housing related factors than for the animal related factors. This was no surprise, as there was more variation in the animal related factors compared to the housing related

factors within each shed. The less variation within each shed, the higher probability of selecting a sample that is an exact representation of the farm. Hence, the precision of the method are affected by the variation within each shed, and thereby also by the systematics on the farm. The model farm had more variation within each shed than most farms have. We, therefore, expect that samples selected with this method on other farms, will have an equal or higher precision than in this study.

But how representative are the samples selected with this new method? Every section in the WelFur-Mink sample represents 6.7% of the total sample in the growth period and 5% in the winter and lactation period. It will, therefore, only in rare cases be possible to select a sample that is a 100% exact representation of the animals and their housing conditions. This means that an Error of ±3.35 percentage points in the growth period is as exact as the method can be. When including a ±2.5 percentage point margin, 72% of the calculated Errors were within this range, leading us to conclude that the presented accuracy is acceptable in the context of WelFur-Mink. MADM is the mean absolute deviation from the median, hence; there are samples outside this range. If we look at the sample range, then a range of 6.7 percentage points is as exact as the method can be. When including a 5 percentage point margin, only 22% of the samples ranges were within this range. However, the distributions of the samples showed that most of the samples were centred round the sample medians with fewer samples further away from the median. The probability of selecting the samples that are the furthest away from the actual farm value is therefore smaller than selecting a sample closer to the actual farm value. This, and the expectation that samples selected with this method on other less complicated farms will have the same or a higher accuracy and precision than in this study, leads us to conclude, that the accuracy and precision of the presented method are acceptable for the use in WelFur-Mink.

The order of the sheds may affect which sheds are included in the sample, as the number of cages in use are summarised shed by shed. However, only the selection of a few sections differed between the tested shed orders, and this did not affect how representative the samples were. Similar neighbouring sheds (e.g. two-row sheds) was summarised consecutively while the starting point within each group of similar sheds and the order of the groups varied between the tested shed orders. If the sheds had been summarised in a completely random order, there would have been a larger variation in the number of sections to sample in each shed. However, the risk of not sampling any sections in the smallest sheds would also have increased. If shed order is random, is it also random if the small sheds are positioned in the shed order just where the sample unit is reached.

The sections in the sample are distributed between the sheds based on the number of cages in use in each shed. In extreme situations, a systematic variation on the farm may collide with the systematic in the sampling. If the sampling procedure e.g. suggests selecting a section in every second shed, and the farmer keeps a certain type of animals in every second shed, this type of animals will be overrepresented in the sample. This, and similar situations, can be dealt with by changing the order the sheds are summarised in, when the systematics at the farm is discovered.

In conclusion, we found that the representativeness of the new sampling method is acceptable for the use in WelFur-Mink. The method has proved feasible in practice and observer bias is avoided. We suggest that this method can be used in WelFur-Mink and when selecting representative samples of mink for other purposes.

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Neighbour effects confirm that stereotypic behaviours in mink are heterogeneous

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Abstract

Stereotypic behaviours (SBs) are prevalent across mink farms around the world and they appear in a variety of forms (e.g., route-tracing along the cage wall, scrabbling [i.e., scratching the cage walls], as well as performing stationary forms like head-bobbing). Despite its heterogeneity, SBs are usually analyzed as a single, broad category. We were interested in testing the hypothesis that scrabbling (and not other SBs) is influenced by the close proximity of neighbouring animals. We conducted two studies to test this hypothesis. The first study focussed on where SBs were performed within the home cage (are SBs performed close to or away from neighbours?). The second study focussed on SB time budgets (are SBs reduced by removing neighbours?). The results partially supported the hypothesis as scrabbling was the only SB performed towards neighbours (although only when mink were younger), and removing neighbours reduced the time spent performing both scrabbling and stationary SBs. Implications of the findings are discussed in terms of potential motivations to perform natural behaviours.

Keywords: conspecifics, scrabbling, welfare, husbandry, housing

Introduction

Stereotypic behaviours (SBs) are repetitive behaviours that are more common in barren environments than enriched, and thus known or suspected to be caused by motivational frustration and/or central nervous system dysfunction (Mason, 2006). SBs are common across mink farms around the world, and typically involve route-tracing and, less often, "stationary" forms (e.g., head-twirling) (Mason, 1993). Farmed mink may also repeatedly scratch at the cage walls ("scrabbling"), sometimes apparently directing this at neighbouring mink in adjacent cages. Although many studies investigate how to reduce the performance of SBs once they have developed (e.g., through the use of environmental enrichments: Dallaire *et al.*, 2012; Díez-León *et al.*, 2013), it is still not completely understood as to what initially causes SBs. Our two studies thus focus on the causation of SBs in young farmed male mink. In particular, we conducted two studies to test the hypothesis that scrabbling (but not other SBs) represents frustrated attempts to reach neighbours. Study 1 investigated whether SBs performed near and/or on the shared cage wall were affected by the proximity of neighbours and Study 2 investigated the effects of removing neighbours on the time spent performing SBs.

Material and Methods

We housed 32 young males in minimally-enriched cages (*W*60cm x *L*75cm x *H*45cm), with an exterior nestbox attached (*W*21cm x *L*25cm x *H*30 cm), between two non-experimental mink of random sex. Neighbours were visually obstructed from each other via a plastic cage wall. These cages were situated in an indoor facility that contained an artificial lighting system that followed "summer" sunrise and

sunset times (14L:10D) to minimize any confound of sexual motivation. Focal observations were used for SBs, which included route-tracing (i.e., pacing and its variants), stationary forms (i.e., SBs occurring in a single spot, e.g., head-twirling, head-bobbing, whole-body bobbing, and head-weaving), and scrabbling.

Behavioural data for Study 1 were collected during 6-8 day periods between 08:00h to 12:00h (always pre-feeding) when subjects were 7, 10, and 12 months old. For Study 2, baseline behavioural data were collected during a 6-day period when mink were 10 months old, and behavioural data for the neighbour removal manipulation were collected during a single day (two days after baseline observations were completed). In Study 1, we compared the proportion of each type of SB performed near each shared cage wall when the relevant neighbour in the adjacent cage was nearby (i.e., the neighbour was adjacent to the subject) versus when that neighbour was distant (i.e., the neighbour was in the nestbox or on the opposite side towards the subject if in the home cage). We predicted that only scrabbling would be performed close to neighbours. Study 2 compared SB time budgets before and immediately after removing one of each subjects' neighbours, to test the prediction that this would reduce scrabbling. Neighbours were removed from the home cage by voluntarily entering a wire-mesh tower that was attached to their home cage, which eventually led to a larger, enriched cage (*W*120 cm x *L*75 cm x *H*45 cm, please see Díez-León *et al.*, 2013 for diagram and details on this additional cage). Neighbours returned to their home cage after morning observations were completed for Study 2. Due to how this facility was set up, only one of the two neighbours had access to the enriched cage, thus the manipulation only involved removing one of our subjects' two neighbours (thereby resulting in one empty adjacent cage for each subject rather than two empty adjacent cages).

Results and Discussion

In Study 1, Wilcoxon Signed-Rank tests revealed that at 7 months, the total number of scrabbling occurrences that were performed on the shared cage wall was higher when male neighbours were nearby (*mdn*=0.46, *IQR*=0.74) than when distant (*mdn*=0, *IQR*=0.22, *W*=17, $p<0.05$), although this became non-significant when 10-12 months old. Scrabbling on the shared cage wall was not significantly affected by female neighbours' proximity. Other SBs' performed on the shared cage wall were also unaffected by neighbour proximity when subjects were 7 months old; but at 10-12 months, the total number of stationary SBs' occurrences that were performed near the shared cage wall was lower when male neighbours were nearby (*mdn*=0, *IQR*=0.21) versus distant (*mdn*=0.48, *IQR*=0.67, *W*=0, $p<0.05$), and the same held for route-tracing (although a trend) when neighbours of either sex were nearby (*mdn*=0, *IQR*=0) versus distant (*mdn*=0.60, *IQR*=0.39, *W*=0, $p=0.10$).

As predicted, removing neighbours (Study 2) also significantly reduced scrabbling (% of observations) regardless of neighbour sex (baseline *mdn*=0.07, *IQR*=0.09; treatment *mdn*=0.03, *IQR*=0.07, *W*=40, $p<0.05$). Surprisingly, it also reduced stationary SBs (% of observations), regardless of neighbour sex (baseline mdn=0.05, IQR=0.06; treatment *mdn*=0, *IQR*=0.10; *W*=8, $p<0.05$). Route-tracing was unaffected by the manipulation.

Overall, our results revealed that scrabbling by male mink is directed towards male neighbours (although not towards females) when 7 months old, but not when older (i.e., 10-12 months old). They also perform less of this SB if neighbours are removed (regardless of sex). Other SBs were generally performed in locations *away* from neighbours (although removing neighbours did not increase their performance). Hence, these data suggest that scrabbling may originally derive from frustrated attempts to access neighbours. In particular, since mink are naturally solitary animals, scrabbling may

represent territorial aggression, which may also explain why our male subjects only scrabbled towards other males (and not towards females). Indeed, wild mink naturally disperse around 3-4 months of age (Dunstone, 1993) and are thus already adapted to roam by adulthood. As such, the decrease in scrabbling towards other males at 10-12 months old could reflect this pattern (i.e., our male subjects may have already gotten accustomed to each other's presence by young adulthood). Most significantly, if scrabbling does represent a form of aggression, then future studies should elucidate whether proximity to conspecifics is stressful for farmed mink.

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Preferences for different cage heights in farmed American mink *(Neovison vison)*: taller is not better

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Abstract

Regulations assume that taller mink cages are better. European cages must be 46 cm tall minimum, while recent Canadian recommendations require cages to be at least 38 cm high. We tested the hypotheses that mink prefer lower heights for feeding (Exp. 1), but higher ones for other behaviours (Exp. 2). Exp. 1 housed 64 2.5 month-old black male-female pairs in 75Lx61W cages. Cage height was 46 cm, except for a modified feeding strip (15Lx61W) accommodating four different heights: 25, 38, 46 or 53 cm. Mink habituated to feeding from each height for 4 weeks. Food was then delivered to all heights and feeding observed, a procedure repeated at 3, 4, 5, 6 and 7 months. Exp. 2 gave 32 11 month-old mink (16 female, 16 male) free access to a compartment whose ceiling was set to 13 cm for 16 animals, and 52 cm for the other 16. Every 3 weeks this was progressively lowered/raised by 13 cm, the proportion of time spent in this compartment being assessed each third week. Exp. 1 showed that females preferred to feed from the lowest height ($p<0.01$), as did males once 5 months-old ($p<0.01$). Exp. 2 revealed no overall preference for any height, as overall they spent the same amount of time in the compartment regardless of its height (females even seemed to prefer the lowest height: $p<0.05$). Thus at group-level, mink do not prefer taller cages.

Keywords: cage size, behaviour, preference

This study is currently under review for publication in Applied Animal Behaviour Science as: Díez-León, M., Quinton, M., Mason, G. How tall should a mink cage be? Using animals' preferences for different ceiling heights to improve cage design. Full details on Methods and Results, as well as a comprehensive Introduction and Discussion can be found there.

Introduction

In both Europe and North America, regulations regarding cage dimensions for mink assume that taller cages are better for mink welfare. For example, in Europe, on-farm audits under the Welfur scheme give the best scores to cages taller than 49.5 cm (EFBA 2013). A recent revision of the Canadian Code of Practice for the Care and Handling of Farmed mink therefore recommended disallowing cages under 38 cm high (NFACC/CMBA 2013). However, these recommendations assume that mink are motivated to stand up on their hind legs. They also disregard the fact that young mink need to climb to feed from the top of the cage, if the ceiling is high, in a way that they might find aversive, since this is not a feeding posture mink would adopt in the wild. Finally, these recommendations are also focused exclusively on external height, ignoring the fact that in North America, some cages are provided with 'drop-in' nest boxes (as opposed to nest boxes outside of the cage) which reduce the internal height of the ceiling within the cage. Given the lack of welfare-relevant research in both Europe and North America regarding the best cage heights for mink (Finley *et al.* 2012), we ran two complementary

experiments aimed at testing the hypotheses that mink prefer lower heights for feeding (Exp. 1), but higher ones for other behaviours (Exp. 2).

Material & Methods

Experiments took place at the Michigan State University Experimental Fur Farm (Exp.1: July-November 2014; Exp. 2: March-June 2015). For Exp. 1, we housed 64 2.5 month-old male-female pairs of the black colour-type in cages (75Lx61Wx46H cm) whose front top portion (15Lx61W cm) had been modified to accommodate four different feeding heights: 25, 38, 46 or 53 cm. These heights were chosen to represent the range of heights currently in use in both Europe and North America, and their position in each cage was randomised across mink in a way that avoided confounds with the room layout. Mink were habituated to feeding from each of the four different heights for a period of at least 8 days. Mink were fed fresh food approx. *ad libitum*. Food was then delivered to all four heights simultaneously (same amount than on a regular day but divide in four), and feeding was observed live for 30 minutes after food delivery. This procedure was done once a month and repeated when subjects were 3, 4, 5, 6 and 7 months old. Food leftovers after 5h were also recorded each time.

