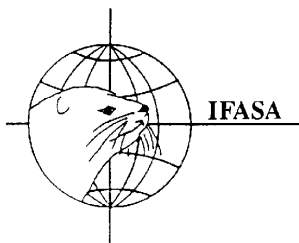
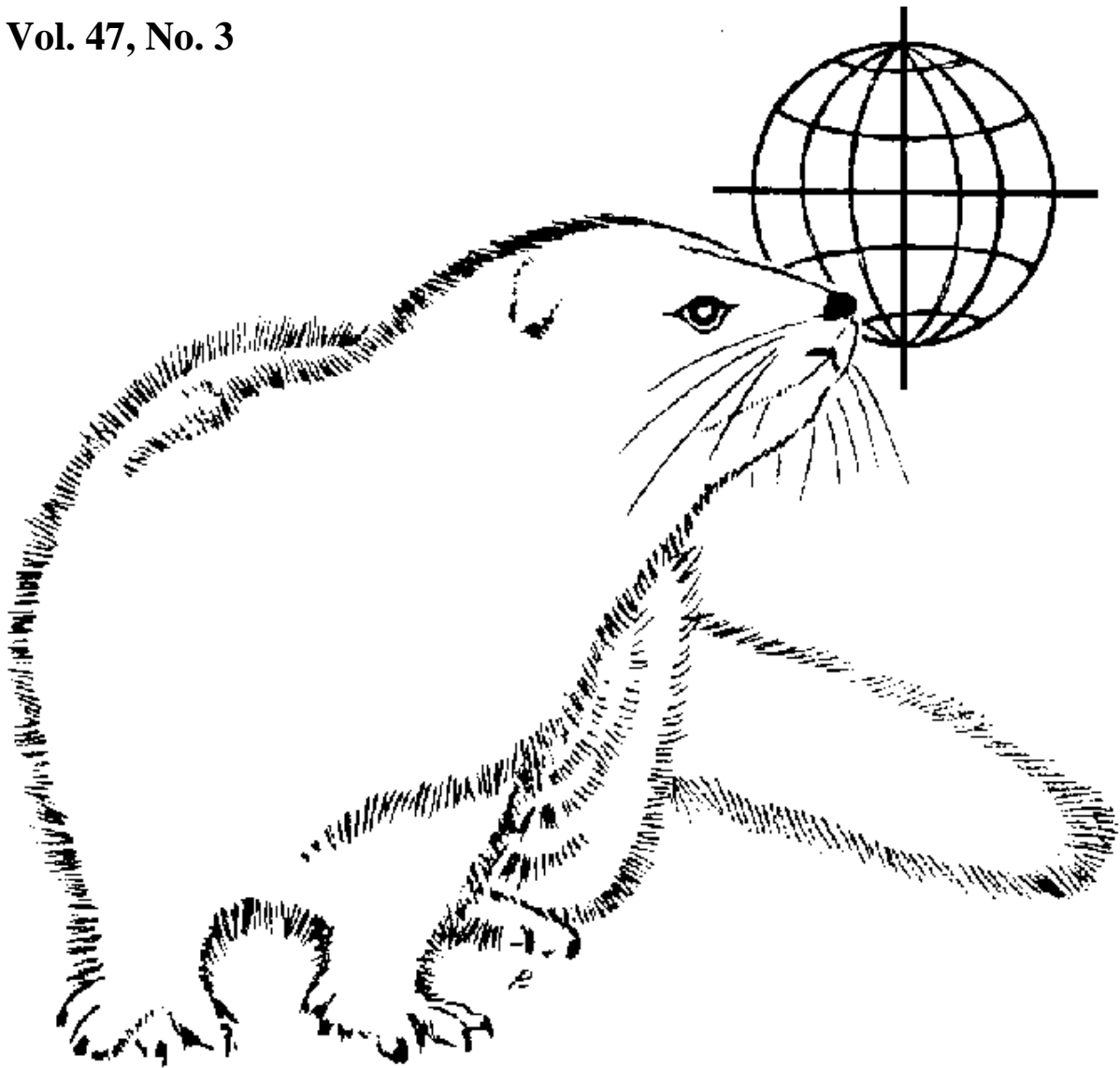


# SCIENTIFUR

SCIENTIFIC INFORMATION IN FUR ANIMAL PRODUCTION

Vol. 47, No. 3



INTERNATIONAL FUR ANIMAL SCIENTIFIC ASSOCIATION

**SCIENTIFUR** scientific information for those involved in fur animal production is published by the International Fur Animal Scientific Association (IFASA).

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**SCIENTIFUR** is published as four issues per year (one volume).

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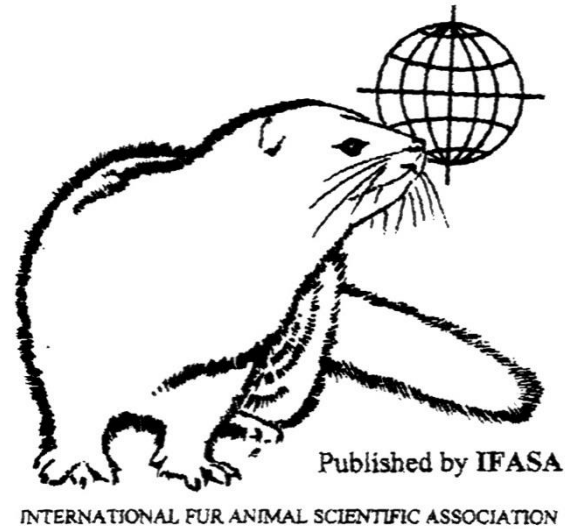
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SCIENTIFUR  
ISSN 0105-2403  
Vol. 47, No. 3



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## Notes from the Editor

The Welfur Assessment System is in line with the Welfare Quality project developed to evaluate the quality of animal welfare in cattle, pigs and poultry with support from the European Commission. In the Welfur project, there has initially been a focus on welfare assessment for mink and foxes. After publishing protocols for these species, the development of a Welfare protocol for the numerically smaller Finnracoon was initiated. In this volume of *Scientifur*, an article is published that describes the work on the elaboration of the protocol for on-farm welfare assessment for the Finnracoon. The assessment is based on the results of the work with mink and foxes, as many of the selected measures are less well documented in Finnracoon due to less research in this species. The protocol for the Finnracoon will be revised when more scientific evidence is available.

A study of behavior of Chinchilla - another numerically minor fur animal species – is reported among the abstracts in this volume of *Scientifur*. It was found that larger cage size and enrichments may improve welfare in the Chinchilla.

Despite the great focus and effort in research and industry for good animal welfare for fur animals, there is great opposition to fur animal production in Europe. A large number of animal welfare organizations have come together to not only ban fur production in the European Union, but also to ban the import of fur from third countries.

At government level, at a meeting of European agriculture ministers in late June 2023, a coalition of European Union member states supported a potential ban on fur farming. Thus, the European Commission was asked to consider the banning of the breeding of fur-bearing animals under the upcoming revision of

animal welfare rules expected in September this year. Arguments against were that the welfare of fur animals can and should be developed on the basis of the latest scientific knowledge and best practice, without it being necessary to ban the industry.

It is always a pleasure to present information about PhD-theses within fur animal research in *Scientifur*. The abstract from a Spanish PhD-thesis “Application of molecular biology to the study of epidemiology and eradication of Aleutian mink disease: environmental detection and phylogenetic studies” defended at University of Santiago de Compostela in 2021 is published in this volume of *Scientifur*.

The PhD deals with important issues related to Aleutian Mink Disease Virus (AMDV). The study shows e.g. the possibility of using qPCR in mapping the environmental contamination with AMDV. It provides knowledge on the distribution of AMDV in different elements and areas of infected farms. It points with a focus on farm visits, to the need to use certified personal protective equipment (PPE) to avoid spread of AMDV. Finally, it is shown that molecular characterization has the potential to reveal the geographical, historical and commercial conditions that form the background for the spread of AMDV on farms.

An abstract shows some of the consequences of AMDV. Even when asymptomatic viral infections are observed effects on reproduction may be seen.

Vivi Hunnicke Nielsen

Editor *Scientifur*



## Development and structure of the WelFur on-farm welfare protocol for the Finnraccoon

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### Abstract

As a response to consumers' increased awareness of animal welfare issues, on-farm welfare assessment protocols have been developed for many farm animal species. The present paper describes the selection of the measures for the on-farm welfare assessment protocol for the Finnraccoon (*Nyctereutes procyonoides*). The protocol was structured on the four welfare principles and 12 more detailed criteria defined in the Welfare Quality® and used also in the WelFur for mink and foxes. Since Finnraccoons are typically raised in the same farms with mink and foxes in partly similar housing facilities, the protocol resembles much those of mink and foxes. Although many of the selected measures are not throughout documented, due to low scientific interest towards the species, the WelFur Finnraccoon protocol provides a good tool to pay attention the welfare of Finnraccoons on private farms. The protocol will be subjected to revision when more scientific knowledge is available.

Keywords: Animal welfare, Behaviour, Feeding, Finnraccoon, Health, Housing

### Introduction

On-farm welfare assessment protocols are tools for enhancing animal welfare through certification and related advisory actions. During the past 15 years welfare assessment protocols have been developed for farm animal species, like cattle, pigs, laying hens and broilers in the Welfare Quality® project (Blokhuys et al., 2013), horses, donkeys, sheep, goat and turkeys in the AWIN project (Canali et al., 2015), and mink and foxes in the WelFur project (Mononen et al., 2012). All these protocols use the Welfare Quality® approach, ie the same four welfare principles and 12 welfare criteria, and prefer using animal-based (outcome based) measures to input based (resource- and management-based) measures (Keeling et al., 2013).