Exp. 2 gave 32 mink from Exp. 1 (16 females :16 males, 11 months old) free access to a large, enriched compartment (see Díez-León et al. 2013 for details) whose ceiling was set to 13 cm for 16 animals (8 females : 8 males), and 52 cm for the other 16. This ceiling was progressively lowered or raised by 13 cm every 3 weeks, and each third week the proportion of time spent in this compartment (both engaged in active and inactive behaviour) and whether the mink were observed standing upright while in there were live-recorded.

Data were analysed using General Linear Models and transformed when necessary (e.g. proportions were arcsine square root transformed). In Exp. 1, feeding preferences were assessed by running two repeated measures models, one that had '% of feeding observations' as the dependent variable, and feeding height, sex, age, and their interactions as blocking factors; another one with % of food left over as the dependent variable, and feeding height, age, and their interactions as blocking factors. Cage was also included in all three models as a random factor. In Exp. 2, ceiling height preferences were assessed by running a model with '% of time spent in the compartment' as the dependent variable, and ceiling height, sex, order of height presentation, and their interactions as blocking factors. Individual mink was nested in both sex and order, and treated as random.

Results

Results from Exp. 1 showed that females always preferred to feed from the lowest height ($p<0.01$). For males, this same preference emerged once they were 5 months old ($p<0.01$). Analyses of food leftovers yielded similar results, with food being consumed faster from the lowest heights. Results from Exp. 2 revealed no group-level preference for any ceiling height, as mink seemed to spend the same amount of time in the compartment regardless of its ceiling height. Females seemed to spend more time in the compartment when the ceiling height was at its lowest, but only if this lowest height was presented last ($p<0.05$).

Discussion

It appears then that mink do not prefer taller cages, and indeed prefer to feed from lower heights, not just as juveniles, but even once they have reached adult size. Preference to feed from lower heights can

be explained in two ways: i) that mink find standing upright or climb to feed aversive, perhaps because these postures are more energetically demanding and/or they increase the risk of falling when young; and ii) that mink prefer to feed using more natural postures (cf. Dunstone 1993). Also, while mink were observed to stand upright, they did not seem to preferentially use cage heights that allowed for this posture to be adopted. This might indicate that they are not motivated to stand upright. However, it is also possible that when the ceiling of the compartment was low, mink chose to stood upright in their home cages. Therefore, minks' motivation to stand upright needs to be investigated further. Future research should also look at long-term welfare implications of different cage heights when choice is not offered.

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Mink behaviour, reproduction and welfare is influenced by nest box material and access to additional drinking nipples in the maternity unit

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Aiming to improve kit survival in mink, we investigated four factors: (i) bedding materials (a. straw, b. straw+lambswool, c. EasyStroe product), (ii) timing for straw provision (a. early, b. medio, c. later after mating), (iii) timing for transfer into the maternity unit (March 23, April 20), and (iv) nest box insertion (a. stone brick, b. EasyBrick). These treatments were distributed across 480 double-mated brown 1st-year dams in 2015. Effects of an additional drinking nipple were tested in a subset of animals from postnatal day 18 to dam separation day 56. Mink used the prevalent nesting materials; however, with higher nest score for straw+lambswool. We did not detect major differences between bedding types in the reproductive output; however, the thermal challenge may have been relatively low. Medio timing for straw provision increased the kit mortality. EasyBrick in the nest box decreased kit mortality when the bedding material was straw. Based on video recordings, we demonstrated concurrent onset of eating and drinking in mink kits, when given access to additional drinking water close to the nest box. The extra drinking nipple reduced the number of injured kits, decreased preterm dam-litter separation, and thus improved welfare towards the end of the lactation period.

Keywords: kit survival, maternal behaviour, *Neovison vison* aka *Mustela vison*, ontogeny, nest building

Introduction

Based on in-nest 24h video recordings of births, we observed in average 9.6 kits born, with 6.2 alive one week after birth, close to the number present at 8 weeks. Thus, the early kit mortality – primarily occurring before day 3 after birth – may represent a major limitation for the number of kits per delivering dam realised in the production (Malmkvist *et al.*, 2006).

Mink is – relative to other production animals such as pigs, cattle and horses – born underdeveloped. Although their birthweight is comparable to that of pigs (ca. 0.5% of the dam weight), mink kits are unable to see and hear until approximately 28 days of age. The young mink has not reached the adult hearing capacity even after 8 weeks after delivery (Brandt *et al.*, 2013). Moreover, the kits have limited ability to defend their body temperature individually during the first weeks of life; consequently hypothermia is considered a life-threatening risk. Farm mink has a high capacity for delivering kits, however, also a high risk to loosing these again following e.g. inadequate maternal care, management or nesting environment (cf. Martino and Villar, 1990; Schneider and Hunter, 1993; Malmkvist *et al.*, 2006; Bækgaard *et al.*, 2007; Malmkvist and Palme, 2008, 2015).

Even before birth, several factors have been shown to influence the dam maternal care, the number of kits born and their survival. For example, birth problems may contribute to the early kit mortality. The duration of parturition in a group of dams with no kit mortality was in average 5.2 h, whereas dams with kit mortality used in average 10.1 h; both groups delivering identical number of liveborn kits

(Malmkvist *et al.*, 2007). Birth problems – e.g. in terms of prolonged birth duration, high variation in the interbirth-interval and stillbirths – are influenced both by the body condition score of the dam and her access to suitable nesting material. Dam in intermediate body condition delivered fewer stillborn kits than did fat dams (Malmkvist and Palme, 2008); a result also reported across several Danish Farms (Bækgaard *et al.*, 2007). Dams with free access to straw in the period prior to delivery had fewer birth problems than dams with access to wood-shavings only. Further different types of nesting material – and not the body condition score – influenced the dam stress hormone concentration, maternal care and mortality in live-born kits in this investigation (Malmkvist and Palme, 2008). These results underpin the importance of the nesting environment for mink prior to the delivery. However, only few systematic investigations have been performed on how to manage the nesting environment of mink prior to the delivery.

One study reported that the death risk for a kit was approximately 4 times higher for a kit born in a nesting environment with barley straw only, compared to a born in an environment with supplemental nesting material (i.e. barley straw, wood wool, and wool from Angora rabbits), given as free choice to the pregnant dams. This last group responded with a low mortality rate among the liveborn kits (ca. 5 %; Lund and Malmkvist, 2012).

This conference presentation is a summary of several experiments performed at our (Aarhus University) research farm, aiming to improve the number of surviving kits by several management procedures.

Material and Methods

We investigated four factors: (i) bedding materials (a. barley straw, b. barley straw+lambswool, c. EasyStroe experimental product from Dansk Dyrestimuli A/S DK-8900 Nykøbing Mors, Denmark; this experimental product was based on the commercial product Easystroe - c 85% wheat and 15% rapeseed heat treated straw cut into 1-1.5 cm pieces, www.easy-stroe.dk, with the addition of flax), (ii) timing for straw provision (a. early, b. medio, c. later after mating), (iii) timing for transfer into the maternity unit (March 23, April 20), and (iv) nest box insertion (a. stone brick, b. EasyBrick from Dansk Dyrestimuli, DK-8900 Nykøbing Mors, Denmark; EasyBrick is a pressure moulded brick of finely cut straw – c 85% wheat and 15% rapeseed; www.easy-stroe.dk). These four treatments were evenly distributed across 480 double-mated brown 1st-year dams in 2015 kept under standard cage conditions in one 10-row housing facility at research centre Foulum, DK-8830, Aarhus University, Denmark (Table 1). Effects of an additional drinking nipple (from Hedensted-Group A/S, DK-8722 Hedensted, Denmark) were tested in a subset of animals from postnatal day 18 to dam separation day 56.

Each nest box held a wooden bottom plate and a wire insertion with Easytroe as bottom material. All nest box lids were additionally covered with shredded barely straw during the experimental period, until the feeding of kits on the nest box lid from Day 28. The dams were randomly allocated to the six treatment groups. We had loggers (Ibutton) in 75% of the nest boxes for the measurement of temperature and relative humidity every 15 minute.

At the onset of the study, each dam received 80 g of nesting material according to Table 1. Hereafter nesting materials were given three days every week in case more than 50% of the material was used. No kit transfer between litters or rescue of cold kits was allowed in the experimental groups (cf. also Castella and Malmkvist, 2008). All dead kits within D0 (Day of birth) and D7 were autopsied and determined whether live- or stillborn using lung flotation test, and signs of lesions, abnormalities registered.

Table 1. *The six experimental groups of dams*

Group	Transfer to birth cage, date	Period of nesting material provision	Type of Nesting material	Number of mated dams
1	March 23	'Early' March 23-D7	Shredded barley straw	80*
2	March 23	'Medio' April 10-D7	Shredded barley straw	80*
3	March 23	'Late' April 20-D7	Shredded barley straw	80*
4	April 20	'Late' April 20-D7	Shredded barley straw	80*
5	March 23	'Medio' April 10 -D7	Free choice: Shredded barley straw + lambswool	80*
6	March 23	'Medio' April 10-D7	Easy-stroe**	80*

*Half of which had stone brick, and the other half with Easybrick in the nest box bottom
**Easy-stroe is an experimental product based upon Easystroe with addition of flax (*Linum usitatissimum)*.

For the drinking nipple experiment, a subset of dams with litter size at 6-11 kits at postnatal Day 18 was selected. Half of these were in cages with an additional drinking nipple (product developed by Hedensted-Group) placed outside the nest box opening. These litters were observed until separation from the dam at day 56 after birth, including video recordings of drinking behaviour of dam and kits.

Results and discussion

Mink used the prevalent nesting materials; however, with higher nest score for experimental group 5 (cf. table 1), having access to both barley straw and lambswool. We did not detect major differences between bedding types in the reproductive output. We cannot exclude that one reason for the discrepancy to earlier findings – the positive effects of using Angora rabbit wool on survival – could be due a different thermal challenge this particular year and type of relatively closed housing facility to mink. The timing of transfer of mated dams into the birth cage was selected based on previous findings (Malmkvist *et al*., 2015). Medio timing for straw provision increased the kit mortality. Likewise, previous studies have demonstrated that that transfer of mink dams to a novel cage around April 10 elicit an increased stress response compared to earlier or later timed management (Malmkvist and Palme, 2015). This period coincide with the time of implantation, peak levels of progesterone, and the period of early blastula growth (Stoufflet et al., 1989). Thus, one explanation for our result could be that mink in general are sensitive towards changes/stressors during this period, with long term negative effects also on kit survival. This is important knowledge for practice; however, we suggest that further studies are needed on this important topic to fully understand the causation behind the results.

Danish farmers traditionally fix a stone brick inside the nest box prior to the birth season, which may aid the kits to reach the fed when later placed upon the nest box lid. EasyBrick rather than a traditional stone brick in the nest box decreased kit mortality during the first week when the bedding material was straw. We speculate that the main reason for this result could be a positive effect of EasyBrick (pressure moulded straw) over a stone brick on the local in-nest climate. To evaluate this hypothesis, data on in-nest temperature and relative humidity around parturition for the two groups (EasyBrick vs. stone) are to be analysed statistically. Based on video recordings, we demonstrated concurrent onset of eating and drinking in mink kits, when given access to additional drinking water close to the nest box. If given the opportunity, kits were observed drinking around the same age as the onset of eating. This is new

knowledge, as previous kits were considered to eat weeks before drinking, however, this was based on observations with the drinking water is placed further away from their early position in the nest. The extra drinking nipple reduced the number of injured kits, decreased preterm dam-litter separation, and thus improved welfare towards the end of the lactation period.

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Diurnal drinking patterns of blue foxes *(Vulpes lagopus)* in winter: a pilot study

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Abstract

All fox farms, for example in Finland, do not have year-round automatic freeze-proof watering systems, but in sub-zero temperatures the foxes are watered manually. In winter, the manual watering is typically carried out by filling the water cups once a day, soon after the feed delivery. This practice is based on the assumption that the foxes' motivation to drink is weak at other times of the day than a few hours after feeding. We tested this assumption in February-April with five blue fox vixens who had ad libitum access to water that was provided from freeze-proof nipple drinkers. Drinking and eating behaviour were measured from video recordings with continuous recording, and the data included altogether 14 24-hour animal days. The drinking frequency varied from 0 to 76 and total drinking time from 0 to 21 min per 24 hours. The daily pattern of drinking was probably partially governed by the feeding time: 44 % of all drinking bouts were observed within the three-hour period after feeding, which is 3.5 times more than by chance ($P < 0.001$, binomial test). However, water consumption studies are needed to confirm the importance of continuous access to drinking water in blue foxes in winter.