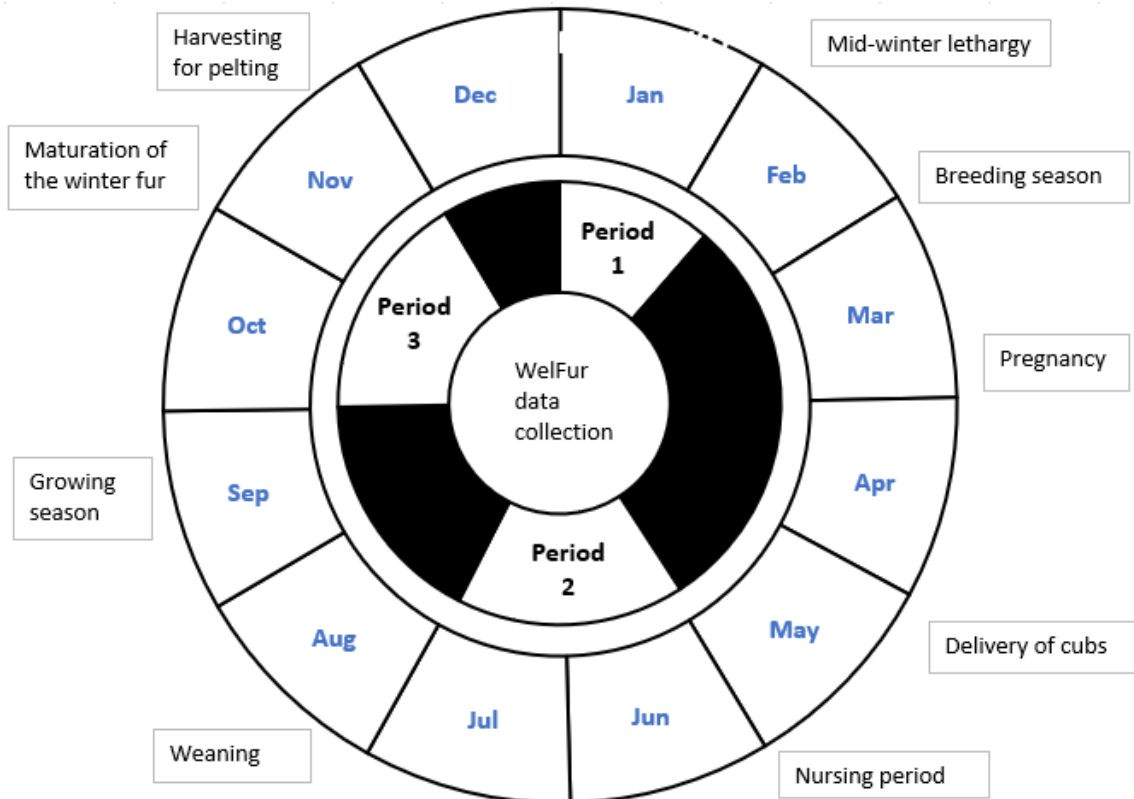
The Finnraccoon (*Nyctereutes procyonoides*) is one of the species farmed for their fur. In Europe, Finnraccoons are raised in Finland, nowadays, on less than 100 farms. The annual pelt production has been around 50 000 – 150 000 during the last 10 years (Fifur tilastot 2022). The farming history of the Finnraccoon dates back to 1970s (Lykkeberg, 1978; Slaska et al., 2010). Farming started with animals captured from the wild, which are called raccoon dogs. The species is a medium-sized canid, and its housing facilities and management on the fur farms resemble much those of farmed foxes, blue foxes (*Vulpes lagopus*) and silver foxes (*V. vulpes*). However, there are also differences stemming from the differences in the biology between the species. *N. procyonoides* is, for example, the only canid species performing a facultative, intermittent, superficial hibernation in winter (Mustonen & Nieminen, 2018), making the animal undemanding in winter.

In the present paper, we describe the WelFur on-farm welfare assessment protocol for the Finnraccoon (see WelFur, 2020). We provide a short description of the structure of the protocol including an account of the choice of the welfare measures. This is followed by a brief overview of the development process.

### Data collection windows and methods

Due to seasonal reproduction of the Finnraccoons, farming follows a strict annual cycle similarly in all farms (Figure 1). Fur animals typically live their whole life, from birth to death, in the same farm, and animals are not routinely transported to specialised farms at any time during their lives. In Finnraccoons, the mating season occurs in late winter. The cubs are born after 60 days of pregnancy (Valtonen et al., 1977) in early summer. The cubs are nursed by their mother, until weaned after the end of the lactation period in late summer at the age of 2-3 months. Most of the animals are pelted at the time when the winter fur

**Figure 1.** Annual cycle of the Finnraccoon farm and the WelFur data collection windows within each of the three Periods.



matures at the onset of winter. During mid-winter, here are only breeding animals on the farms.

In WelFur, it was recognised that, to be able to cover the whole production cycle of a fur farm in one protocol, three data collection periods are needed (Mononen et al., 2012). These periods represent mid-winter, (Period 1), nursing period in summer, (Period 2) and growing-furring period in autumn (Period 3) (Figure 1). It has been demonstrated in mink, that the overall score for the farm cannot be based on the assessment of one period only, but data from all Periods are needed (Henriksen et al., 2016, see also seasonal effect of pasture on the welfare assessment in cows: Burow et al., 2013). Each of the three periods challenges the welfare of the animals in a characteristic way. Therefore, data collection method or categorization may differ between the three periods, and not all measures are needed in all periods (Table 1).

In Period 1, data collection window is specified to January-February to cover the wintering circumstances after the end of the pelting season, but before

the mating season starts (Figure 1). During that time, Finnraccoons are seasonally inactive, even lethargic, due to the facultative passive wintering strategy (Mustonen & Nieminen, 2018). Due to animal welfare reasons, and to maintain the feasibility of the assessment (no need to chase the animals up to their feet), a walking assessment method is used, resembling the transect walk used in the welfare assessment of broilers (e.g. BenSassi et al., 2019a). In this method, no individual cages/animals are sampled for detailed inspection, but animals are observed from 12 rows (of shed house/barn) while passing by. The transect walk has been proved reflecting environmental inputs and detecting production outcomes (broiler: Ben Sassi et al., 2019a, b), but the outcome can differ from the assessment of individuals in some variables (broiler: Marchewka et al., 2013; turkey: Marchewka et al., 2015). The assessment method requires further investigation in the Finnraccoon. Due to the uncertainty in gathering data from the lethargic animals, Period 1 has only half of the weight of Periods 2 and 3 in the score calculation model (WelFur, 2020, p 95).



The data collection window during the nursing period (Period 2) was set to June-July (Figure 1). At that time majority of the cubs are at the age of 4 weeks or older. This minimises the disturbance of the females at the time of the delivery and first weeks after that. Since the primiparous females typically breed later than the multiparous females, the selected data collection window ensures that the primiparous females have the youngest cubs during the data collection, and thus the collected data captures better the challenges with the early lactation and nursing. Multiparous females typically have less challenges; and some of their cubs, born early in the season, may already have been weaned, which enables the inclusion of the weaning process into the data collection. A total of 120 housing units are randomly sampled for the detailed assessment. The sample includes breeding females, their cubs, breeding males and already weaned juveniles.

In autumn (Period 3), the most optimal data collection window is October-November, just before pelting season (Figure 1). At that time, the animals are fully grown, and many of the heritable (and contagious) health issues have become visible. This represents the health condition and net welfare of the animals close to the end of their lives (except in the case of breeding animals). Random sample of 120 housing units and animals from those is selected for detailed assessment as in Period 2. The sample includes both the animals to be pelted (mainly juveniles), and breeding animals (nulliparous and multiparous).

Undoubtedly, there are also other critical periods in the welfare of Finnraccoons. These include procedures used during the mating season (eg heat detection, use of artificial insemination and related handling), parturition and euthanasia. Data collection during these possibly critical periods was rejected, since these occur within a short period of time, making the data collection window very narrow. The outcome of these procedures is, however, indirectly measured in the protocol. For example, killing of animals is covered by questions, and unfriendly handling of the animals affects the human-animal relationship in general, and can be then **captured by using behavioural tests**.

### **Selection of the measures**

The WelFur assessment is planned to be started in the morning, and it can, at the maximum, take one working day on farm. Although, one-day assessment has been considered too long and therefore, challenging

the implementation of the WQ scheme (cattle: Andreassen et al., 2014), in the implementation of WelFur in all European mink and fox farms, it was not regarded an insuperable issue. Therefore, the maximum duration of the assessment was not shortened in the Finnraccoon. Although quite generous, the maximum duration of one working day set some limits to the number and type of measures. In the selection of the measures, the aim was to find at least one good measure to describe each of the 12 welfare criteria defined in the WQ® (Welfare Quality, 2009). The validity, reliability and feasibility are important aspects of good welfare measures (Veissier et al., 2013). However, since the welfare of Finnraccoons have not gained much scientific attention, the knowledge is limited in this species. Expert knowledge elicitation process was implemented to fill in the gaps to increase the knowledge in the Finnraccoon, and in many cases, the limited knowledge from the same-size canines, ie blue fox and silver fox, could be applied. Full testing of validity and reliability could not be required for the measures.

A total of 25 measures were included in the WelFur Finnraccoon protocol (Table 1). Of these, 11 are output measures, which show the animal's direct reaction to the housing and management and the rest 14 are input measures, which indicate the potential provided by the housing facilities and management practices. Output measures are valued over input measures, "because animals, as nonverbal beings, express their internal state through behaviour" (Hausberger et al., 2020) and physical state. In the WelFur Finnraccoon protocol, the input measures were selected to supplement the output measures, and when no appropriate output measures were available.

### **Welfare measures within each welfare criterion**

In this section, we briefly introduce the measures describing each of the 12 welfare criteria and provide a short justification for the selection of the measure.

#### ***Absence of prolonged hunger***

Finnraccoons have an effective internal control of appetite, body condition and activity level as a habituation to the fluctuation of the availability of food in the wild (Mustonen & Nieminen, 2018; Niiranen et al., 2021). In autumn, the endocrinological control of appetite leads to feed intake exceeding the maintenance, which ensures retention of subcutaneous fat reserves (Farm: Korhonen, 1987a; Nieminen et al., 2002; Mustonen, 2003; wild: Kauhala, 1992). Since the

**Table 1.** Welfare measures within the welfare principles and criteria in the WelFur Finn raccoon on-farm welfare assessment protocol, including the data collection Periods when the measures are taken and the base-ment of the measure (input or output based).