Keywords: thirst, watering system, animal behaviour, animal welfare

Introduction

All fox farms, for example in Finland, do not have year-round automatic freeze-proof watering systems, but in sub-zero temperatures the foxes are watered manually (Ahola *et al.*, 2014). Typically, animals are provided warm water into water cups once a day, soon after being fed with fresh feed which contains 65-70 % water (Hernesniemi, 2000). This watering practice is based on the assumption that the foxes' motivation to drink is weak at other times of the day than a few of hours after feeding. The freezing rate of the drinking water in the cups depends on the amount of ice in the cup before the water delivery, the temperature of the water delivered and the ambient temperature. In practise, the animals may have access to drinking water only for a few hours per day during the coldest winter periods, which may compromise the foxes' welfare.

There are only a few studies on the water requirements of farmed foxes in winter. Daily water consumption of adult blue foxes *(Vulpes lagopus)* and silver foxes *(Vulpes vulpes)* ranges from 100 to 600 ml per day in winter (Dille *et al.*, 1998). Blue and silver foxes with freeze-proof watering systems have more dilute urine than animals that are offered 500 ml water in water cups once a day (Moe *et al.*, 2000).

In our pilot study, we measured the drinking behaviour of blue fox vixens in winter to test the hy-

pothesis that blue foxes' daily pattern of water intake would depend on the timing of the feed delivery.

Material and methods

The experiment was carried out on farm located in Kannus in Western Finland, approximately 63°54'00"N and 23°55'00"E. The behaviour of five breeding blue fox vixens (three primiparous and two multiparous) was video recorded for three 24-hour periods ('animal days') in February-April 2014. Infrared lights were used in the dark hours to facilitate seeing.

The foxes had ad libitum access to water that was provided from freeze-proof nipple drinkers. The foxes were fed with fresh fox feed (water content 65-70 %) that was delivered between 8 am and 1 pm. The first and last recordings included only one feed delivery, but during the first recording in February the animals were fed twice, i.e. in the both mornings of the 24-hour recording.

Drinking (mouth-contact to the drinking nipple) and eating (eating feed or mouth-contact to the feeding plate) behaviour were analysed with continuous recording, and general activity (animal on its four feet) with instantaneous sampling (1 min sampling interval length; Martin & Bateson 2007). Due to technical problems in February the data presented consists only of 14 animal days.

We report here, firstly, the total daily frequencies and durations of drinking. Secondly, we compare the drinking frequency to the total activity of the animals (the Pearson correlation, r). Thirdly, we illustrate the daily distribution of drinking bouts in each hour of the day. Finally, we test the relationship between the timing of the feed delivery and drinking by comparing (the binomial test) the drinking frequency after feeding in one-hour and three-hour time windows to the theoretical even daily distribution of all drinking bouts.

Results

The frequency of drinking bouts per 24 hours varied from 0 to 76, mean ± SD being 27 ± 22 (n=14 animal days). The corresponding figures for total daily drinking time were 0 to 20.9 min and 6.4 ± 5.7 min. Drinking frequency had a moderate correlation ($r = 0.61$; $p<0.05$) with daily total activity that ranged from 5.3 to 31.4 % of time (mean ± SD: 17.9 ± 7.8 % of time).

Fig. 1 illustrates that drinking bouts seem to be clustered more around the feed delivery hours than the other hours of the day. A more detailed analyses showed that the 30-min periods during which the animals were fed and the next 30 min period after that covered 14.9 % of the daily total 376 drinking bouts which is 3.6 times more ($p<0.001$, the binomial test) than by chance. When this time window was expanded to three hours, the corresponding figures were 43.7 % of the daily total 261 drinking bouts (N = 9 animal-days in the analysis: the 24-hour recording with the two feed deliveries ignored) and 3.5 times the chance level ($p<0.001$).

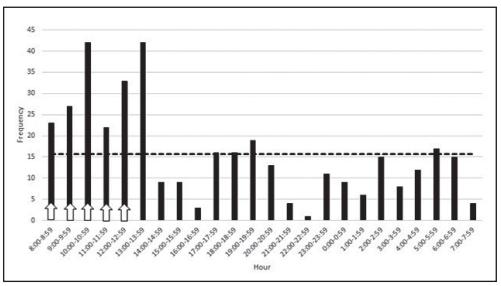

Figure 1. *The frequency of drinking bouts per each hour of the day by five female blue fox vixens in February-April. The data is pooled from all 14 animal days of the study. The open arrows indicate the hours of feed delivery for the observation days. The dashed vertical line represents the theoretical frequency if the bouts would have been distributed evenly across the hours.*

Discussion

The present pilot study showed that there was marked variation between the foxes and recording days in the frequency of drinking behaviour. One fox did not drink at all during one observation day, whereas another fox was in contact with the water nipple for more than 20 min per 24 hours. Abnormal behaviour (stereotypic behaviour or polydipsia) cannot be ruled out as a reason in the latter case. No health problems that could lead to decreased or increased water consumption were recorded in our experimental foxes.

The total frequency of drinking correlated with the general activity of the animals, but one cannot be sure of the causal relationship between these two. Instead, it seems that the daily pattern of drinking behaviour is, at least partially, governed by the feeding time. However, our simple experimental set-up does not allow very firm conclusions, since it cannot be ruled out that the mere time of the day rather than feed delivery is crucial in triggering the drinking behaviour.

Measuring behaviour with continuous recording is laborious (Martin & Bateson 2007). In addition, measuring drinking behaviour has some special challenges, since it may be difficult to see from video recordings when a fox is truly drinking, i.e. that there is really water intake.

We conclude that true water consumption studies with spilled water measurements are needed to confirm the importance of continuous access to drinking water in blue foxes in winter.

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Cortisol and testosterone in circulation are primarily excreted into faeces in male mink

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We examined the excretion of circulating cortisol and testosterone in adult male farm mink in February. Eighteen adult male mink, half of which were castrated in December, were intravenously injected once with tritium labelled cortisol (1st week) and testosterone (2nd week) and excreta sampled for 84 h. We found that faeces are the predominating excretory route of both circulating cortisol (83 %) and testosterone (93 %), with relatively short time lag to maximum concentration: 3.7 h for cortisol, and 7.5 h for testosterone. The minor excretion into urine was relatively quick for both hormones (peak for cortisol: 3.3 h; testosterone: 4.3 h). We did not detect any difference between intact and castrated male mink in the primary route and temporal pattern of excretion of neither cortisol nor testosterone. The excretory signal of testosterone in faeces was prolonged compared to that of cortisol, with radioactivity evident for approximately 20 hours longer after testosterone infusion in the male mink. In conclusion, the high proportion of excretion of cortisol and testosterone into faeces makes non-invasive measurement of hormone metabolites in faecal samples (rather than in urine) of male mink feasible e.g. in studies focussing on HPA-axis activity and responses in male reproductive hormones.

Keywords: male reproduction, *Neovison vison* aka *Mustela vison*, sex hormones, stress

Introduction

Non-invasive measurement of hormones is advantages when aiming to evaluate concentrations without handling the animals. Non-invasive measurement of glucocorticoids – output from the hypothalamic-pituitary-adrenal (HPA)-axis – in faeces may for example be used in the assessment of different type of housing and enrichment on female farm mink (Hansen *et al.*, 2007; Malmkvist and Palme, 2008; Malmkvist *et al.*, 2011). These HPA-axis responses are central in the assessment of stress and may be included in the evaluation of animal welfare (Mormede *et al.*, 2007). The baseline levels of these glucocorticoids (GC) in the blood are not easily obtained in large scale studies since the capture of one animal may arouse other not yet sampled animals housed nearby (Pedersen, 1992), and the handling and blood sampling itself may act as a stressor eliciting secretion of GC into circulation. The active HPA-axis hormone in circulation is cortisol in mink (Mormede *et al.*, 2007). Another hormone of relevance in the production of mink is testosterone, as the primary reproductive hormone in males.

An efficient non-invasive method is developed for mink females in relation to FCM as a valid indicator of cortisol (Malmkvist *et al.*, 2007). Today the measurement of cortisol metabolites in faeces of female mink is running in large-scale at our laboratory (Aarhus University) as a matter of routine. However, detailed information about metabolism and excretion of circulating cortisol and testosterone has not been described previously in male mink. This knowledge is useful in the development of novel non-invasive validated method of measurement. From other species than mink, sex difference has been founds in the excretion pattern (e.g. Palme *et al.,* 2005; Goymann, 2012; Touma *et al.*, 2003; Touma

and Palme, 2005). Therefore, we describe the primary excretion route of (i) cortisol and (ii) testosterone from blood into faeces/urine in adult male mink.

Materials and Methods

We used 9 male sibling pairs (n = 18) of a brown colour type of farmed mink. The mink were housed individually at the AU research according to the standard housing system. The males were born late April the previous year, and one of each sibling pair was castrated on December 3rd (n = 9), whereas it sibling was kept intact (n = 9). The sibling for castration was chosen randomly. The hormone excretion studies (1. Cortisol, 2. Testosterone) were performed during the 3rd to 13th February, i.e. before the mating season in March.

The eighteen animals were weighed and moved from their original cage into metabolic steel cages (dimension: 46 x 32 x 46 cm) with access to a straw covered nest box (18 x 24 x 23 cm) 7 days prior to onset of the sample collection. In addition to natural lighting from windows, the housing facility had full light h 08-20, dim red light h 20-24, and darkness h 24-08.

Two periods of data collection following infusing of hormones were used: 1. February 3rd to February 6th for the cortisol excretion study and 2. February 10th to 13th for the testosterone excretion study. For both periods, we injected each animal Day 0 (hour 0830-0950) once into the front leg vein with 1.0 ml radioactive-labelled hormone dissolved in sterile 0.9% NaCl Solution with 10% (v/v) ethanol.

We collected all urine and faeces delivered during the experimental period of four days, that is hours 0 to +84 relative to the time of injection on Day 0. The sampling intervals were Day 0: continuously hours 08-24, and Day 1 to 3: every hour 8-16, then at hour 20, and 24. The exact injection and sampling times were registered for each animal, and the urine and faecal samples were weighed to nearest 10 mg and stored at -21 °C until analysis for radioactivity (methods as in Malmkvist *et al.*, 2011).

Data were analysed in a linear normal model in the procedure 'Mixed' using the software SAS for calculation. A probability level (P) of 0.05 was chosen as the limit of statistical significance.

Results and discussion

Excretion of infused ^{3}H-cortisol

There was no significant difference between intact and castrated male mink in the total recovery (range 49 – 89% of the amount injected), the proportion of recovered radioactivity excreted in faeces (range 77 – 96%), the time lag until maximum concentration of radioactivity was reached in faeces (range 1.7 – 5.0 h) and in urine (range 1.4 – 5.4 h).

Faeces are the main predominating excretory route of cortisol in males, accounting for 86 % of the recovered radioactivity after a 3H-cortisol injection. This proportion of GC excretion via the faeces is higher than in most other species tested (Palme et al. 2005). The proportion is comparable to excretion in female farm mink, with faeces accounting for 83 % as average for cortisol (Malmkvist *et al.*, 2011). Supplementary to the excretion study, we performed behavioural sampling and HPLC – data not presented – however making the method for non-invasive measurement of cortisol via FCM an promising possibly also in male mink.

Excretion of infused ^{3}H-testosterone

There was no significant difference between Intact and Castrated male mink in the total recovery (range 51 – 105 % of the amount injected), the proportion of recovered radioactivity excreted in faeces (range 84 – 97%), the time lag until maximum concentration of radioactivity was reached in faeces (range 2.4 – 22.4 h) and in urine (range 0.5 – 11.4 h). The proportion of excretion into faeces increased weakly with the percentage of recovered radioactivity ($F_{1,15} = 4.8$, $P = 0.044$). There was a tendency that the time lag until maximum concentration of radioactivity in faeces decreased with the number of defecations per male ($F_{1,15} = 3.9$, $P = 0.068$).

Faeces are the predominating excretory route of testosterone in males, accounting for 93 % the recovered radioactivity after a 3H-testosterone injection versus only 7 % in the urine. This is the first promising step for the development of a non-invasive method based on faeces collection of male mink. The excretion pattern (data not presented) for male testosterone are however more complex and less distinct than for cortisol. We look forward to analyse this further, in order to obtain a trustworthy method for testosterone based on male faecal samples.

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Nipple surface temperatures in mink dams using IR thermography

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The purpose of using IR thermography to measure nipple surface temperature was to quantify signs of increased temperature and inflammations towards the end of the lactation period, which negatively would affect the welfare of the dam. After a period of method development and procedure adjustment, we evaluated surface temperature around nipples in dams at separation from the litter, in two experiments: (1) dams taken away from the litter either day 49 ± 1 (7w, *n* = 185) or day 56 ± 1 (8w, *n* = 189) after birth in 2014, and (2) dams from litters having additional drinking water or not during the lactation period in 2015. The method and result from these two experiments are presented. The progress of using IRT as a tool to evaluate teat health depends on valid methods, and for the first time, we presented a method for a systematic and standardised way to use IRT to evaluate surface temperatures in mink.

Keywords: infrared thermography (IRT), lactation, method development, *Neovison vison* aka *Mustela vision*, weaning

Introduction

We suggest a role for using infrared thermography (IRT) to evaluate indicators of importance for mink production and welfare. However, care should be taken when using thermovison in animal science, as several factors (e.g. type of camera, light conditions, angle and distance, hairiness, moisture, emissivity of the surface, vascularisation and blood perfusion of target area) potentially may influence the surface temperature readings (see e.g. Soerensen, 2014). These factors should be considered, and may particularly be a challenge during sampling on live animals at farms.

In the present study we used IRT pictures to determine the surface temperature around the nipple of dams. The reasoning was to see whether elevated temperatures could be detected, influenced by litter age/size/housing conditions and also linked with signs of nipple problems, such as mastitis, inflammations and wounds. These health problems are sometimes reported increasingly as the lactation period proceeds, and with many young in the litter. Likewise, it is well recognized that both the welfare of the dam and of the kits should be considered in determining the optimal time to move the dam away from her litter in production of fur animals (cf. EU, 1999; DMAF, 2006; NFACC, 2013). Monitoring the health of the dams due to the increased litter burden can thus be important. Infrared thermography (IRT) has previously been suggested for assessing surface temperatures in relation to health in pigs (Soerensen and Pedersen, 2015) and to estimate thermoregulation in sows (including at the udder; Malmkvist *et al.*, 2012) and in neonate pigs (Kammersgaard *et al.*, 2013). Also one mink study (Dawson *et al.*, 2013) indicated that mothers with get-away bunks were less likely to have swollen, red and/or crusting teats and may have higher surface temperatures around 6 weeks after birth. We present a method for a systematic way to use IRT to evaluate surface temperatures around the nipples in lactating mink dams. This method was used in two experiments: (1) dams taken away from the litter either at 7 or 8 weeks after birth in 2014, and (2) dams from litters having access to additional water nipples or not during the lactation period in 2015.

Materials and Methods

On the day for moving the dam from the litter, the dams were captured by hand and held outside the home cage with exposed ventral side allowing the observer to count and score nipples as either visible or active (defined as: visible nipples with swollen area, lack of hair around centre, apparently in use for suckling). Additionally, any wounds, being wet, and signs of inflammation, crustiness were registered for each visible nipple. The nipples were numbered (1-10) starting from the dam's right to her left and hereafter from her back to front legs. Infrared (IR) pictures were taken of the dam's teat region using a calibrated FLIR model P660 IR camera (Flir systems, Wilsonville, Oregon, USA). This model was a micro-bolometer type IR camera with a manufacturer specified accuracy of ± 1 °C, spectral sensitivity of 7.5-13.5 µm making it suitable for temperature measurements in the physiological range. The spatial resolution was 640 x 480 pixels and the thermal resolution (noise equivalent temperature difference, NETD) was 0.03 °C. The lens had an instantaneous field of view of 0.325 mm x 0.325 mm at a 50 cm distance. Experience of using this IR camera and analysis were based on previous validation work with this equipment (Soerensen, 2014).

The mink dam was held in a fixed position in a sampling cradle by two persons – one holding the mink, the other keeping hind legs away – with the dam teat region exposed, in equal distance and position perpendicular to the camera optical axis for a focused thermal image acquired by a third person. During thermal image acquisition, the mink was held in a mobile custom-made wooden box, keeping out sunlight and other infrared radiation coming from the surroundings, as this could otherwise reflect in the investigated mink surface area, influencing the measured surface temperatures. Wet teats were left wet since cooling effect by evaporation was limited inside the enclosing box. The duration for this procedure was up to 30 seconds from time of catching the dam in its home cage. The settings for skin emissivity was 0.98 assuming the infrared skin properties are similar to those found in human and porcine skin (Soerensen et al., 2014), and temperature adjusted to the ambient (range 13.5-23.0 °C) prior to each photo session. Thermal image analysis was performed in the ThermaCAM software (ThermaCAM Researcher Pro 2.10, Flir Systems). Surface temperatures (average, minimum, and maximum) were outputted for a circle of maximum 300 mm2 centred on each visible nipple and if the nipples were densely packed the circle areas were decreased to avoid overlapping nipple areas.

We used first-year brown farm mink dams housed and managed according to standard Danish conditions at the research farm at Aarhus University (DK-8830, Denmark) in 2014 and in 2015 for the two experiments. All mink were housed in one 10-row facility with 1020 cages, each second cage housing a dam with litter until separation.

Experiment 1 with two separation times (7 weeks and 8 weeks) was conducted in a balanced way, randomly allocating the dams to treatment group in equal number within each day of birth (from 23 April to 9 May 2014). The dam was taken away from the litter either at day 49 ± 1 (7w, n = 185) or at day 56 ± 1 (8w, n = 189) after birth. A detailed description of this study can be found in (Malmkvist *et al.*, 2016).

Experiment 2 included a group of dams with and without access to additional drinking nipples (prototype from Hedensted-Group, DK-8722, Hedensted, Denmark), placed close to the nest box opening with water access from Day 18. Dams (n = 2x 30) were taken away from the litter at day 56 ± 1 (8w) after birth.

Results and discussion

The mobile box made it possible to take an IRT picture immediately after capture, within the mink shed, next to the home cage. The sampling procedure involved three persons (one person operating the fixed camera, two persons holding upper and lower part of mink in a 'craddle') and the use of the apparatus functioned well after a period of training. We obtained focussed IRT pictures from c 430 dams; afterwards the pictures were handled in the software to prepare data. Both the sampling and the following data acquisition step need to be standardised to obtain data which can be replicated. In the sampling we used an automatic procedure to equalize the area in mm2 and thus number of pixels in a circle around each active nipple; this procedure is feasible only because we kept an equal angle and distance between the lens and the target area between individuals.

During the development of the sampling procedure, we did a special effort to reduce the time from capture to the first IRT picture; also to reduce time and to make the method feasible in large-scale experiments on farms rather than in a laboratory setting. The time of capture/animal handling may be of importance, as surface temperatures also depend on blood perfusion. Therefore activity and stressors activating the sympathetic nervous systems can lead to a different blood perfusions of the skin (e.g. due to vasoconstriction). However, in the present study – looking at the nipple area in lactating dams – this stressor affect may be of less importance. This could be studied further, also whether IRT can detect differences in the intensity of arousal/emotionality in animals.

Of the 2042 nipples evaluated in 342 dams in experiment 1, there were no signs of wounds, crustiness or inflammation, 1704 were evaluated as active (83%) and 9 (0.4%) as bitten off (NS different between 7w and 8w) but all healed/without a wound. No dams were diagnosed with signs of nursing sickness. The range in maximum surface temperature was 29.1-37.9 °C. The surface temperature of the active nipple area was in average 1.2 °C higher in 7w than in 8w dams ($F_{1,\ 347} = 55.3$, $P < 0.001$), and the mean surface temperature increased with litter size ($F_{1,\ 347} = 43.1$, $P < 0.001$; reported in Malmkvist *et al.*, 2016). Further studies, with dams having nursing sickness and following the development/effect treatments could be interesting.

We suggest that the lower surface temperature around active nipples at 8 weeks are due to reduced blood flow to support the milk producing tissue and by decreased suckling activity of the kits. This is in accordance with a study reporting that the average amount of dam mammary gland tissue is reduced from peak values of 40-45 g at week 4 to around 10 g week 7 and only 5 g week 8 after birth (Pinkalski and Møller, 2014). In line with these results, we found limited evidence for exhaustion in dams being with the litter for 8 versus 7 weeks, and there were no signs of nipple/inflammatory problems.

IRT data from experiment 2 is currently under analysis. Other indicators show that dams fare better in the treatment with additional drinking water close the nest box, and that the kits have an earlier onset of drinking (approx. 11 days compared to control group). This demonstrate for the first time a concurrent onset of eating and drinking behaviour in young mink, based on video observation (data not presented). Consequently, in case the burden is reduced in the dams, we predict reduced surface temperature around the nipples in the group provided with additional drinking water (primarily) for the kits.

We did not wipe of wet teats, although a wet surface can influence the surface temperature (Kammersgaard *et al.*, 2013). Avoiding wiping-off was chosen as we did not want to interfere with the surface temperature (or the dam arousal) by rubbing the dams' nipple area. Additionally, we minimized draft

and thus the influence of evaporative heat loss by using the custom-made enclosing box during sampling. Still we believe that it should be noted whether the nipple was visible wet or not, and this can be done after the taken picture to avoid further delay in the procedure.

In conclusion, we believe that IRT as a method has proven useful and may have further potential to be used in mink.

Influencing factors and type of equipment should be carefully considered, as with any method. We suggest the method of having a relatively closed apparatus (thus controlling radiation sources, and minimizing evaporative chilling caused by draft on wet surfaces) and keeping the mink in a standardised position a short, combined with a camera suitable for measurement in the physiological range, can be used to evaluate the surface temperature around the nipple area in lactating dams.

At the conference, we hope to get the possibility to discuss further relevant use of IRT for indicators of value for the welfare and production of farmed fur animals.

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Where hot males chill: the preferences of male American mink *(Neovison vison)* for different floor areas during hot weather

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Abstract

In North America, heat stress during summer represents a potential welfare issue. Larger floor areas may be beneficial, allowing spread-out postures during rest. Canadian industry-recommended floor areas are now 2225cm^2 for males, compared to 2550cm^2 in Europe. We therefore tested whether European floor areas improve mink welfare during summer. Focusing on Pastel, melatonin-implanted pair-housed males, we hypothesised that mink raised with North American (NA) cages will show more heat stress than mink raised with European (EU) cages; and that EU cages are preferred. We raised 64, non-related male pairs from weaning in EU or NA cages. For 7 hot (>27°C) and 7 cooler days (<27°C), we collected data on heat dissipation (via thermal imaging), contact between cagemates, and behavioural signs of heat stress (e.g. panting). After 5 weeks, we removed every other pair. Remaining pairs were given free access to their neighbouring cage (EU pairs now having access to NA cages and *vice versa*), and preferences were assessed by recording time spent in either cage. Preliminary results show no preference for either EU or NA cages. Analyses on heat stress-related variables are on-going. This research will help determine the welfare relevance of floor area for mink, an important issue for cage size regulations.

Keywords: heat stress, cage size, welfare, behaviour

Introduction

A recent revision of the Canadian Codes of Practice for the Care and Handling of Farmed mink recommended increasing current cage floor areas to 2225cm^2 for males (NFACC/CMBA 2013). These new standards fall short of European standards, which set floor areas at a minimum of 2550cm^2. Although mink raised in North America tend to be smaller in size than European mink, it is possible that larger floor areas than currently recommended could be beneficial for their welfare. Larger floor areas might be particularly important during North American summers: daily averages of 30°C are common in some mink-keeping regions (e.g. Ontario) making heat stress a concern, especially for melatonin-implanted individuals. We therefore conducted an experiment to test the hypotheses that mink raised in North American (NA) cages will show more signs of heat stress than mink raised in European (EU) cages; and that EU cages are preferred

Material & Methods

We selected melatonin-implanted, Pastel males for this experiment. These represent the population most at risk of heat stress at this time of the year due to their large size and their already-developing winter fur. We raised 64, non-related male pairs from weaning in EU (n=32 pairs) or NA (n=32 pairs) cages. Both types of cages were 46cm high, but had floor areas of either 2550cm^2 (EU) or 2225cm^2

(NA). Cage types alternated across the rooms (for a description of the facility, see Díez-León et al. 2013). After a 2 week habituation period, for 7 hot (>27°C) and 7 cooler days (<27°C), we collected thermal imaging data (using a FLIR T300 camera) on heat dissipation via exposed areas on their paws, as well as data on physical contact between cage mates when resting, % of food eaten c.5h after food delivery, and behavioural signs of heat stress (e.g. panting, decreased activity levels; cf. Williams 1986). After 5 weeks, we removed every other pair. Remaining pairs were then given access to their neighbouring cage via a connecting tunnel at the top of the cage, so that pairs that had been raised in EU cages now had access to NA cages and vice versa. The preferences of the two mink in each pair could then be assessed by recording the time they spent in either cage. After another 2 week habituation period, and in order to assess cage preference, time spent in either cage then quantified for 6 hot (>27°C) and 7 cooler days (<27°C).

Results & Discussion

Analyses on heat stress-related variables are on-going. Preliminary results from the 'preference' phase of our study suggest that mink used both cages equally, regardless of ambient temperature. Our final results should help determine the relevance of cage size *per se* to heat stress in mink.