Principle	Criteria	Definition	Measure	Peri-ods*	Base
<b>1. Good feeding</b>	1. Absence of prolonged hunger	Animals should not suffer from prolonged hunger, i.e. they should have a suitable and appropriate diet.	Body condition	1, 2, 3	Output
			Availability of nutritional fibre	1, 2, 3	Input
	2. Absence of prolonged thirst	Animals should not suffer from prolonged thirst, i.e. they should have a sufficient and accessible water supply.	Continuous water availability: type of watering system, availability of potable water	1, 2, 3	Input
<b>2. Good housing</b>	3. Comfort around resting	Animals should have comfort when they are resting.	Opportunity for allohousing	2, 3	Input
			Resting shelter	1, 2, 3	Input
	4. Thermal comfort	Animals should have thermal comfort, i.e. they should neither be too hot nor too cold.	Cleanliness of the fur	1, 3	Output
			Protection from exceptional hot weather	2	Input
			Protection from wind	1, 3	Input
	5. Ease of movement	Animals should have enough space to be able to move around freely.	Possibility for horizontal movement	1, 2, 3	Input
Possibility for vertical movement			1, 2, 3	Input	
<b>3. Good health</b>	6. Absence of injuries	Animals should be free of injuries, e.g. skin damage and locomotory disorders.	Difficulties in moving	1, 2, 3	Output
			Skin lesions and other injuries to the body	1, 2, 3	Output
	7. Absence of diseases	Animals should be free from diseases, i.e. farm managers should maintain high standards of hygiene and care.	Bent feet	2, 3	Output
			Diarrhoea	1, 2, 3	Output
			Other disease	1, 2, 3	Output
			Mortality	1, 2, 3	Output
	8. Absence of pain induced by management procedures	Animals should not suffer from pain induced by inappropriate management, handling, killing or surgical procedures (e.g. castration).	Emergency killing	1, 2	Input
Killing at farm at the end of Period 3			3	Input	
<b>4. Appropriate behaviour</b>	9. Expression of social behaviours	Animals should be able to express normal, nonharmful, social behaviours (e.g. grooming).	Social housing of juveniles	2, 3	Input
	10. Expression of other behaviours	Animals should be able to express other normal behaviours, i.e. it should be possible to express species-specific natural behaviours such as foraging.	Stereotypic behaviour	1, 2, 3	Output
			Fur chewing	1, 3	Output
			Availability of straw	1, 2, 3	Input
			Opportunity to use activity object	1, 2, 3	Input
			Quality of the available area	1, 2, 3	Input
	11. Good human-animal relationship	Animals should be handled well in all situations, i.e. handlers should promote good human-animal relationships.	Voluntary approach test	1, 3	Output
	12. Positive emotional state	Negative emotions such as fear, distress, frustration or apathy should be avoided whereas positive emotions such as security or contentment should be promoted.			

\* Period 1 refers to data collection before the start of breeding period (January-February), Period 2 refers to the data collection during the cub nursing period (June-July) and Period 3 refers to the data collection during the growing season (October-November).

economic value of the pelt is related to the size of the skin, which, in turn, is directly dependent on the size and obesity of the animal (blue fox: Kempe, 2018), the farmers utilise animals' willingness to eat at the end of the growing season to produce larger skins. Although overfeeding, and subsequent obesity tend to be problems in fur animals to be pelted, in all species (Kempe, 2018), prolonged hunger may also result from poor management of feeding.

Due to the adaptation to the scarcity of food in the wild, the appetite of the Finnraccoon is poor in winter. The voluntary feed intake is lower than the maintenance, if sufficient subcutaneous fat reserves exist (Korhonen, 1988a). Finnraccoons may not eat despite of the available feed, and therefore, *ad libitum* fed animals lose weight during winter (Mustonen et al., 2004; Koistinen et al., 2018). They can even tolerate total food deprivation for weeks without deleterious effects to their metabolism or welfare and, obviously without feeling of hunger (Asikainen, 2013; Kinnunen et al., 2016; Niiranen et al., 2021a). It seems that, in winter, Finnraccoons with sufficient subcutaneous fat reserves, have absence of hunger by nature, and they lose weight regardless of the feeding regime. Those, without sufficient subcutaneous fat reserves, may suffer of prolonged hunger if not fed appropriately through winter.

In spring, the voluntary feed intake returns to maintenance (Korhonen, 1988a). Appetite is good also during cub nursing period in summer. To avoid prolonged hunger, sufficient feeding of breeding females is very important. Some females with large litters may still end up to a low body condition due to high energy demands of lactation. In summer, there is a high risk of prolonged hunger in the breeding females.

*Body condition*, used as a measure of prolonged hunger in several on-farm assessment protocols (foxes: WelFur, 2015a; cattle: Welfare Quality, 2009), is used to detect too lean animals to illustrate the criterion *Absence of prolonged hunger* also in Finnraccoons. The body condition scoring validated for blue foxes (Kempe et al., 2009), and commonly utilised also in Finnraccoons, was adapted to the WelFur Finnraccoon protocol. This visual method is more feasible in the on-farm circumstances than those requiring the weight and body dimensions, like the *Obesity index* (Korhonen et al., 1982) and *Body Mass Index* (e.g. Nieminen et al., 2004) developed for the Finnraccoon. Only three categories are used to secure

the robustness, instead of the five categories used in foxes (WelFur, 2015a).

The body condition of the animal describes only the quantitative aspects of hunger (undernutrition – whether the animal has gained enough energy). There are also other aspects of hunger, like malnutrition, which describes inappropriately balanced diet (Kyriazakis & Tolcamp, 2018). The qualitative aspect of the “hunger” is included by measuring *Availability of nutritional fibre* in the WelFur Finnraccoon protocol. Finnraccoons are often fed with the feed designed for mink and foxes, although feeding habits of the wild raccoon dog (eg Kauhala & Auniola. 2001; Sutor et al., 2010), digestive tract physiology (Brudnicki et al., 2001) and better digestibility of carbohydrates (compared to *Vulpes vulpes*: Gugolek et al., 2014) show that the Finnraccoon is a more omnivorous species than foxes and mink. Therefore, there is a risk that the Finnraccoons, fed with the feed designed for the nutritional requirements of the more carnivorous species, suffer lack of sufficient source of fibre, and consequently qualitative hunger. This could be mitigated by using feed designed for nutritional requirements of the Finnraccoon, adding fibre (eg oat meal) to the commercially available mink/fox feed or by providing source of fibre (eg straw) besides the main feed. These both options are assessed in the WelFur Finnraccoon protocol.

#### ***Absence of prolonged thirst***

There are no detailed studies on the water requirements or drinking behaviour of Finnraccoons. Small scale behavioural observations show that Finnraccoons drink several times a day (Korhonen, 1988b; Korhonen et al., 1991). The consumption of water is at highest in summer and autumn (Bielański et al., 1996), but the need for drinking water may be suppressed in winter due to changes in metabolism (Asikainen, 2013).

There are several behavioural (eg Kyriazakis & Savory, 1997) and physiological (Pritchard et al., 2008) measures of thirst, but none of these is feasible to be used in an on-farm welfare protocol. In the WelFur Finnraccoon protocol, the criterion *Absence of prolonged thirst* is assessed by using a measure labelled *Continuous water availability*, which combines input variables from the management of watering on farm.

One issue measured in the *Continuous water availability* measure is type of the watering system.

Automatic and manual watering systems, similar in all species, are used on fur farms. The commonly used automatic watering system enable drinking liquid water at will at any time. The main challenges for water availability are accidentally broken individual nipples and freezing of the system during the winter due to the lack or failure of the frost protection system. If a more labour-intensive manual watering system is used (as a main watering system or while the automatic watering system is frozen) stockpersons provide water to a cup once or a few times a day. In this case, an open water source is available, which enables a more natural drinking behaviour than the nipple, but water may not be available for a long period of time, since the animals may quickly consume (or waste) all the provided water and freezing of the water is fast in temperatures below zero (°C). In blue foxes, it has been demonstrated that providing 0.5 l drinking water once a day in autumn may cause prolonged thirst (Moe et al., 2000). This may also be the case in the approximately same-size Finnraccoon, and therefore water should be provided more often. In silver foxes, it has been shown that ice or snow can replace liquid drinking water to some extent (Moe et al., 1999), and therefore, although illegal (Ministry of Agriculture and Forestry, 2011), access to snow or ice is better for the animal than no water at all. Therefore, when assessing the type of the watering system, the functioning of the water point is assessed in automatic watering systems, and the number of water provisions is measured if manual water supply is used, and availability of ice is observed in winter.

Besides insufficient functioning of the watering system, low frequency of water provision and freezing of the drinking water, access to water might also be challenged due to other reasons like, limited number of water points, slow flow rate of the drinking water or soiling of the drinking water (e.g. cattle: Welfare Quality®, 2009). Due to relatively small group sizes, high social tolerance of the species (e.g. Koistinen et al., 2020) and low duration of daily drinking behaviour, the number of watering points was considered irrelevant in this species. Also, water flow speed was considered irrelevant, due to the relatively small amount of daily water consumption. Therefore, only soiling of the drinking water with faeces is included in the *Continuous water availability* measure.

Finally, water provision of small cubs gained some attention during the development of the protocol. Since the field veterinarians did not recognise cubs' dehydration as a welfare issue in the Finnraccoon, it

was decided not to include extra water source of cubs into the first version of the protocol. There is some indication in mink kits, that they benefit from extra water source close to the nest (Henriksen et al., 2018). In Finnraccoon, extra source of drinking water is not typically provided, but different from mink, the nest box always situates close to the water nipple/cup ensuring easy access to the water from the nest box (in which, the small cubs mostly stay). Based on the authors' personal observations cubs quickly learn to operate the water nipple by observing the mother's drinking behaviour.

### ***Comfort around resting***

Similar to the crepuscular activity rhythm of the wild conspecifics (Kauhala et al., 2007; Rudert et al., 2011), the activity of Finnraccoons peaks in sunrise and sunset, but is slightly adjusted according to the human activities (eg feeding) on the farm (Korhonen, 1988c; Ahola et al., 2007; Koistinen et al., 2018). Majority of the resting occurs during the night-time (dark hours), long resting bouts dominating the behavioural repertoire, indicating sleeping. During the daytime, resting bouts are frequent, but shorter.

Juvenile Finnraccoons prefer allohuddling in contrast to resting alone (Ahola et al., 2007; Koistinen & Korhonen, 2018; Koistinen et al., 2018, 2020) and they willingly utilise various shelters, like nest box, while resting (Koistinen et al., 2018; Koistinen et al., 2020). They do not necessarily rest inside the shelter, but also against the shelter or against any solid wall/construction. Also, wild conspecifics rest together in secluded areas, but not necessarily in a nest (Kowalczyk & Zalewski, 2011). Farmed foxes do prefer resting on high locations, like on platforms (eg Koistinen & Korhonen, 2013), which are also often mounted in the cages of Finnraccoons. However, Finnraccoons do not quite consider the platform as a resting site (Koistinen & Korhonen, 2018).