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WelFur Fox: Inter-period consistency and inter-assessor repeatability of animal-based measurements of the principle Appropriate behavior

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Abstract

The WelFur fox assessment includes three production periods and according to the current fox protocol all measurements are not taken in all periods. The justification of this was studied for the four animal-based measurements of the Appropriate behavior principle: Stereotypic behavior, Fur chewing, Feeding test and Temperament test. We clarified with correlation analyses whether all the four behavioral measurements should be taken in all periods or would it be enough to make the measurements only in one period. For the 16 correlations calculated from the percentage data correlation co-efficient ranged from 0.29 to 0.59 ($p<0.05$, N=69-81), whereas for the ten correlations based on the sub-scores this range was from 0.25 to 0.57 ($p<0.05$, N=69-81). Thus, carrying out all these measurements in all periods in the WelFur fox protocol should be considered. Our second aim was to study inter-assessor repeatability by comparing correlation from the pair-wise period comparisons in the cases when one welfare assessor did all the three visits to a farm and in the cases when the visits were made by three different assessors. These analyses indicated that assessor's individual views may have a marked impact on the results from the less objective measurements (i.e. Stereotypic behavior and Temperament test).

Keywords: animal welfare, animal behavior, on-farm welfare assessment, farmed foxes

Introduction

The WelFur fox assessment includes three production periods (P): breeders before the breeding season (P1), females while nursing their cubs (P2) and both breeding animals and juveniles during the growing season (P3) (WelFur 2015). However, according to the current fox protocol all measurements are not taken in all periods. In the case of the behavioral measurements of Appropriate behavior principle – Stereotypic behavior is measured in all three periods, Fur chewing in P1 and P2 and Feeding test and Temperament test (Stick test) only in P1. It was reasoned that Fur chewing is too challenging to assess in P2 during moulting and according to Feeding test and Temperament test results from one period could reflect the situation also in other periods. However, in the Fur Farm 2020 project all of these four measurements were carried out in all three assessing periods. Inter-period correlations of the behavioral measurements were expected to be relatively strong, meaning that the results for each measurement are expected to be similar on the particular farm in every period. Assessor's individual experience may have an impact on the results from the less objective measurements (i.e. Stereotypic behavior and Temperament test). The present study focuses on the four animal based behavioral measurements and our aims were:

1.To clarify with correlation analyses whether all these measurements should be taken in all periods

('weak correlation hypothesis'), or would it be enough to take the measurements ('strong correlation hypothesis') only in one period.

2. To study inter-assessor repeatability by comparing correlation coefficients from the pair-wise period comparisons in the cases when one welfare assessor did all the visits to a farm and in the cases when the visits were made by three different assessors. We hypothesized that the former between-period correlations were higher than the latter.

Material and methods

The data was collected in the Fur Farm 2020 project 2012-2014 in two production periods (6 farms) or in all the three production periods (77 farms). The assessments were carried out by 13 assessors. The following behavioral data was used in the analyses:

Stereotypic behavior, i.e. the percentage of active foxes with stereotypic behavior, all periods;
Feeding test, i.e. the percentage of foxes that ate within 30 s in the presence of the assessor, all periods;
Fur chewing, i.e. the percentage of foxes with marks of fur chewing, P1 and P3 and
Temperament test, i.e. the percentages of foxes which were interpreted curious, uninterested or aggressive, all periods.

In addition to these percentages, sub-scores calculated according to the WelFur fox protocol (WelFur 2015) were used in the statistical analyses.

The inter-period Pearson correlation (rp) coefficients were calculated from the percentage data (n=69-81 depending on the measurement) and from the sub-score data for the four behavioral measurements. The inter-assessor repeatability of the four behavioral measurements were tested by comparing r-values based on percentage data from farms assessed in all three periods by one and the same assessor (n=17) and from farms which were assessed by a different assessor in each of the three periods (n=31)

Results

The differences when comparing the coefficients of results and sub-scores were rather marginal: the difference ($r_{percentage}$ minus $r_{subscore}$) ranged from -0.004 to 0.151 (Table 1). Thus, using sub-scores did not bring any added value to the correlation analyses. However, confirming this was necessary, since the transformation of the percentage onto sub-scores is done in a non-linear manner.

For the 16 correlations calculated from the percentage data the correlation co-efficient ranged from 0.29 to 0.59 ($p<0.05$, N=69-81; Table 1), whereas for the ten correlations calculated based on the sub-scores it ranged from 0.25 to 0.57 ($p<0.05$, N=69-81). All the correlations were significant and positive, i.e. there was some consistency between the results from the three periods. However, the level of correlations were only from low to moderate, which indicates that results from one period cannot be used for predicting reliably the results from another period.

On the other hand, the inter-assessor repeatability analyses indicate that the assessor's individual experience may have a marked impact to the results. The between-periods correlation coefficients for Stereotypic behavior and Temperament test were clearly higher when all three assessment visit were done by one assessors as compared to the cases when visits were made by three different assessors (Table 2).

Table 1. *The four animal-based measurements under the principle Appropriate behavior and their period-wise Pearson correlation coefficients (r) based on the original percentages (n=69-81) and the sub-scores calculated from these percentages. Note that the three percentages from the temperament test are combined into one sub-score.*

Measure	**r: percentages**			**r:sub-scores**		
	P1-P2	P1-P3	P2-P3	P1-P2	P1-P3	P2-P3
Stereotypic behavior	0.585 ***	0.453 ***	0.569 ***	0.518 ***	0.389 ***	0.571 ***
Fur chewing		0.402 ***			0.251 *	
Feeding test	0.362 **	0.336 **	0.287 *	0.359 **	0.335 **	0.286 *
Temperament test: interpreted as curious	0.294 *	0.501 ***	0.444 ***	0.270 *	0.505 ***	0.430 ***
Temperament test: interpreted as uninterested	0.322 **	0.501 ***	0.467 ***			
Temperament test: interpreted as aggressive	0.377 **	0.554 ***	0.492 ***			

(*)=p<0.05 (**)=p<0.01 (***)=p<0.001

Table 2. *The Pearson correlation coefficients (r) between periods for the four animal-based measurements from the farms which were assessed by a different assessor in each of three periods and from the farms which were assessed by the same assessor in every three periods.*

Measure	**r: different assessor (n=17)**			**r: same assessor (n=31)**		
	P1-P2	**P1-P3**	**P2-P3**	**P1-P2**	**P1-P3**	**P2-P3**
Stereotypic behavior	0.240	0.288	0.460	0.754 ***	0.430 *	0.735 ***
Fur chewing		0.539 *			0.428 *	
Feeding test	0.409	0.401	0.242	0.499 **	0.188	0.388
Temperament test: interpreted as curious	-0.236	0.145	-0.372	0.638 ***	0.701 ***	0.729 ***
Temperament test: interpreted as uninterested	-0.194	0.156	-0.356	0.625 ***	0.694 ***	0.741 ***
Temperament test: interpreted as aggressive	-0.046	-0.055	-0.145	0.213	-0.052	0.419 *

(*)=p<0.05 (**)=p<0.01 (***)=p<0.001

Discussion

Although there was positive significant correlation between the periods for all four animal-based measurements of the Appropriate behavior welfare principle, the correlations were rather low. Thus, carrying out all these measurements in all periods in the WelFur fox protocol should be considered.

The inter-assessor repeatability was lower for the less objective measurements. Feeding test and Fur chewing can be regarded to be more objective measurements whereas Stereotypic behavior and Temperament test are considered as more subjective measurements. In these less objective measurements there is a higher risk that assessor's subjective experience has a marked impact on the results. This should be taken into account in the assessor training.

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The temperament test of mink is affected by a shelf in front of the cage, but not the test person's position

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Abstract

The so-called 'stick test' is used for evaluation of the temperament of farmed mink and is included in the welfare assessment WelFur-mink. Different housing conditions might challenge the procedure of the temperament test. Present study tested the hypothesis that the position and distance of the human tester as well as a shelf close to the test area influences the temperament score in a stick test. The temperament of 600 pair housed (male and female) brown juvenile mink, divided into two groups of 150 cages were investigated. One group (75 cages) was tested with or without the assessor bending over the cage. The other group was tested with or without the animals having access to an extra shelf positioned in the front of the cage. There was no effect of the test person's position on the classification of the minks' temperament, while the animals with access to a shelf close to the test area were more explorative than tested without the shelf. We can therefore reject the hypothesis that the test person's position during the stick test affects the test results, while the hypothesis that animals with access to a shelf are more explorative can be accepted.

Keywords: animal welfare, stick test, temperament, welfare assessment, WelFur

Introduction

The so-called "stick test" is a simple and valid test of the temperament in mink (Kirkden *et al.*, 2010; Malmkvist, 1996). The original procedure has been simplified for on farm use, e.g. for selection of explorative animals in farmed mink (Hansen, 1996; Hansen and Møller, 2001). In short, it is tested how the animals react when a wooden tongue spatula is put into the front of their cage through the wire netting. The mink can be categorized as explorative, fearful, aggressive or uncertain. The stick test is included in WelFur-mink which is a welfare assessment system for farmed mink (Mononen *et al.*, 2012; Møller *et al.*, 2015). In WelFur-mink, fearful animals are negatively and explorative mink are positively associated with welfare (Møller *et al.*, 2015). The stick test is therefore included as a measurement to evaluate the mink's temperament and, hence, the expected human-animal relationship on mink farms.

The stick test is included in all three seasonal production periods and the WelFur protocol should be applicable to all farming conditions in Europe. The stick test is therefore going to be applied in conditions that differ from those it was developed in. Different housing conditions on different farms have therefore challenged the normal procedure of the 'stick test' and made is necessary to adjust the testing procedure to new situations. Due to the amount of straw in front of the cage or closed cage fronts, there might be situations where it is difficult to see the animals from the front, and it might be necessary to look down on the animals from the top of the cage while performing the stick test trough the top of the cage. However, this might be an additional stressor for the animals, and may influence their reaction to

the wooden tongue spatula in the test. It is therefore relevant to investigate if the position and distance of the human tester (assessor) influences the temperament score.

Shelfs have been introduces as cage enrichments in many farms, while the position of shelfs in the cage differ. On some farms, a shelf is positioned in the back of the cage, on others in front of the cage, from where the animals easily can reach the feed. The shelfs may also take different forms, e.g. flat, bunks or tubes hanging from the top of the cage. The position of the shelf may affect the minks' reaction in the stick test. A shelf might function as a "safe place" for the animals, and make it easier for both animals in the cage to get access to the wooden spatula. It is therefore also relevant to investigate if access to a shelf in front of the cage affects the minks' reaction to the tongue spatula in the test. We therefore hypothesized that the position and distance of the human tester as well as a shelf close to the test area influences the temperament score in a stick test.

Material and method

The study population was 600 Brown juvenile mink. They were housed in 300 cages in male-female pairs in two two-row sheds. The study was conducted in October and November 2016 at the research farm at Aarhus University Foulum in Denmark. The cages were conventional Danish production cages of wire mesh (L x W x H: 90 cm x 30 cm x 45 cm) with a nest box attached in front of each cage. The cages had a 15 cm wide shelf attached in the back of the cage. Also, there were one small plastic tube and straw both in the nest box and on top of the nest box.

Two treatments were tested:

1. The assessor bending over and looking down on the nest box opening, 15 cm above the wire mesh while doing the stick test
2. An additional shelf formed as a bunk positioned in the front part of the cage

The additional shelves were placed in the cages seven days before the test. The shelves were of plastic mesh (Uniq Farm System, GVA), 40 cm wide and 22 cm deep, big enough for two animals. The animals were divided into two groups with 300 animals (i.e. 150 cages) in each. Group 1 was used for the test of treatment 1 (human position) and had animals situated in two different sheds. Group 2 was used for the test of treatment 2 (extra shelf) and had animals in only one shed. Each group was divided into two subgroups of 75 cages each, being both control-group and test-group. The animals were tested two times with seven days between the two test days. The study was conducted as a crossover design, where the same animals were their own control.

Stick test procedure

The animals were excluded from their nest box before the test started. The assessor was standing in front of the cage. After about 10 seconds the test started and the assessor put a wooden tong spatula (150x18 mm) into the cage through the wire mesh, in the middle of the front of the cage, about 4 inches from the top. The mink's reaction was registered as explorative if the mink sniffed, bit gently or in other way explored the spatula, fearful if the mink moved away from the stick without coming closer than 15 cm, aggressive if the mink made a fast and intense attack at the stick and maintained the bite, and undecided if the animal could not be described by one of the other behaviours mentioned or did not have any stable reaction. After 30 seconds the test was finished. The two mink in the cage were tested simultaneously.

Statistical analysis

Data usable for further statistical analysis were collected from 73 cages for both subgroups in the test

of human position, and 73 cages and 75 cages for the sub-groups in the test of extra shelf in front of the cage. The effect of the different treatments, and interactions with sex and date of assessment, on the prevalence of 'explorative', 'undecided', 'fearful' and 'aggressive' animals were analysed using logistic binomial mixed models. Cage and animal id was included as random variables. The analyses were performed with the package lme4 of the statistical software R (R Core Team 2015). The models were reduced using backwards-stepwise elimination of explanatory variables that were non-significant using a significant level of 5 %.