The main resting time occurs outside the timing of the assessment and the time-consuming nature of the observation of resting behaviour dispute the feasibility of the output measures of the resting behaviour. Therefore, input measures, which determine the possibilities of using the preferred resting options, are justified. Due to high preference for allohuddling and using of resting shelters, *Comfort around resting* is measured by *Opportunity for allohuddling* and *Resting shelter*. These measure whether the animal has opportunity to allohuddle with conspecifics and whether it can use a resting shelter. The value of the

shelter increases with increasing protection provided, based on the number of walls (from 1 to 4 walls) in the shelter.

### *Thermal comfort*

Finnraccoons are adapted to the annual variation in the ambient temperature through physical, physiological and behavioural mechanisms. They have stocky body shape (Ward & Wurster-Hill, 1990). Autumnal retention of subcutaneous fat (Korhonen, 1988a, b; wild: Kauhala, 1992) further decreases body surface-mass ratio before winter (Mustonen & Nieminen, 2018). The body temperature is maintained through insulative winter fur (Korhonen & Harri, 1986), white adipose tissue (Nieminen et al., 2005, Niiranen et al., 2021b) and muscular shivering (Korhonen et al., 1985, Niiranen et al., 2015). The ambient temperature affects body temperature, physical activity, and shelter use (Mustonen et al., 2007, 2012; Kowalczyk et al., 2008). Finnraccoons may remain inactive, hibernate superficially for weeks during the cold periods (Mustonen & Nieminen, 2018). Their paws have been considered sensitive to frost bites (Korhonen & Harri, 1989), but there is some indication of peripheral heterothermy in the foot pads (Käkelä & Hyvärinen, 1996). In early spring, the Finnraccoon pelage is optimal for capturing solar radiation in the sunshine, through postural adjustment, ie basking in the sun (Harri & Korhonen, 1988). Shedding of the insulative winter fur occurs in spring, and thereafter, the less insulative summer pelage secures thermal comfort also in summer (e.g. Xiao, 1995).

Since Finnraccoons are raised in outdoor sheds (buildings without solid walls), they are vulnerable to the ambient temperature and some other aspects of weather conditions. Regardless of the various adaptations to the variation of the ambient temperature, they may need some protection from extreme weather conditions to maintain thermal comfort. The roof of the shed prevents exposure to the snowfall, rain and most (or all) direct sunshine. The housing facilities *per se* do not typically provide much protection from wind, but various shelters and wind shields can be provided to enable behavioural adjustment during cold and windy weather. In spring, access to sunshine for performing basking behaviour may be valuable, whereas in summer, an additional protection from direct sunshine may be needed. The design of the shed affects the air flow inside the shed; if the ridge of the roof is open, it ensures better air movement under the roof and prevents heating of the air inside the shed. Watering the roof of the shed and

sprinkling the air inside the shed can also prevent the air temperature “inside the shed” from increasing too high. Unfortunately, the detailed welfare effects of any of the methods have not been measured.

Dirty and wet fur does not protect the animal from cold temperatures as efficiently as dry fur (Korhonen, 1987b). Although, cleanliness of the animal is often used to indicate comfort around resting, (eg foxes: WelFur, 2015a), in the Finnraccoon, cleanliness of the fur was considered better indicating thermal comfort during the cold periods, ie autumn and winter than resting comfort. Therefore, the criterion *Thermal comfort* is illustrated by using a risk factor for thermal comfort ie *Cleanliness of the fur* in autumn and winter and the input measures *Protection from exceptional hot weather* in summer and *Protection from wind* in autumn and winter. The measure *Protection from exceptional hot weather* covers three variables: whether the design of the shed enables air circulation inside the shed, protection from direct sunlight (availability of sufficient shade and/or roofing) and the farmers routine to sprinkle the roofs of the sheds or the air inside the sheds in hot weather. The measure *Protection from wind* covers the protection from the wind outside the housing facility (stand of trees or built constructions) and in-cage wind shield.

Also, serious fur chewing (a self-injurious behaviour) and so-called samson fur, (Korhonen & Harri, 1984) may affect the thermal comfort of the animal in cold and windy weather. In both cases, the cover hairs may be missing from some areas (fur chewing) or totally (samson fur), but the underfur, which mainly determines the insulative capacity of the fur, remains typically intact. These were not, however, included in criterion *Thermal comfort*. Fur chewing primarily represent abnormal self-injurious behaviour and is included within the criterion *Appropriate behaviour*. Samson fur, in turn, is included in the criterion *Absence of diseases*. Furthermore, since Finnraccoons with Samson fur are not kept as breeding animals, there are not kept on the farm during winter.

Finally, new-born cubs are in special situation regarding thermal comfort since they are heterothermic. Their body temperature drops without extraneous heat source, but they easily recover from low body temperatures (Harri et al., 1991). During their first weeks of life, they are totally dependent on the thermal protection provided by the nest box and their mother. In the nest boxes designed for Finnraccoons,

a high doorstep at the entrance hinders the small cubs from emerging the next box. However, if the cub ends up outside the nest box, the cub cannot usually return to the nest box itself. If the mother does not retrieve the cub, the farmer must move the cub back to the nest box. The input measures, like type of the nest box and the surveillance practices to detect cubs outside the nest box, could be included in the later version of the protocol.

### ***Ease of movement***

Wild racoon dogs roam in home ranges ranging from 0.5 to 4 km<sup>2</sup> (eg Kauhala et al., 2010; Söld et al., 2017; Schwemmer et al., 2021). A mean daily distance travelled of 3-4 km has been reported (Herfindal et al., 2016). The minimum cage size for an individual Finn raccoon set by European convention (1999) is 0.8 m<sup>2</sup>. The minimum length and width of the cage are 100 cm and 75 cm, respectively. Since the body length of the Finn raccoon is approximately 70 cm (Koistinen et al., 2018), the minimum cage size enables lying down, rising up, turning around and taking some steps. Due to limited number of studies of the topic, the optimal cage size in relation to the welfare of the Finn raccoon is not well known. Small changes in the cage size (from 0.6 m<sup>2</sup> to 2,5 m<sup>2</sup>) and animal density in the current housing facilities do not systematically affect the production variables (like weight gain, fur quality), physiological welfare variables (like haematological variables) and general behaviour of the Finn raccoon (eg Korhonen & Harri, 1988; Koistinen et al., 2018, 2020). However, juveniles housed in groups of six in a larger total area perform less stereotypic behaviour than the ones housed in pairs in smaller cages, with the same available area per animal (0.6 m<sup>2</sup>) (Ahola et al., 2007), which may simply indicate that the slightly larger available area better enable other physical activity than stereotypical movements.

The minimum required height of the cage is 70 cm (European Convention, 1999), which enable some stretching upwards. The height of the cage, besides the possibility to stretch upwards, is not crucial to Finn raccoons since they do not prefer high places (Koistinen & Korhonen, 2018) and neither do their wild conspecifics (Rudert et al., 2011). However, two floor cages have been tested in Finn raccoons, and they do also use the upper floor of the cage (Mohaibes et al., 2008), but this may be just seeking for more space to move around, and not particularly need to stay in a high location.

The criterion *Ease of movement* is measured by using the output measures of *Possibility for horizontal movement* and *Possibility for vertical movement*. The ease of movement is evaluated according to the behavioural repertoire enabled by the available area. As described above, the horizontal available area at the legislative minimum, only enable lying down, rising up, turning around and taking a few steps. In the other end of housing facilities may enable wider behavioural repertoire of locomotion, like walking, running, and locomotor play. The three categories of the WelFur Finn raccoon protocol, describe the behaviours enabled by the available area, *ie* taking a few steps, walking a distance corresponding to the body length of the animal and enabling wider repertoire of locomotor activities. In the *Possibility for vertical movement*, it is evaluated whether the animal can stretch upwards or not in the provided area.

### ***Absence of injuries***

Various accidents, poor-quality housing facilities (eg spikes in the net, broken feeding trays) (Nordgren, 2017) and handling devices may cause skin lesions and injuries in Finn raccoons. Agonistic behaviour is uncommon and therefore, group housing is not typically risk factor for injuries (Koistinen et al., 2020). Skin lesions and injuries are not common in Finn raccoons, since the prevalence is < 1% of animals (Koistinen et al., 2013). Although massive fur may make assessment challenging, inspection of the leather side of the pelts after pelting has not revealed a higher number of skin lesions or injuries (Hänninen et al, 2002; Mohaibes et al., 2008; Koistinen et al., 2020). Moving difficulties may be caused by various accidents. Obesity may cause some moving difficulties in autumn, as has been demonstrated in blue foxes (Kempe, 2018). Moving difficulties has been reported occurring in 2% of Finn raccoons in autumn (Koistinen et al., 2013).

*Absence of injuries* is assessed by two measures, *Skin lesions and other injuries to the body* and *Moving difficulties*. The skin lesions are not divided into categories based on their size, but fresh and already healed injuries are kept in separate categories.

### ***Absence of diseases***

Although the wild raccoon dog is recognised as a vector of several infectious diseases, of which many are zoonotic (e.g. Söld et al., 2014), and a number of diseases are reported in Chinese farms (e.g. Yang et al., 2021), the European Finn raccoons are seldom diseased and do not often need veterinary care

(Nordgren, 2017). This is probably due to high hygienic standards on the farms. The most common diseases in Finnraccoons are various diarrhoeas, in up to 27% of animals in autumn (Koistinen et al., 2013). Other common disease is the contagious FENP (Fur animal epidemic necrotic pyoderma) with variable and painful symptoms (Nordgren, 2017). Bent feet, which is caused by carpal joint laxity, is a common problem in blue foxes (Kempe et al., 2010) and has been documented also in the Finnraccoon (Koistinen et al., 2013). Although the aetiology of bent feet is not well understood, it is considered unnatural and potentially painful condition. In recent years, dwarf growing Finnraccoons have appeared on farms, but the aetiology and welfare effects of this condition are unclear (Koistinen et al., 2020; Toppari, 2020). Annual mortality is low, typically less than 1% (unpublished data of authors).

For the WelFur Finnraccoon protocol, the measures for the criterion *Absence of diseases* were selected with the aid of veterinarians. Four output measures are used, namely *Diarrhoea*, *Bent feet*, *Other disease*, and *Mortality*. In the measurement of diarrhoea, three categories are used, no diarrhoea, loose faeces and diarrhoea. In the case of *bent feet*, the tool developed to evaluate the carpal joint angle in blue foxes (Kempe et al., 2010) was adopted, but the number of categories was reduced to three, and other unnatural foreleg conformations were included in the worst category. All other symptoms indicating diseases were lumped into the *Other disease* measure. Mortality is checked from the recordings of the farm. Mortality is divided into animals found dead and euthanised due to disease or other poor health condition. This enables inclusion of the farmer's attitude, ie whether the farmer detects the sick and injured animals, and treats them accordingly (including euthanasia). Also, the quality of the mortality data (whether it is solid and systematically collected) is recorder.

#### ***Pain induced by management practices***

Since the body of Finnraccoons is not mutilated (no castration or marking etc.), there are no other management procedures which may cause pain in Finnraccoons than killing. The almost solely used killing method in Finnraccoons is through body electrocution, which has been proved fast and effective in blue foxes (Korhonen et al., 2009). Both, emergency killing and killing for pelting are carried out by using the same method. Only killing of small cubs may deviate from this.

*Emergency killing* and *Killing at farm level at the end of P3* are measured to describe the criterion *Absence of pain induced by management procedures*. Besides inspection of the killing device, the former includes detailed inspection of the documents in relation to the killing of animals for pelting (in Period 3). These documents include the species-specific standard operation procedure (SOP) for killing Finnraccoons (see Korhonen & Huuki, 2013), certification of the competence (education) for killing and annually renewed certificate (or testing) of the correct functioning of the killing device. *Emergency killing* may be needed at any time, and therefore the presence and functioning of the emergency killing method is evaluated in other periods, separately for cubs, when present.

#### ***Expression of social behaviour***

In the wild, raccoon dogs live in pairs and in small family groups (Ward & Wurster-Hill, 1990). They do not actively defend a territory but tolerate or ignore the conspecific around their home range (eg Drygala et al., 2008). Also, Finnraccoons are social animals. Social communication includes for example persistent allohuddling, play, allogrooming and greeting rituals (Korhonen et al., 1991; Koistinen & Korhonen, 2018; Koistinen et al., 2020). Due to a high social tolerance within a family and sibling groups, agonistic interactions are seldom observed (Kasanen et al., 2000; Koistinen et al., 2020). Instead, singly housing jeopardises the welfare of juvenile Finnraccoons. On farm conditions, social housing determines the animals' possibility to express social behaviours, therefore *Social housing of juveniles* is used as an input measure of criterion *Expression of social behavior*. In this measurement it is measured whether juvenile animals are raised in pairs or in groups, which enable social communication.

Weaning age, measured in the WelFur mink (WelFur, 2015b), was considered also in the Finnraccoon, but rejected. Finnraccoon cubs are typically weaned at the age of 2-3 months. Early weaning at the cubs' age of 5 weeks compromises the health and welfare of both, the cub and the mother (Zon et al., 1989), whereas late weaning may be beneficial (Kasanen et al., 2000). In the wild, flexible dispersion patterns are observed (Herfindal et al., 2016), and some cubs may spend their first winter with their parents. The data collection window in Period 2 is placed at the end of the nursing period, when some of the cubs have been already separated from their mothers, therefore

health (e.g. mortality of weaned cubs) and behavioural (e.g. stereotypical behaviour of the mother) measures will detect the outcome of the possible challenges in weaning.

### ***Expression of other behaviour***

Finnraccoons manipulate regularly various activity objects, like bones, wooden blocks and straw (Koistinen et al., 2017, 2018, 2020). They also willingly utilise the cage furniture, like nest box and a large tube (Koistinen et al., 2020). Both, the activity objects, and furniture provide complexity to the otherwise monotonous housing facilities and enable more variable behavioural repertoire than the barren cage. The preference of Finn-raccoons for various objects and furniture items is not known. According to the Finnish legislation (Ministry of Agriculture and Forestry, 2011) Finn-raccoons must be provided with a platform and activity material.

Not optimal housing may cause abnormal behaviours. In Finn-raccoons, stereotypic behaviour (SB) and fur chewing has been reported. Stereotypic behaviour is observed 0-3% of time (Ahola et al., 2007; Koistinen & Korhonen, 2018; Koistinen et al., 2018). Most of the stereotypic activity in the Finn-raccoon is locomotor stereotypy, but also persistent scratching, oral manipulation (extensive liking or biting of an object, other than food or toy) and head twirling (repeated twirling of head against wall or ceilings) are observed in a lesser extend (Koistinen & Korhonen, 2018; Koistinen et al., 2018). The occurrence of stereotypic behaviour is integrated into diurnal activity rhythm and possibly also to human activities (e.g. feeding regime) on the farm (Ahola et al., 2007).

Fur chewing is a self-injurious behaviour observed in fur animals, in which the animal chews own fur (Malmkvist & Hansen, 2001). Only cover hairs are typically affected and underfur remains intact. In blue foxes, fur chewing starts typically from (the tip of) the tail and other body parts are affected later (Koistinen et al., 2021). Finn-raccoons have very short tail, and due to stocky body shape, they do not quite reach their tail. Instead, the first signs of fur chewing may be observed anywhere in the body where the animal can reach. Fur chewing is seen most in late autumn and in winter (Koistinen et al., 2021), which may be partly because it is hard to observe during moult in spring and from the tenuous pelage in summer. In autumn, fur chewing is observed in 0-3% of Finn-raccoons (Koistinen et al., 2013, 2018).

The criterion *Expression of other behaviours* is measured by using three input measures of the behavioural potential provided by the housing facilities, i.e. *Complexity of the available area*, *Opportunity to use activity object* and *Availability of straw* and; two output measures of behavioural deprivation, i.e. *Stereotypic behaviour* and *Fur chewing*. In the input measures, the housing facilities are observed for the complexity and access to activity materials and objects. The rationale is that the more complex is the housing facility and with more activity material and objects, the wider behavioural repertoire it enables, and the better it is for the welfare of the animal. The observation of the stereotypic behaviour is carried out, by using a larger sample of animals than for other measures, when it tends to remain at the highest level in the dawn, before the morning feeding (Ahola et al., 2007). Fur chewing behaviour can be observed from the consequences, since they remain easily visible in the fur of the animal for a long period of time (until next moult).

### ***Good human animal relationship and Positive emotional state***

Regardless of the short domestication history, Finn-raccoons are calm and curious in the presence of human. Due to the lack of sufficient positive welfare measures, the criteria *Good human animal relationship* (HAR) and *Positive emotional state* are partly combined in the WelFur Finn-raccoon protocol (see WelFur, 2020, p 88). Therefore, a measure combining some elements from both criteria was selected.

In fur animals, stick test, feeding test, human presence/approach test and "hand catch" test and their numerous variations (reviewed by Rekilä, 1999; Kirkden et al., 2010) have been used to test HAR and/or temperament of the animal. Stick test is a novel object test, validated to test temperament in mink (Malmkvist & Hansen, 2002), and used in the WelFur mink (WelFur, 2015b). The Feeding test is validated in blue foxes (Rekilä, 1999) and used as a HAR test in the WelFur fox (WelFur, 2015a). It measures whether the animal is brave enough to eat in the close proximity of human. These tests were not selected for the Finn-raccoon, since the stick test does not quite measure human animal relationship, and the outcome of the feeding test may be dependent on the season in the Finn-raccoon (due to fluctuating appetite, eg Korhonen, 1988a). A simply *Voluntary approach test*, resembling the "human test" described by Pedersen and Jeppesen (1990) for foxes, was selected, alt-



though not validated in Finnraccoons. In the *Voluntary approach test*, animal is faced with a situation whether to approach an unfamiliar human or not. It is expected that, a confident animal, with positive expectations of human contact will approach, whereas a fearful animal and any animal with negative expectations of human contact do not voluntarily approach. Similar tests are used for example in horses, donkeys (Dalla Costa et al., 2015), goats (Battini et al., 2016) and turkeys (Erasmus & Swanson, 2014).

### **An overview of the WelFur Finnraccoon protocol development process**

The development of the WelFur Finnraccoon protocol was initially launched simultaneously with the WelFur mink and fox protocols already in 2009 (Mononen et al., 2012). First, literature was reviewed to identify potential welfare issues and to make a preliminary list of potential measures. However, due to higher uncertainty, it was soon decided to slow down the process in the Finnraccoon and prioritize the development of the protocols for mink and foxes. Only after publishing the WelFur mink and fox protocols, more intensive development of the WelFur Finnraccoon protocol was started.

The development of the WelFur Finnraccoon protocol started as a structured, sequential waterfall project, but later, when there was a need to speed up the process, it ended up of being a flexible agile project with iterative cycles, common in software development (Reiff & Schleger, 2022). The latter part of the development process enabled repeated testing on farms and subsequent systematic utilization of data and experiences accumulated. Because of that, it was evident that the measures, data collection methods and the related WelFur mobile data collection application (WMA) also taking care of the stratified sampling on farm (Ojala et al., 2021) all worked well in practice when finalised. This end part of the process differs from the development of the WelFur mink and fox (see Mononen et al., 2012), in which, numerous modifications to the measures and data collection had to be done after implementation of the scheme, because of limited testing experiences on private farms.

To scrutinize the protocol versions and to support the development process, an External Reviewer Committee (ERC) with three members, experienced in the fur animal production and on-farm welfare assessment in other farm animals, was nominated at the later part of the process. ERC followed and quidded the development closely until the end of the process.

When the measures were selected, an expert panel of five persons familiar with the species (two independent animal welfare scientists, a veterinarian, a scientist working for the industry and a farmer) was established for interpreting how the welfare data are transformed into the measure scores, and how the measure scores are aggregated into the criterion scores. The panel had preliminary data from farms, collected during the development process, available while doing the scoring. The work of the panel was facilitated by an external scientist who had participated in the development of Welfare Quality® scoring systems. The scoring was based on the consensus view of the panel, and not based on individual opinions of the panellists. The aggregation of the criterion scores to principle scores is done in the WelFur Finnraccoon protocol with the same Choquet integrals as in the fox and mink protocols (WelFur, 2020, p 52 onwards).

### **Discussion**

The welfare of Finnraccoons has been overlooked, while the main interests of fur animal welfare science has concentrated on the more numerous species. The WelFur Finnraccoon protocol encourages the farmers and the whole industry to pay more attention to the welfare of Finnraccoons. This is important and has major animal welfare implications. Many of the selected measures anticipate testing of validity and reliability, supported by scientific study in this species. Therefore, revision of the protocol as the knowledge of the species increases is necessary.