Results

Around 47 % of the animals in the test of human position, and over 50 % of the animals in the test of an extra shelf were registered as exploratory in the study (Figure 1). In the test of an extra shelf 45 of 296 animals were lying on the shelf wile tested, and all, except from one animal, were registered as explorative. There were no animals registered as aggressive.

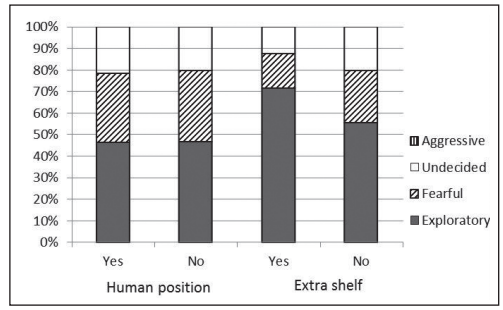

Figure 1. *Percentage of exploratory, fearful, undecided and aggressive animals with and without the assessor bending over the cage while doing the stick test (Human position) and with and without a shelf in front of the cage (Extra shelf).*

There was no significant effect of human position on the prevalence of exploratory animals (χ^2=0.0082, P>0.10), fearful animals (χ^2=1.1727, P>0.10) or undecided animals (χ^2= 0.0132, P>0.10). There were no interactions between the treatment and sex (exploratory:χ^2=0.0008, P>0.10; fearful: χ^2=1.3954; P>0.10; undecided: χ^2=1.5069; P>0.10) or the date of assessment (exploratory: χ^2=2.8502, P>0.05; fearful: χ^2=0.2957, P>0.10; undecided: χ^2=1.3644; P>0.10).

There was a significantly higher prevalence of exploratory animals and lower prevalence of fearful and undecided animals when the animals had access to an extra shelf (exploratory: χ^2=29.048, P<0.0001; extra shelf: 71.6 %; no extra shelf: 55.4 % and fearful: χ^2=28.755, P<0.0001; extra shelf: 15.9 %; no extra shelf: 24.3 % and undecided: χ^2=7.2956, P<0.001; extra shelf: 12.5 %; no extra shelf: 20.3 %), and the animals that were lying on the shelf while tested (22 females and 23 males) were more explorative than when tested without the shelf (χ^2=41.588; P<0.0001; on shelf: 97.8 %; without: 40.0 %). There were no interactions between the treatments and sex (exploratory: χ^2=0.1485, P>0.10; fearful: χ^2=0.1714, P>0.10; undecided: χ^2=0.6289, P>0.10) or the date of assessment (exploratory: χ^2=3.113, P>0.05; fearful: χ^2=0.7634, P>0.10; undecided: χ^2=1.124, P>0.10).

Discussion with conclusions

The assessor bending over the cage did not affect the response of the animals in this study. The hypothesised more threatening position of the test person will, therefore, not affect a WelFur-score or the selection of animals for breeding by increasing the prevalence of fearful animals. In WelFur, the prevalence of non-explorative animals has a negative effect on the welfare score, and fearful animals have the largest negative effect (Møller *et al.*, 2015). The position is, therefore, not expected to have any influence on the WelFur assessment. We reject our hypothesis that the test person's position during the stick test affects the result in terms of exploratory, undecided or fearful animals.

Animals were tested as more explorative when having access to a shelf in the front of the cage than without the additional shelf. The effect of the shelf did not depend on the sex or the date of assessment. We therefore accept our hypothesis that a shelf close to the test area influences the temperament score in a stick test.

The animals that were using the additional shelf when tested were all, except one, exploratory, but not necessarily assessed as exploratory when not having access to the extra shelf. This shows that the shelf is more than a feeding place for the animals, and that the shelf may function as a place of refuge, just like the nest or a plastic tube (Hansen *et al.*, 2007). It is a challenge to test the general temperament of these animals with the stick test, both in on-farm selection of exploratory animals and in WelFur assessments.

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Effects of vitamin supplement on foreleg welfare, growth and fur properties in blue fox *(Vulpes lagopus)*

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Abstract

The aim was to clarify to which extent multivitamin supplement in feed influences on foreleg welfare and production result in juvenile blue foxes. Study groups were: (1) basic feed ; (2) basic feed with addition of multivitamin mixture; (3) basic feed with extra addition of B vitamins. Initial body weights of animals in groups averaged 8.34-8.50 kg. Final body weights were 15.98-16.09 kg. Foreleg bendiness did not differ between groups either in September (score points 2.61-2.71) or in November (score points 2.31-2.40). Foreleg bendiness tended to be more pronounced ($P<0.1$) in September compared to November. The breaking strength of radius varied from 363.0 to 405.1 N and, correspondingly, breaking strength of ulna from 286.0 to 314.9 N , respectively. Ash and dry matter contents of radius was similar in all groups. Grading of fur from live animals did not show any statistical difference in size, mass, purity or cover of hair coat between groups. Significant difference was found in color of fur which was best in Group 3 ($P<0.05$). Evaluation of raw skins at pelting did not reveal any statistical differences in fur properties. In conclusion, studied vitamin supplement do not enhance foreleg welfare and production result during growing-furring season.

Keywords: Vitamin, growing-furring, bone condition, animal welfare

Introduction

Body size of farmed blue foxes *(Vulpes lagopus)* has increased markedly during the last decades. Also leg weakness and foot bending has become more common. Vitamin D regulates calcium and phosphorus metabolism and may have a certain influence on mineralization of bones (Jensen, 2014). Pronounced foreleg bending in foxes may be an indication of such a phenomenon (Korhonen *et al.* 2014). As concerns general wellbeing and growing- furring process, deficiency of water-soluble B vitamins may be as essential as that of fat-soluble vitamins A and D (Berestov *et al.* 1984; Schweigert and Buchholz, 1995; Koskinen and Sepponen, 2012). Further understanding on vitamin requirements are needed, for instance, because earlier data are based on studies made in smaller-sized foxes than housed today. The aim of the present study was to clarify to which extent multivitamin supplement in feed influences on foreleg welfare and production result in juvenile blue foxes during growing-furring season.

Materials & Methods

The experiment was performed at the Research Station of Kannus during the period September -November 2011. Diet groups were: Group 1, Vit 0: basic fox feed without any extra addition of vitamins; Group 2, Vit 100: basic fox feed with addition of specific multivitamin mixture; Group 3, Vit B: basic fox feed including extra addition of B vitamins only (Koskinen and Sepponen 2012). Vitamin content of group 2 was the following: A-vitamin 3500 IU/kg; D3-vitamin 350 IU/kg; E-vitamin 80 mg/kg;

thiamine 30 mg/kg: riboflavin 6 mg/kg; niacin 10 mg/kg; pantothenic acid 4 mg/kg; pyridoxin 3 mg/kg; biotin 0.1 mg/kg; B_{12} 0.02 mg/kg. Vitamin content of group 3 was the following: thiamine 16 mg/kg: riboflavin 6.4 mg/kg; niacin 10.4 mg/kg; pantothenic acid 5.6 mg/kg; pyridoxin 2.2 mg/kg; biotin 0.032 mg/kg; B_{12} 0.010 mg/kg; K 0.08 mg/kg (Koskinen and Sepponen, 2012). Groups were formed on Sept 15.

Each group comprised 35 males and 35 females, housed in pairs. Animals were juvenile blue foxes born in May. Groups were made genetically equal so that one cub from same litter was placed in each group. Housing cages were 105 cm long x 115 cm wide x 70 cm high. Each cage had a wire-mesh platform (105 cm long x 25 cm wide) and a wooden block for chewing (diameter 7 cm, length 35 cm). Animals were weighed with a Mettler SM 15 balance, accuracy ± 10 g. Bending of feet was evaluated according to scale 1-5, where 5 is normal, healthy feet and 1 was highly bend (Koskinen and Sepponen, 2014). Fur evaluations based on scaling and evaluation by the Saga Furs Ltd, Vantaa, Finland. Color of fur was based on scale 1-3 where 1 was light and 3 dark (Koskinen and Sepponen, 2014).

Diets were the same throughout the study. Main ingredient of the diets were slaughterhouse offal (45.4%), fish offal (7%), fish meal (3.5%), Baltic herring (4%), feather meal (7%), Fox carcass (8.7%) cereal mixture (14.5%) ,soya oil (0.2%),and water (9%) (Koskinen and Sepponen, 2012). Freshly mixed feed was supplied twice a day. Chemical compositions of diets are in Table 1. Watering was automatic ad libitum.

Table 1. *Chemical compositions and calculated metabolizable energy (ME).*

Variable		Vit 0	Vit 100	Vit B
Dry matter (%)		41.8	41.8	43.9
Ash (%)		4.7	4.2	4.5
Crude protein (%)		12.1	12.1	12.8
Crude fat (%)		13.2	13.3	14.3
Crude carbohydrates (%)		11.8	12.2	12.3
Metabolizable energy (MJ/kg)		7.6	7.7	8.1
From ME (%):	protein	24.0	23.8	23.8
	fat	62.3	62.2	62.9
carbohydrates		13.7	14.0	13.3

Results

Summary of results are in Table 2. Initial body weights of animals in groups averaged 8.34-8.50 kg. Final body weights were 15.98-16.09 kg. Significant differences in weights were not found between groups. Foreleg bendiness did not differ between groups either in September (score points 2.61-2.71) or in November (score points 2.31-2.40). Foreleg bendiness tended to be more pronounced ($P<0.1$) in September compared to November. The breaking strength of radius varied from 363.0 to 405.1 N and, correspondingly, breaking strength of ulna from 286.0 to 314.9 N , respectively. Ash and dry matter contents of radius was similar in all groups. Grading of fur from live animals did not show any statistical difference in size, mass, purity or cover of hair coat between groups. Significant difference was found in color of fur which was best in Group 3 ($P<0.05$). Evaluation of raw skins at pelting did not reveal any statistical differences in fur properties.

Table 2. *Feed intake, body growth, bending score and breaking strength of foreleg, and dry matter (DM) and ash content of radius. Data are as mean ± SD. Statistical significance: P<0.05. Means with same uppercase letters are not significant.*

Variable		Vit 0	Vit 100	Vit B
Feed intake (g/day/cage)	1945 ± 55	1943 ± 48	1939 ± 61	
Body weight, kg:	Sept 15	8.4 ± 0.8	8.5 ± 0.7	8.3 ± 0.7
	Oct 25	12.5 ± 1.0	12.4 ± 1.0	12.6 ± 1.2
	Nov 21	16.1 ± 1.3	16.0± 1.2	16.1 ± 1.4
Bending of feet:	Sept 19	2.71 ± 0.60	2.61 ± 0.60	2.66 ± 0.68
	Oct 24	2.40 ± 0.6	2.40 ± 0.57	2.31 ± 0.56
Breaking strength (N):	radius	364 ± 82	363 ± 82	405 ± 85
	ulna	303 ± 68	286 ± 57	315 ± 24
±DM (%),	radius	66.8 ± 2.3	67.1 ± 1.8	67.9 ± 1.9
Ash (g/kg, DM),	radius	443 ± 17	432 ± 12	432 ± 18
Live animals:	Size	3.93 ± 0.73	3.84 ± 0.85	3.79 ± 0.81
	Color	1.93 ± 0.39 a	2.01 ± 0.47 ab	2.14 ± 0.49 b
	Mass	2.99 ± 0.58	3.09 ± 0.61	3.09 ± 0.65
	Purity	4.11 ± 0.75	4.19 ± 0.67	4.24 ± 0.71
	Cover	4.30 ± 0.64	4.41 ± 0.67	4.40 ± 0.67
Raw skins:	Weight, g	847 ± 62	859 ± 68	839 ± 59
	Length, cm	1319 ± 43	1313 ± 42	1304 ± 40
	Mass	7.16 ± 1.66	7.36 ± 1.36	6.97 ± 1.45
	Cover	7.01 ± 1.40	7.13 ± 1.32	6.84 ± 1.30
	Quality	7.24 ± 1.53	7.44 ± 1.32	7.11 ± 1.35

Animals ate the offered feed well in all study groups. It seems that vitamin contents of feed did not affect palatability of the feed negatively. Feed consumption in all groups was also similar, which was reflected as a similar weight gain in groups. Vitamin composition of diets did not affect negatively on dry matter or ash contents of bones.

Discussion

A well-balanced vitamin supplement is a prerequisite for a good growth, fur development and well-being in farmed foxes (Berestov *et al.* 1984; Schweigert and Buchholz, 1995). At the beginning of the study, animals in treatment groups were same-sized. Body weights were also normal compared to previous knowledge (Korhonen *et al.* 2005; 2014). As our results showed weight gain in all groups from mid-September onwards was also similar. Final body weights measured here were comparable to those found in previous studies (Koskinen and Sepponen, 2012; Korhonen *et al.* 2014). This clearly shows that used vitamin supplement had no effect on weight gain. Animals ate the offered feed well in all study groups. It seems that, vitamin contents of feed did not affect palatability of the feed negatively. Feed consumption in all groups was also similar, which was reflected as a similar weight gain in groups.

Breaking strength of leg bones have been generally used as an indicator of bone condition and strength in farmed foxes (Koskinen and Sepponen, 2012; Korhonen *et al.* 2005). Typically either tibia or ulna has been evaluated. In the present study, we measured breaking strength of both ulna and radius bones. It is known that some vitamins may have direct or indirect effects on growth of skeleton and bones. Particularly vitamin D regulates calcium and phosphorus metabolism and thus may have an influence on mineralization of bones (Jensen, 2014). Low Ca:P ratio in the diet has been found to increase bending of foreleg and moving difficulties in juvenile foxes (Korhonen *et al.* 2014). In the present study, Group 2 (Vit 100) contained extra amount of vitamin D. Therefore, positive effects on breaking strength would be expected to be seen particularly in Group 2. However, the results showed that vitamin contents in the diets did not affect breaking strength at all. Also ash and dry matter content was similar in groups. This indicates a normal mineralization of bones in study groups. The amount of bending was not very pronounced in any each of the study groups. This tempts us to conclude that different vitamin contents of feed do not essentially influence foreleg bendiness.

Development of proper fur coat requires enough protein and fat in the diet. Also several vitamins are needed (Berestov et al. 1984). Unfortunately, it is partly unknown which are the most essential vitamins for proper fur maturation process. Our results here showed that vitamin contents of feed studied had minor effects on fur properties in juveniles. Only difference found was in grading of live animals. The color of fur was better in animals with vitamin B supplement compared to control feed. Conclusion is that studied vitamin supplement do not enhance foreleg welfare and production result.

Acknowlegements

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Comparative electrophysiology for euthanasia in mink *(Neovison vison)* and blue fox *(Vulpes lagopus)*

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Abstract

The mink *(Neovison vison)* typically has an elongated body shape, short legs and sexual dimorphism. Due to high surface-to-mass ratio, it has to sustain higher resting metabolic rate than other mammals of the same body weight. The blue fox *(Vulpes lagopus)* is well-adapted to tolerate extreme conditions because of its excellent physiological and morphological characteristics. The aim was to compare electrophysiological variables during killing in these physiologically very different species. Eight farm-raised mink and 15 blue foxes were employed here. Before stunning, heart rates and breathing frequency of mink were higher than those of blue foxes. Time for absence of electroencephalography (EEG) and brainstem auditory evoked responses (BAER) were same order magnitude in both species. Stunning time in foxes was very quick compared to mink. Stunning time in foxes was actually so short that it seems that animals do not suffer during killing procedures. The absence of BAER was same order of magnitude in both species. The time when all life activity is gone from animal body was similar for mink and blue fox. Euthanasia methods (electric stunning, CO) applied to farmed foxes and mink can be considered proper for humane killing.

Keywords: Stunning, electrophysiology, killing, Farmed fur animals, Welfare

Introduction

Absence of pain is crucial for proper wellbeing of animals (AVMA 2007). While sensitivity to pain is related to consciousness and brain function, essential is to understand what occurs to brain during killing procedure (Korhonen et al. 2009; 2012). During 2006-2011 several experiments were carried out to clarify killing efficiency and electrophysiology in farmed mink *(Neovison vison)* and blue fox *(Vulpes lagopus)*. Comprehensive data were collected for both species separately. These animal species are many ways different, however. For example, housing conditions, body size and physiology differs clearly (Korhonen *et al.* 1983). The mink typically has a small, elongated body shape, short legs and sexual dimorphism. Due to high surface-to-mass ratio, it has to sustain higher resting metabolic rate than other mammals of the same body weight (Brown and Lasiewski 1972; Moors 1977). The blue fox is a medium-sized canid which is well-adapted to tolerate extreme conditions because of its excellent physiological and morphological characteristics (Scholander *et al.* 1950; Korhonen *et al.* 1983). Species-specific differences are also reflected to means how mink and foxes are handled and euthanized. The aim of the present paper is to compare main physiological variables during killing procedures in these physiologically different species.

Materials & Methods

Eight farm-raised mink and 15 blue foxes were employed here. Mink were euthanized by gas (CO) and

foxes by electric shock. Electroencephalography (EEG) was evaluated with the aid of the polygraphic lead in the EEG equipment. The brainstem auditory evoked responses (BAER) were recorded after earplug-loudspeakers had been placed deep in the external ear canal. For details of methods see original publications of Korhonen *et al.* (2009; 2012).

Results

Before stunning, heart rate and breathing frequency of mink were higher than that of blue fox (Table I). Time for absence of electroencephalography (EEG) and brainstem auditory evoked responses (BAER) were same order magnitude in both species. Stunning time by electric shock in foxes was very quick compared to CO killing of mink. Electrophysiological recordings (EEC) revealed that after stunning, EEC was not able to recognize any brain activity after 60-120 and 51-137 seconds in foxes and mink.

Table I. *Basic physiological data (mean, with minimum and maximum) for mink and blue foxes. Body weights are in grams, others are in seconds.*

Variable	Mink	Blue fox	P
Number of animals	8	15	
Sex	males	females	
Body weight	1970 (1665-2298)	8520 (7480-10360)	<0.001
Heart rate[a]	194 (90-294) (	57 40-88)	<0.05
Breathing frequency[a]	40 (16-72)	17 (13-48)	<0.05
EEC absent[b]	86 (51-137)	103 (60-120)	ns
BAER absent[b]	176 (139-213)	151 (1-250)	ns
Stunning time	75 (56-135)	3.6 (2.3-5.2)	<0.001

[a]pre-stunning; [b]after the start of killing procedure

Discussion

The results showed that pre-stunning heart rate and breathing frequency are markedly higher in mink than in blue fox. This finding well coincides with the known physiological fact that basal metabolic rate of mink is high compared to blue fox (Korhonen et al. 1983). Heat loss from the body of mink is high due to its high surface-to-mass ratio (Brown and Lasiewski, 1972). Therefore, mink has to sustain high basal metabolic rate to keep its body temperature in normal range of homeothermic animal (Korhonen *et al.* 1983).

Killing of foxes is carried out by electric shock while mink are killed by gas. As the results showed stunning time of these species is different due to different killing methods. Essential question here is: how ethical these two killing methods are? Do they cause animals unnecessary suffer or not? Sensitivity to pain is essentially related to consciousness and function of brain. The central nervous system is the

center of consciousness (AVMA 2007). Killing of animals should result in rapid loss of consciousness followed by ultimate loss of brain function (Korhonen et al. 2012). Essential here is that killing occurs with minimal pain and distress. Pain is that sensation that results from nerve impulses reaching the cerebral cortex via ascending neural pathways. Pain can be experiences only when the central cortex and subcortical structures are functional (AVMA 2007). Therefore, the functional state of brain is essential for animal wellbeing during killing procedures.

Although stunning time in mink and blue fox is very different, the results showed that absence of EEC and BAER are not. These are very interesting findings. Pain is related to function of EEC. After stunning, brain activity in both species is starting to diminish rather quickly, i.e. between 86 and 103 seconds. Stunned animals are unconscious and, therefore, cannot feel pain or distress. Furthermore, it takes on average only 86-103 seconds before brain activity is totally lost. Particularly in foxes stunning time is so short that it can be expected that animals do not feel pain at all during killing procedure. Also in the mink it seems evident that experience of pain is minor because consciousness is lost rather quickly as the results showed. Therefore, euthanasia methods used in this study can be considered ethical.

Brainstem auditory evoked responses (BAER) are important variable during killing procedure because it indicates when all life activity is gone from animal body. Latest it is lost from brainstem (Korhonen *et al.* 2012). The present results showed that absence of BAER is same order of magnitude in both species. So, although stunning time is shorter in blue fox, the time lapse while final life activity is gone from body is similar in mink and blue fox. Euthanasia methods of mink and blue fox can be considered proper for humane killing.

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Evaluation of domestication changes in the American mink (Neovison vison) based on the track of a forelimb.*

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Abstract

In Poland, American mink lives in a natural environment as a non-indigenous species. According to some authors in the 1980's there were introductions of minks from fur-farms what resulted in modifications of genetic structures in the population of wild minks. They also claim that up to this day it is not known how the process of local population's augmentation ran or in what ways farm mink influenced the population of the wild ones. The aim of study was defining the differences between wild and currently farmed American mink. The study is based on the track of a forelimb left by both types of minks. The wild and farm animals were obtained from the same area in Poland. Modelling clay was used to show differences in the track size between the wild and the farm mink. Artificial imprints of left front paws were made and photographed. The area of the metacarpal pad imprint ranged between 128,28-167,98 mm^2 for the farm mink and between 75,99-106,1 mm^2 for the wild mink. Therefore, it can be assumed that finding an imprint with area above 120 mm^2 means that it was left by a farm mink.

Keywords: Mink domestication, mink forelimb, wild mink, habitat

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Introduction

American mink is an animal which has precious and valuable fur that has been desired by people for many years. It used to occur in the wild in North America, but due to its attractive fur it became a farm animal. Breeding American minks dates back to the XIX century, but its real rise began in XX century. The development of farming allowed its legal regulation what resulted in recognizing American mink as a domesticated species and livestock in Polish legislation.

Animal husbandry led to changes which helped to achieve features especially desired by men. Not only animals' body size, weight, features and colour of fur have undergone these changes, but also their psychological characteristics. All those transformations within the species are typical examples of changes which appear as a result of domestication (Filistowicz and Kuźniewicz 1999).

In Poland, American mink lives in a natural environment as a non-indigenous species which occupied the ecological niche left by its European ancestor - European mink *(Mustela lutreola)*. In the 1920's and 1930's American mink was brought to fur-farms in Europe, but it was not introduced in Poland until the 1950's, when the intensive growth of its breeding began in other European countries.

The first signals of American minks appearing in the wild date back to 1962-1963 (Ruprecht *et al.*,

1983). Most likely they were moving populations of minks from Russian fur-farms, where between 1933-1977 they were released into the wild (Birnbaum, 2006). In those years Russians introduced 21 300 minks, which developed into herds of wild American minks. The biggest population of minks was set free in Latvia in 1944. Thanks to favourable European climate this species adopted in our ecosystem and using the wildlife corridor it moved through east and middle parts of Poland to the west.

According to some authors (Brzeziński and Marzec 2003, Zalewski *et al.* 2010, Zalewski *et al.* 2011) in the 1980's there were introductions of minks from fur-farms what resulted in modifications of genetic structures in the population of wild minks. They also claim that up to this day it is not known how the process of local population's augmentation ran or in what ways farm minks influenced the population of the wild ones.

In order to elucidate this issue, research defining the differences between wild and currently bred in farms American minks were undertaken. They were based on the track of a forelimb left by both types of minks.

Material and methods

The study included minks caught in their natural habitat (10 male and 22 female) and taken from one fur-farm (10 male and10 female). Both places where the biological material was obtained were in the same area, i.e. Western Pomerania in Poland.

The study material was divided into four groups, i.e. wild females, farm females, wild males and farm males. The animals were weighed with an accuracy of 1g. Modelling clay was used to show differences in track's size between the wild and the farm mink. Artificial imprints of left front mink's paw was made in it and photographed. The images were specially processed and measured with a computer program Axio Version Rel. 4.8.2. by Zeiss.

Fig. 1. *Places where the measurements of the mink's forelimb were made.*

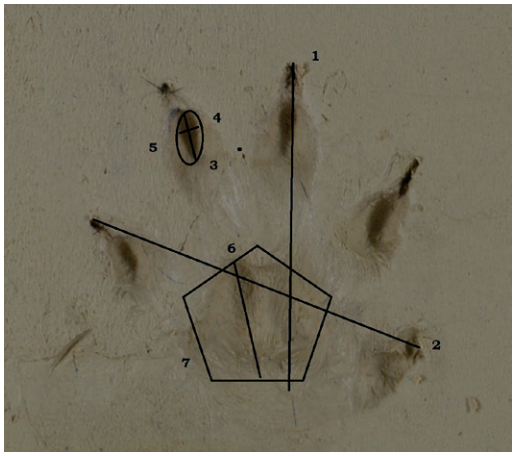

Fig. 2. *Minks' tracks imprinted in modelling clay: NDZ – wild minks, H – farm minks*

Seven measurements were made for the tracks obtained this way (Fig. 1.): vertical distance of the track – track's height; horizontal distance of the track – track's width; height of the second digital pad; width of the second digital pad; surface of the second digital pad; height of the metacarpal pad; surface of the metacarpal pad.

The obtained data were statistically assessed using the computer program Statistica 9.1 with the use of multi-factor analysis of variance and Duncan's range test.

Results and discussion

In table 1 the results of mink's body weight were collated regarding the sex and origin.