On-farm welfare assessment protocols of other species (e.g. horse: Hausberger et al., 2020, cattle: Andreassen et al., 2014; de Graaf, 2017), including WelFur protocols for mink and foxes (Kleibert et al., 2020; Müller et al., 2021) have been criticised. All protocols tend to struggle with the same challenges in the selection of the measures and weighing of the data. It has been shown that regardless of the similar welfare statements, different welfare protocols (using different measures) may end up to different outcomes (cattle: de Rosa et al., 2019). The selection of the welfare measures is a sensitive work. All aspects of the multifaceted animal welfare concept should be covered, and the welfare program should meet the public's understanding of animal welfare (Heise & Theuvsen, 2018). The accumulating data from farms, future discussions and criticism will show how did we manage to solve these tasks in the WelFur Finnraccoon protocol and how should we modify the protocol in future from the societal perspective.

Animal welfare is defined as the subjective experience of the animal (eg Mellor, 2016). At the measure level, this is considered when using output measures. But combining data from various measures into a farm level scoring violates the definition of the individual perspective of the welfare experience (Lundmark et al., 2015). Detailed information of individual animals disappears into the calculation system. This means that a farm may get a good overall score, although the welfare of individual animal is poor. The individual perspective of welfare experience should be better included in the assessment and scoring in future.

Better use of modern technology should be considered in future animal welfare assessment protocols. For example, video recording of undisturbed behaviour could be used to detect comfort around resting, stereotypic behaviour and positive social behaviours. Use of computer vision to detect animal behaviour have already been successfully piloted in silver foxes (Schütz et al., 2022).

In conclusion, despite of the scientific uncertainty, the protocol provides a good tool to start evaluating and improving the welfare of Finnraccoons on private farms. With the aid of advisory work, measurements of animal welfare ensure improvements in animal welfare on Finnraccoon farms.

#### Declaration of interests

The authors declare no conflict of interest.

#### Acknowledgements

We are grateful to the Finnraccoon farmers, veterinarians and other stakeholders for their massive input to the development of the WelFur Finnraccoon protocol. The personnel of Research Farm Luova Ltd. are acknowledged for their continuous testing and evaluation of the measures. The external review committee is acknowledged for the constructive discussions and gently guidance throughout the process. The authors are grateful to Dr. Hannu Korhonen for his indispensable work in sharing his deep perspectives to Finnraccoon as a unique animal species. Dr. Jussi Peura is acknowledged for his constructive comments to the earlier version of the MS.

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**BREEDING, GENETICS AND REPRODUCTION****Coat color inheritance in American mink**

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**Background**

Understanding the genetic mechanisms underlying coat color inheritance has always been intriguing irrespective of the animal species including American mink (*Neogale vison*). The study of color inheritance in American mink is imperative since fur color is a deterministic factor for the success of mink industry. However, there have been no studies during the past few decades using in-depth pedigree for analyzing the inheritance pattern of colors in American mink.

**Methods**

In this study, we analyzed the pedigree of 23,282 mink extending up to 16 generations. All animals that were raised at the Canadian Center for Fur Animal Research (CCFAR) from 2003 to 2021 were used in this study. We utilized the Mendelian ratio and Chi-square test to investigate the inheritance of Dark (9,100), Pastel (5,161), Demi (4,312), and Mahogany (3,358) colors in American mink.

**Results**

The Mendelian inheritance ratios of 1:1 and 3:1 indicated heterozygous allelic pairs responsible for all studied colors. Mating sire and dam of the same color resulted in the production of offspring with the same color most of the time.

**Conclusion**

Overall, the results suggested that color inheritance was complex and subjected to a high degree of diversity in American mink as the genes responsible for all four colors were found to be heterozygous.

*BMC Genomics*. 2023 May 4; 24 (1): 234.  
Doi: 10.1186/s12864-023-09348-8.

**Population genomics of American mink using genotype data**

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Understanding the genetic structure of the target population is critically important to develop an efficient genomic selection program in domestic animals. In this study, 2,973 American mink of six color types from two farms (Canadian Centre for Fur Animal Research (CCFAR), Truro, NS and Millbank Fur Farm (MFF), Rockwood, ON) were genotyped with the Affymetrix Mink 70K panel to compute their linkage disequilibrium (LD) patterns, effective population size ( $N_e$ ), genetic diversity, genetic distances, and population differentiation and structure. The LD pattern represented by average  $r^2$ , decreased to  $<0.2$  when the inter-marker interval reached larger than 350 kb and 650 kb for CCFAR and MFF, respectively, and suggested at least 7,700 and 4,200 single nucleotide polymorphisms (SNPs) be used to obtain adequate accuracy for genomic selection programs in CCFAR and MFF respectively. The  $N_e$  for five generations ago was estimated to be 76 and 91 respectively. Our results from genetic distance and diversity analyses showed that American mink of the various color types had a close genetic relationship and low genetic diversity, with most of the genetic variation occurring within rather than between color types. Three ancestral genetic groups was considered the most appropriate number to delineate the genetic structure of these populations. Black (in both CCFAR and MFF) and pastel color types had their own ancestral clusters, while demi, mahogany, and stardust color types were admixed with the three ancestral genetic groups. This study provided essential information to utilize the first medium-density SNP panel for American mink in their genomic studies.

*Front Genet.* 2023 May 9; 14: 1175408.  
Doi: 10.3389/fgene.2023.1175408.  
*eCollection* 2023.

## The Genetic Diversity of Mink (*Neovison vison*) Populations in China

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The American mink (*Neovison vison*) is a semi-aquatic species of Mustelid native to North America that is now widespread in China. However, the knowledge of genetic diversity of mink in China is still limited. In this study, we investigated the genetic diversity and identified significant single nucleotide polymorphisms (SNPs) in mink populations of five different color types in three different mink farms in China. Using double-digest restriction site-associated DNA sequencing, we identified a total of 1.3 million SNPs. After filtering the SNPs, phylogenetic tree, Fst, principal component, and population structure analyses were performed. The results demonstrated that red mink and black mink grouped, with separate clustering of all other color types. The population divergence index (Fst) study confirmed that different mink populations were distinct (K = 4). Two populations with different coat colors were subjected to the selection signature analysis, and 2300 genes were found to have a clear selection signature. The genes with a selection signature were subjected to Gene Ontology (GO) categorization and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, the results revealed that the genes with a selection signature were enriched in the melanogenesis path-

way. These study's findings have set the stage for improved breeding and conservation of genetic resources in real-world practical mink farming.

*Animals (Basel).* 2023 Apr 27; 13 (9): 1497.  
Doi: 10.3390/ani13091497.

## Asymptomatic viral infection is associated with lower host reproductive output in wild mink populations

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Many endemic viruses circulate in populations without hosts showing visible signs of disease, while still having the potential to alter host survival or reproduction. Aleutian Mink Disease Virus (AMDV) circulates in many American mink (*Neovison vison*) populations in its native and introduced ranges. In this study, we analysed how AMDV infection in female American mink affects the reproduction of a feral population. Females infected with AMDV delivered significantly smaller litters (5.8 pups) than uninfected females (6.3 pups), meaning their litter size was reduced by 8%. Larger females and yearling females had larger litters than smaller and older females. There were no significant differences in whole litter survival between infected and uninfected females; however, offspring survival until September or October within litters of infected females was 14% lower than that within those of uninfected females. This negative link between infection and reproductive output means that Aleutian disease could seriously affect the wild mink population. This study increases our understanding of the threats posed by the spread of viruses to wildlife from farm animals or humans, highlighting that viruses circulating in wildlife, even in the absence of clinical manifestation, can be important drivers of population dynamics in wildlife.

*Sci Rep.* 2023 Jun 9; 13 (1): 9390.  
Doi: 10.1038/s41598-023-36581-8.

## NUTRITION, FEEDING AND MANAGEMENT

### Effect of *Cyberlindnera jadinii* supplementation on growth performance, serum immunity, antioxidant status, and intestinal health in winter fur-growing raccoon dogs (*Nyctereutes procyonoides*)

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#### Introduction

This study aimed to investigate the effects of *Cyberlindnera jadinii* supplementation on the growth performance, nutrient utilization, serum biochemistry, immunity, antioxidant status, and intestinal microbiota of raccoon dogs during the winter fur-growing period.

#### Methods

Forty-five 135 ( $\pm 5$ ) day-old male raccoon dogs were randomly assigned to three dietary groups supplemented with 0 (group N),  $1 \times 10^9$  (group L) and  $5 \times 10^9$  CFU/g (group H) *Cyberlindnera jadinii*, with 15 raccoon dogs per group.

#### Results

The results showed that *Cyberlindnera jadinii* in groups L and H improved average daily gain (ADG) and decreased feed-to-weight ratio (F/G) ( $P < 0.05$ ). No significant difference was found in nutrient digestibility and nitrogen metabolism among the three groups ( $P > 0.05$ ). Compared with group N, serum glucose levels were lower in groups L and H ( $P < 0.05$ ). The levels of serum immunoglobulins A and G in group L were higher than those in the other two

groups ( $P < 0.05$ ), and the levels of serum immunoglobulins A and M in group H were higher than those in group N ( $P < 0.05$ ). Supplementation with *Cyberlindnera jadinii* in groups L and H increased serum superoxide dismutase activity, and the total antioxidant capacity in group H increased compared with group N ( $P < 0.05$ ). The phyla Bacteroidetes and Firmicutes were dominant in raccoon dogs. The results of principal coordinate analysis (PCoA) showed that the composition of microbiota in the three groups changed significantly ( $P < 0.05$ ). The relative abundance of Campylobacterota was increased in the H group compared to the N and L groups ( $P < 0.05$ ). The relative abundance of *Sarcina* was increased in group L compared with the other two groups ( $P < 0.05$ ), while the relative abundance of *Subdoligranulum* and *Blautia* were decreased in group H compared with the other two groups ( $P < 0.05$ ). Also, the relative abundance of *Prevotella*, *Sutterella* and *Catenibacterium* was higher in group L ( $P < 0.05$ ) compared with group H.

#### Discussion

In conclusion, dietary supplementation with *Cyberlindnera jadinii* improved growth performance, antioxidant activity, immune status, and improved intestinal microbiota in winter fur-growing raccoon dogs. Among the concentrations tested,  $1 \times 10^9$  CFU/g was the most effective level of supplementation.