Table 1. *Wild and farm minks' body weight (g).*

	female		male	
	Wild	**Farm**	**Wild**	**Farm**
mean ± SD	752* ± 121,5	1415* ± 258,3	1388* ± 271,8	2757* ± 406,4
Min -Max	599,5 - 945	1110 - 1870	960 - 1870	2130 - 3460
V%	16,15	18,26	19,58	14,74

*differences statistically significant between origin of minks with p ≤0.01

American minks are characterized by distinct sexual dimorphism, therefore, it was necessary to divide the groups additionally according to their sex. Comparison of body weight within sex showed that the farm population was twice as heavy as the one living in the wild. Maximum body weight of wild living minks was lower than the minimum value of that trait among farm animals. The diversity of the trait was on approximate level in all tested groups. This study shows that there are substantial differences in both examined populations of animals: wild and farm. The differences statistically significant with p≤0,01 were shown between all tested groups apart from the group of wild male and farm female.

Seven measurements of the tracks imprinted in modelling clay were made and then set in tables with a division into: made on the whole track (table 2), made on the second digital pad (table 3), made on the metacarpal pad (table 4).

The height of the mink's track varied between 24,3 – 44,2 mm showing big diversity within the sex as well as the origin (Table 2). Similar differences were observed in the width of the track which varied between 21,06 – 42,28 mm. Differences within sex, between wild and farm minks, were for the track's height 21,1% (females) and 21,4% (males), and for the track's width 25% (females) and 21,8% (males). The smallest difference was between the farm female's track and the wild male's track (height – 8,8%, width – 9,4%), where the farm female's track was higher and wider than the wild male's one. The measurements' differences were statistically verified between all the tested groups.

The measurements of the second digital pad showed that although the whole track was bigger, the digital pads of farm females were smaller in size (width and surface) than those of the wild females in most of the cases. However, both of the male groups had similar sizes of the digital pads. In the female group the differences were 8,1% for height, 39% for width and 45,9% for the track's surface. In males' group the biggest difference was in the height of the second digital pad and amounted 12,2% in farm males' favour.

Table 2. *Measurements of the whole mink's forelimb track*

sex		height (mm)		width (mm)	
		Wild	Farm	Wild	Farm
female	**mean ± SD**	28,2* ± 2,02	35,74* ± 1,71	23,81 ± 2,14	31,77 ± 1,6
	Min -Max	24,33 - 31,5	33,43 - 38,18	21,06 - 28,19	29,32 - 33,87
	V%	7,16	4,78	8,99	5,04
male	**mean ± SD**	32,58* ± 2,46	41,45* ± 2,14	28,78 ± 2,25	36,79 ± 2,77
	Min -Max	28,43 - 35,71	37,9 - 44,2	24,6 - 31,59	33,83 - 43,28
	V%	7,55	5,16	7,82	7,53

*statistically significant differences with p ≥0,01 between minks origin

Table 3. *Measurements of the second digital pad of the mink's forelimb*

sex		height (mm)		width (mm)		surface (mm2)	
		Wild	Farm	Wild	Farm	Wild	Farm
female	**mean ± SD**	9,09^{A}±1,17	8,35^{B}±1,39	5,28±0,84	3,22±0,86	37,55±8,55	20,32±9,07
	Min -Max	7,19-10,67	6,6-10,68	3,4-6,25	1,69-4,62	18,54-48,06	10,13-35,65
	V%	12,87	16,65	15,91	26,71	22,77	44,64
male	**mean ± SD**	9,07±1,68	10,33^{AB}±0,88	5,51±1,22	5,65±1,09	40,26±17,01	43,38±12,12
	Min -Max	5,67-12,13	8,69-11,51	3,43-8,19	4,07-7,13	16,07-69,22	25,48-68,88
	V%	18,52	8,52	22,14	19,29	42,25	27,94

Table 4. *Measurements of the metacarpal pad of the mink's forelimb*

Sex		height (mm)		surface (mm2)	
		Wild	Farm	Wild	Farm
female	**mean ± SD**	9,47±1,17	10,58±1,97	75,99±7,25	128,28±27,19
	Min -Max	7,44-10,95	7,35-13,72	61,58-87,52	94,13-171,64
	V%	12,35	18,62	9,54	21,2
male	**mean ± SD**	10,73±1,66	12,04±2,52	106,1±24,27	167,98±41,72
	Min -Max	8-13,86	7,17-16,07	54,63-147,42	109,16-232,1
	V%	15,47	20,93	22,87	24,84

The measurements of the metacarpal pad's height showed that it is lower over about 10% among wild animals within sex . The smallest height of the trait was observed among farm females (7,35cm) and the biggest among farm males (16,07 cm). Great diversity of the trait among all tested groups (12,35-20,93%) indicates its instability in both tested populations. The differences between the wild females group and the farm males were statistically verified with p≥0,01.

The measurements of metacarpal pad's surface showed that the wild living animals have decidedly smaller surface of metacarpal pad and the differences between them reach 41,2% among males and 36,8% among females. Between all the tested groups the study showed highly statistically significant

differences with p≥0,01. Interestingly, in the scope of the trait, the wild males as well as females got lower values than the farm animals. The difference between the farm females and the wild females was 22,18 cm2 (i.e. 17,3%). Statistically significant differences (with p≥0,01) were shown between all the tested groups apart from the group of wild males and farm females where the differences were statistically insignificant with p≥0,05. Therefore, the obtained results can be a foundation for estimating the origin of a track.

It seemed important to draw proportions between the made measurements. They were collated in Table 5. Basing on these calculations, it can be estimated that the shape of the track left by a mink sustains its proportions irrespective of the origin and the sex. However, distinct changes occurred between the track's height and the width of the second digital pad, which mostly differed between wild and farm females (respectively 5,21 and 11,96). The reference of the track's height proportions to its elements appears to be a better parameter to describe its origin than the analysis of singular measurements.

Table 5. *Proportions between chosen measurements of wild and farm minks' tracks*

	sex	female			male		
		mean ± SD	Min -Max	V%	mean ± SD	Min -Max	V%
HT/WT	wild	1,19±0,04	1,12-1,24	3,36	1,13±0,04	1,08-1,24	3,54
	farm	1,13±0,03	1,08-1,17	2,65	1,13±0,05	1,02-1,19	4,42
HT/HFP2	wild	3,05±0,38	2,61-3,76	10,1	3,74±0,63	2,88-5,34	16,8
	farm	4,38±0,67	3,16-5,34	15,3	4,03±0,34	3,63-4,71	8,4
HT/WFP2	wild	5,21±0,37	4,45-5,82	7,1	6,27±1,34	4,28-10,19	21,4
	farm	11,96±3,69	7,31-20,5	30,85	7,56±1,3	5,88-9,53	17,2
HT/HMP	wild	2,99±0,37	2,55-3,67	12,4	3,09±0,36	2,46-3,78	11,7
	farm	3,5±0,77	2,52-4,77	22	3,59±0,83	2,66-5,58	23,1
HFP2/WFP2	wild	1,73±0,23	1,42-2,13	13,3	1,69±0,3	1,24-2,52	17,75
	farm	2,71±0,57	2,21-4	21,03	1,88±0,31	1,52-2,37	16,5

HT – height of track; WT – width of track; HFP2 – height of second digital pad;
WFP2 – width of second digital pad; HMP – height of metacarpal pad

Conclusions

1. Basing on the metacarpal pad imprint left by a mink, it is possible to assess its origin, i.e. if it is a wild living animal or a runaway from a farm. It can be assumed that finding an imprint of a surface above 120 mm^2 can be a foundation to establish the track's origin as the farm mink's.
2. A good indicator to establish a track's origin, if it is wild or farm mink's, is analyzing the proportions between the track's height and the height or the width of the second digital pad. If the proportions exceed the second digital pad's height four times or its width seven times, then the mink's track points to its farm origin. Lower results may be a foundation to describe a mink's origin as wild.

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Preliminary results on Finnraccoons' interaction with a bone enrichment

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Abstract

The aim of the study was to measure the interaction with a bone enrichment in the Finnraccoon. The interaction with a half of a cattle femur was recorded in eight pairs of juvenile Finnraccoon females in September and November. The total duration of interaction with the bone was 8.1 ± 4.6 minutes and 4.5 ± 2.0 minutes (mean ± SD) during the six hours of continuous recording in September and November, respectively. The total duration of the interaction with the bone decreases slightly from September to November ($P < 0.05$, Wilcoxon Signed Rank Test). Extrapolated to a 24-hour day, a pair of Finnraccoons utilized the bone for 33 minutes in September and 18 minutes in November. The most common type interaction with the bone included both pawing and oral activities. The results show that juvenile Finnraccoons utilize bone enrichment relatively much, but the duration of the interaction decrease slightly as the autumn proceeds.

Keywords: animal welfare, behaviour, environmental enrichment, *Nyctereutes procyonoides*

Introduction

Finnraccoons *(Nyctereutes procyonoides ussuriensis)* are farmed for their fur in a few European countries in housing conditions very similar to those of farmed foxes *(Vulpes lagopus and V. vulpes)*. Research on the welfare of Finnraccoons is a topical issue due to ongoing development of an on-farm welfare assessment protocol for Finnraccoons, comparable to those already published for foxes and mink (WelFur 2015a, b).

Since bones are valuable enrichment objects for farmed foxes (Ahola *et al.* 2010, Hovland *et al.* 2016, Koistinen et al. 2009), the aim of the present preliminary study is to document the level of interaction with a bone enrichment in Finnraccoons. The experiences from farms show that Finnraccoons interact with a bone enrichment, but to the best of our knowledge, interaction with a bone enrichment has not been previously documented.

Materials and Methods

The subjects of the experiment were eight pairs of Finnraccoon females. The sister pairs were housed from weaning in July (body weight 2.6±0.3 kg, mean±SD) to December (13.6±1.1 kg) in the research farm of Luova Ltd. (Kannus, Finland) in an outdoor shed in standard cages (floor area 1.2 m^2, height 70 cm) furnished with a platform. Each pair of Finnraccoons had also an already used (by other animals) half of a cattle femur (weight 0.4-0.7 kg, length 16-30 cm) as an enrichment. Water was available through an automatic watering system throughout the study, and feeding was close to *ad libitum*.

The behaviour of the Finnraccoons was video recorded for 24 hours in early September and late November. The interaction with the bone was analysed for 15 mins of each hour of the day (e.g. 1:00-1:15, 2:00-2:15 etc.) by using continuous recording (Martin and Bateson 2007). Thus, the total duration of analysis was 6 hours for each pair of Finnraccoons on both observation days. After initial analysis of the recordings the "intentional" interactions with the bone with the minimum duration of three seconds were divided into six exclusive categories (Table 1).

The total daily durations of various interactions with the bone were compared between the two observation days by using Wilcoxon Signed rank test (SAS, Version 9.4).

Table 1. *The type of the interaction with the bone, description of the interaction, the mean (±SD) duration (s) of the interaction with the bone during the 6 hours of analysis in September and November, and the statistics (Wilcoxon Signed Rank test).*

Type of interaction	Description	September (s)	November (s)	Statistics
Total		491 ± 279	270 ± 121	P = 0.039
Oral	Sniffing, licking, gnawing, poking and carrying of the bone	139 ± 110	131 ± 76	P = 0.945
Paws	Pawing and scratching of the bone	4 ± 6	2 ± 3	P = 0.500
Both oral and paws	Combination of the above types of interaction	305 ± 296	130 ± 120	P = 0.055
Elimination	Defecation and urination on the bone	15 ± 30	8 ± 23	P = 0.500
Social	Both animals interact with the bone simultaneously	28 ± 28	0 ± 0	-
Other	Any other interaction with the bone	1 ± 1	0 ± 0	-

Results and discussion

Finnraccoons continued interacting with the bones throughout the study, but the total duration of interaction decreased from September to November (Table 1). The bones were utilized for a total of 491 s (i.e. 8 min) and 270 s (i.e. 4.5 min) during the six hours of observation in September and November, respectively. Assuming that the animals were using the bones evenly across all hours of the day, these results correspond to 33 min and 18 min of interaction in a day (24 hours). This is slightly less than the 43-57 min of interaction with a "fresh" cattle femur in a day in August-November reported for juvenile blue fox pairs (Koistinen *et al.* 2009). If we speculate further that both Finnraccoons in the cage interacted with the bone to the same extent (the individuals could not be identified from the video recordings), then our results show that one Finnraccoon interacted with the bone for 16-17 min a day in September and 9 min a day in November.

The Finnraccoons typically changed between various types of interaction (e.g. licking, gnawing, poking and pawing) very fast and several times within a bout of interaction with the bone. Frequent alteration between the use of paws and mouth was the most common type of interaction with the

bone, and tended to decrease from September to November (Table 1). Typically, this behaviour could have been called play with the bone. Bones stimulate play behaviour also in blue foxes (Koistinen *et al.* 2009). Also pure oral activities (mainly gnawing) was commonly observed, and its level remained stable through the autumn.

Scent marking is very important for the social life of *Nyctereutes procyonoides* (e.g. Korhonen *et al.* 1991), and also in our study elimination on the bones was observed (Table 1). Social interaction with the bone and 'other interaction with the bone' were observed only in September.

Our preliminary data show that Finnraccoons utilize even the already worn bones for various activities. A more comprehensive analysis of the interaction with a bone enrichment will be provided in future.

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