*Front Vet Sci.* 2023 May 11; 10: 1154808.

Doi: 10.3389/fvets.2023.1154808. eCollection 2023.

## BEHAVIOUR AND WELFARE

### Defining hippocampal area CA2 in the fox (*Vulpes vulpes*) brain

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*Hippocampus. 2023 Jun; 33 (6): 700-711.*

*Doi: 10.1002/hipo.23546. Epub 2023 May 9.*

### **The effects of age, size, and cage complexity on the behaviour of farmed female chinchillas (*Chinchilla lanigera*)**

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Even though chinchillas have been farmed for a century, there are not many studies concerning their behaviour in captivity or their optimal housing conditions, both of which are important factors in the assessment of their welfare. This study aimed to evaluate the effect of different cage types on chinchillas' behaviour and their reactions towards humans. Female chinchillas (n = 12) were kept in three types of cages: standard with a wire floor (S); standard with a deep litter floor of shavings (SR); and enlarged with a deep litter floor of shavings (LR). Animals spent 11 weeks in each type of cage. The chinchillas' reactions toward humans were observed via intruder test. Ethograms were prepared based on round-the-clock video recordings. The activity of the chinchillas was compared, taking into account the different cage types and the animals' varying responses to the hand test. The generalized ordered logistic regression model was used to ascertain whether cage type has an

effect on a chinchilla's behaviour towards humans. To compare the time distribution of various activity between chinchillas, the non-parametric Scheirer-Ray-Hare test was used. Animals kept in LR cages presented significantly less timid reactions in comparison to those kept in S and SR cages. The chinchillas spent most of their time resting (68% of the day), in locomotion (23%), and eating or drinking (8%); they spent only 1% on grooming behaviour. Cage enrichment generally reduced the fear of humans. However, the average chinchilla response to the hand test was classified in each type of cage as "cautious". Analyses of the ethograms indicated that the chinchillas were active mostly during the dark stage of the day. In conclusion, the larger cage size and its enrichment (particularly litter) reduced the fearfulness and passivity of the animals, which could be evidence of better welfare conditions.

*Sci Rep. 2023 Apr 14; 13 (1): 6108.*

*Doi: 10.1038/s41598-023-32516-5.*

### **The brain of the silver fox (*Vulpes vulpes*): a neuroanatomical reference of cell-stained histological and MRI images**

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Although the silver fox (*Vulpes vulpes*) has been largely overlooked by neuroscientists, it has the potential to serve as a powerful model for the investigation of brain-behavior relationships. The silver fox is a melanistic variant of the red fox. Within this species, the long-running Russian farm-fox experiment has resulted in different strains bred to show divergent behavior. Strains bred for tameness, aggression, or without selection on behavior present an excellent

opportunity to investigate neuroanatomical changes underlying behavioral characteristics. Here, we present a histological and MRI neuroanatomical reference of a fox from the conventional strain, which is bred without behavioral selection. This can provide an anatomical basis for future studies of the brains of foxes from this particular experiment, as well as contribute to an understanding of fox brains in general. In addition, this can serve as a resource for comparative neuroscience and investigations into neuroanatomical variation among the family Canidae, the order Carnivora, and mammals more broadly.

*Brain Struct Funct.* 2023 Jun; 228 (5): 1177-1189.  
Doi: 10.1007/s00429-023-02648-5.  
Epub 2023 May 9.

## HEALTH AND DISEASE

### A comparison of sampling and testing approaches for the surveillance of SARS-CoV-2 in farmed American mink

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Surveillance for SARS-CoV-2 in American mink (*Neovison vison*) is a global priority because outbreaks on mink farms have potential consequences for animal and public health. Surveillance programs often focus on screening natural mortalities; however, significant knowledge gaps remain regarding sampling and testing approaches. Using 76 mink from 3 naturally infected farms in British Columbia, Canada, we compared the performance of 2 reverse-transcription real-time PCR (RT-rtPCR) targets (the envelope [E] and RNA-dependent RNA polymerase [RdRp] genes) as well as serology. We also compared

RT-rtPCR and sequencing results from nasopharyngeal, oropharyngeal, skin, and rectal swabs, as well as nasopharyngeal samples collected using swabs and interdental brushes. We found that infected mink were generally RT-rtPCR-positive on all samples; however, Ct values differed significantly among sample types (nasopharyngeal < oropharyngeal < skin < rectal). There was no difference in the results of nasopharyngeal samples collected using swabs or interdental brushes. For most mink (89.4%), qualitative (i.e., positive vs. negative) serology and RT-rtPCR results were concordant. However, mink were positive on RT-rtPCR and negative on serology and vice versa, and there was no significant correlation between Ct values on RT-rtPCR and percent inhibition on serology. Both the E and RdRp targets were detectable in all sample types, albeit with a small difference in Ct values. Although SARS-CoV-2 RNA can be detected in multiple sample types, passive surveillance programs in mink should focus on multiple target RT-rtPCR testing of nasopharyngeal samples in combination with serology.

*J Vet Diagn Invest.* 2023 Jun 27;  
10406387231183685.  
Doi: 10.1177/10406387231183685.  
Online ahead of print.

### Evaluation of SARS-CoV-2 passive surveillance in Lithuanian mink farms, 2020-2021

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The newly emerged SARS-CoV-2, causing COVID-19 in humans, is also infecting American mink (*Neovison vison*), used in fur production. Since 2020, passive surveillance of SARS-CoV-2 in mink farms was implemented in Lithuania. Here, we describe data from a survey of all 57 active Lithuanian mink farms carried out during November-December 2021 to complement passive surveillance in the country. In all 57 mink farms, nasopharyngeal swab samples were collected from dead or live mink and tested by real-time RT-PCR. Dead mink samples were tested

in pools of 5, while live mink samples were tested individually. In 19 mink farms, blood serum was collected and tested for antibodies to determine previous exposure to the virus. Environmental samples from 55 farms were also collected and tested in pooled samples by real-time RT-PCR. The present survey has detected 22.81% viral RNA-positive mink farms and a high number of mink farms that were exposed (84.21, 95% CI 67.81-100%) to the virus. The increasing exposure of mink farms to the virus due to growing human COVID-19 cases and limitations of passive surveillance could explain the observed epidemiological situation of SARS-CoV-2 in Lithuanian mink farms, compared to the few positive farms previously detected by passive surveillance. The unexpected widespread exposure of mink farms to SARS-CoV-2 suggests that passive surveillance is ineffective for early detection of SARS-CoV-2 in mink. Further studies are needed to reveal the present status in previously infected mink farms.

*Front Vet Sci.* 2023 Jun 9; 10: 1181826.  
Doi: 10.3389/fvets.2023.1181826. eCollection 2023.

**Cryptic SARS-CoV-2 lineage identified on two mink farms as a possible result of long-term undetected circulation in an unknown animal reservoir, Poland, November 2022 to January 2023**

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In late 2022 and early 2023, SARS-CoV-2 infections were detected on three mink farms in Poland situated within a few km from each other. Whole-genome sequencing of the viruses on two of the farms showed that they were related to a virus identified in humans in the same region 2 years before (B.1.1.307 lineage).

Many mutations were found, including in the S protein typical of adaptations to the mink host. The origin of the virus remains to be determined.

*Euro Surveill.* 2023 Apr; 28 (16): 2300188.  
Doi: 10.2807/1560-7917.ES.2023.28.16.2300188.

**Cross-species transmission of coronaviruses with a focus on severe acute respiratory syndrome coronavirus 2 infection in animals: a review for the veterinary practitioner**

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In 2019 a novel coronavirus termed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged from an unidentified source and spread rapidly among humans worldwide. While many human infections are mild, some result in severe clinical disease that in a small proportion of infected people is fatal. The pandemic spread of SARS-CoV-2 has been facilitated by efficient human-to-human transmission of the virus, with no data to indicate that animals contributed to this global health crisis. However, a range of domesticated and wild animals are also susceptible to SARS-CoV-2 infection under both experimental and natural conditions. Humans are presumed to be the source of most animal infections thus far, although natural transmission between mink and between free-ranging deer has occurred, and occasional natural transmission between cats cannot be fully excluded. Considering the ongoing circulation of the virus among people, together with its capacity to evolve through mutation and recombination, the risk of the emergence of animal-adapted variants is not negligible. If such variants remain infectious to humans, this could lead to the establishment of an animal reservoir for the virus, which would complicate control efforts. As such, minimising human-to-animal transmission of SARS-CoV-2 should be considered as part of infection control efforts. The aim of this review is to summarise what is currently known about the species specificity of animal coronaviruses, with an emphasis on SARS-CoV-2, in the broader context of factors that facilitate cross-species transmission of viruses.

*N Z Vet J.* 2023 Jul; 71(4): 159-171.

Doi: 10.1080/00480169.2023.2191349.  
Epub 2023 Apr 19.

### Zoonothronotic transmission of SARS-CoV-2 and host-specific viral mutations revealed by genome-wide phylogenetic analysis

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Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a generalist virus, infecting and evolving in numerous mammals, including captive and companion animals, free-ranging wildlife, and humans. Transmission among non-human species poses a risk for the establishment of SARS-CoV-2 reservoirs, makes eradication difficult, and provides the virus with opportunities for new evolutionary trajectories, including the selection of adaptive mutations and the emergence of new variant lineages. Here, we use publicly available viral genome sequences and phylogenetic analysis to systematically investigate the transmission of SARS-CoV-2 between human and non-human species and to identify mutations associated with each species. We found the highest frequency of animal-to-human transmission from mink, compared with lower transmission from other sampled species (cat, dog, and deer). Although

inferred transmission events could be limited by sampling biases, our results provide a useful baseline for further studies. Using genome-wide association studies, no single nucleotide variants (SNVs) were significantly associated with cats and dogs, potentially due to small sample sizes. However, we identified three SNVs statistically associated with mink and 26 with deer. Of these SNVs,  $\sim\frac{2}{3}$  were plausibly introduced into these animal species from local human populations, while the remaining  $\sim\frac{1}{3}$  were more likely derived in animal populations and are thus top candidates for experimental studies of species-specific adaptation. Together, our results highlight the importance of studying animal-associated SARS-CoV-2 mutations to assess their potential impact on human and animal health.

*Elife*. 2023 Apr 4; 12: e83685.

Doi: 10.7554/eLife.83685.

### Identification of intestinal parasites in wild American mink (*Neovison vison*) from Biebrza and Narew national parks (Poland)

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American mink (*Neovison vison*) is an invasive species in the sylvatic environment of Poland. Mink are exposed to different parasite infections as their preys serve as intermediate and/or paratenic hosts. The study aimed to discriminate the pattern of intestinal parasite infections in mink inhabiting Biebrza (BNP) and Narew (NNP) national parks. Gastrointestinal tract examinations revealed *Coccidia*, *Echinostomatidae*, *Taenidae*, and *Capillariidae* parasites. There was no significant difference in the parasite burden of mink, but patterns of infections varied between both localizations. *Coccidia* were found in 3.8% of BNP vs. 6.7% of NNP mink. Fluke prevalence was significantly higher in NNP 27.5% compared to 7.7%

in BNP mink. Tapeworms were only found in 3.4% of NNP mink. Significantly more *Aonchotheca* eggs were found in BNP 34.6% vs. 11.4% in NNP mink. The intensity of coccidiosis and aonchothecosis was low in both parks. Fluke intensity varied between low to moderate (ranging from 1 to 16) in BNP and low to massive (ranging from 1 to 117) in NNP mink. Coinfections of various parasite species were noted in both areas. Morphological and DNA analysis revealed that flukes belonged to *Isthionorpha melis* and tapeworms to *Versteria mustelae*. It was the first isolation of *V. mustelae* in mink of those localizations. In conclusion, our study showed that mink indwelling Biebrza and Narew national parks are moderately infested with parasites. Results suggest that

mink play an important role as a reservoir for parasites endangering endemic mustelids, becoming also a potential risk factor in case of accidental transmissions to farm mink. That is why, more strict biosecurity measures are required to protect farm mink.

*Parasitol Res.* 2023 Jul; 122 (7): 1621-1629.

*Doi:* 10.1007/s00436-023-07864-w.

*Epub* 2023 May 16.





TESIS DE DOCTORADO  
**APLICACIÓN DE LA BIOLOGÍA MOLECULAR AL ESTUDIO DE LA EPIDEMIOLOGÍA Y ERRADICACIÓN DE LA ENFERMEDAD ALEUTIANA DEL VISÓN: DETECCIÓN AMBIENTAL Y ESTUDIOS FILOGENÉTICOS**

PhD THESIS  
**APPLICATION OF MOLECULAR BIOLOGY TO THE STUDY OF EPIDEMIOLOGY AND ERADICATION OF ALEUTIAN MINK DISEASE: ENVIRONMENTAL DETECTION AND PHYLOGENETIC STUDIES**

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LUGO  
2021

**Abstract**

Aleutian Mink Disease Virus (AMDV) has a worldwide distribution and infects mainly the American farmed and wild mink, but also other mustelids and members of the *Musteloidea* superfamily. In most cases, it produces a persistent infection; the consequences of this infection depend on the genotype of mink, the strain of virus, the time of infection, the immune status of the animal as well as the dose and route of infection. In adult mink, it produces a progressive disease mediated by immune complexes; however, in newborn kits lacking maternal immunity it produces acute and generally fatal interstitial pneumonia. All field strains cause disease in almost all Aleutian genotypes (blue mink); however, in other varieties, the course of infection depends to a great extent on the virulence of the strain with inapparent infection (nonprogressive form of the disease) predominant in most cases.

Given that AMDV causes persistent infections and no effective vaccine has been developed to date, the first step for its eradication is the detection and elimination of infected animals (“test and removal”) or culling the entire herd (“stamping-out”). Eradication programs are often frustrated by the reemergence of disease, undermining the enormous investments in them. The uncertain sensitivity of the CIEP test and individual variability in antibody production have been pointed out as possible causes for the failure of eradication systems. Another key factor to address in eradication programs is the environmental burden of

the virus on infected farms, which is ensured by the viral excretion in all body fluids and its high environmental resistance. Therefore, the study of the environmental reservoir by detecting the viral DNA in different zones and elements of the farm is an important step towards the association of fomites with the risk of infection and resurgence of the disease. Furthermore, the genetic characterization of circulating strains and the study of their phylogenetic relationships can be of great help in identifying their origin and the routes of entry into the farms.

With this background, the **objectives** that were set for this doctoral thesis were, in the first place, to study the feasibility of the detection of AMDV in environmental samples by means of qPCR in farms with different sanitary qualifications. This methodology was then used to study the distribution of the virus in the environment of infected farms and its epidemiological implications, serving also to assess two different DNA extraction methods used in this type of environmental samples. In third place, an evaluation of the different protective personal equipment employed during visits to mink farms was carried out using the techniques developed in previous studies. Finally, the molecular characterization and the phylogenetic study of the circulating strains in the farms of southwestern Europe in the period 2012-2020 were carried out. The results obtained from the different studies in this thesis were finally used to make different proposals in order to improve the current disease control and eradication programs.

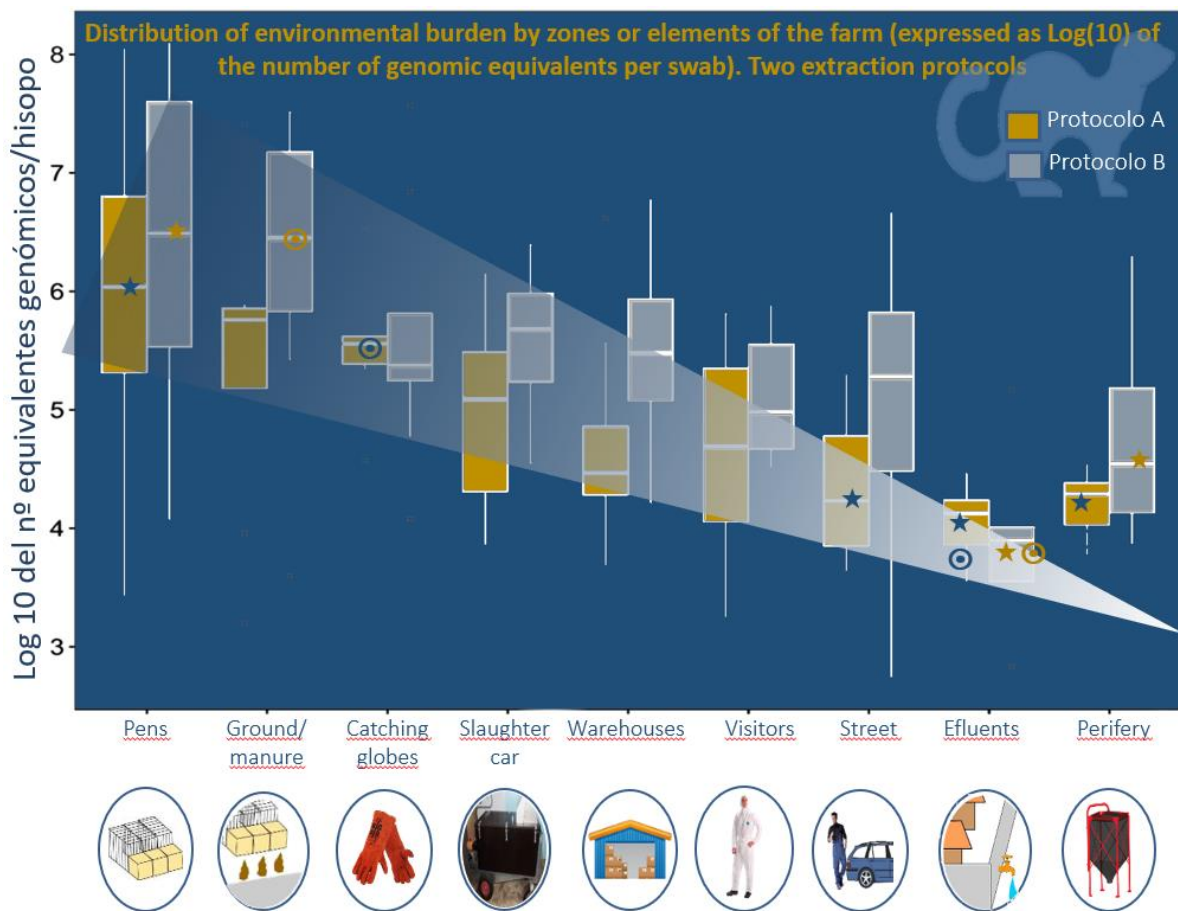
In the **first study** of this thesis, the application of qPCR to the environmental detection of AMDV was analyzed in four types of mink farms classified according to their health status (five negative farms without specific risks, one negative farm recently repopulated after finishing a process of “stamping-out”, one negative farm under a high epidemiological risk known in advance and three positive farms in a phase of epizootic expansion of the disease). This assay was done by using a simple swabbing system as a sampling method for collecting environmental samples. The results of the study showed that all samples collected from the five certified negative farms and those from the newly repopulated negative farm were negative for qPCR; however, the virus was detected in 93.9% of the samples from the three farms with confirmed infection. Samples from the remaining farm (negative for more than ten years but with a known risk of infection due to share a worker with one of the positive farms in this study), were positive in 40% of the cases. In this last farm, it should be noted that after this study was completed, infection was detected by the CIEP test performed routinely within the AD SG control program. Among all positive samples, the number of copies of virus detected fluctuated between  $6.9 \times 10^3$  and  $6.9 \times 10^7$  genomic equivalents/swab, being higher in stool samples or from surfaces in direct contact with animals (cage walls, work gloves) and lower in samples furthest from the animals, such as truck wheels, wheel dips, effluents and silos. These results have represented, to the best of our knowledge, the first demonstration of environmental contamination by AMDV on diverse surfaces associated with mink farms with confirmed infection or with a high and well-defined risk of infection. The negative results obtained in the negative farms, together with the high percentage of positive results from the positive ones, demonstrate the feasibility of studying the environmental contamination by AMDV using qPCR. The positive results also show that environmental contamination encompasses a multiplicity of elements, both in direct and indirect contact with animals, which must be taken into account when assessing risks and designing cleaning and disinfection programs. The positive results in the negative farm at high risk of infection, together with its subsequent seroconversion, clearly illustrate this route of infection. Likewise, the negative results obtained in the newly repopulated negative farm after a “stamping-out” process, were subsequently validated by its health evolution, which suggests that environmental tests can be used to assess the impact of the

cleaning and disinfection processes during the eradication procedure.

The **second study** of this thesis was focused on describing the distribution of AMDV on different elements and surfaces of farms that had suffered a recent epizootic outbreak, also comparing two different methods of DNA extraction from collected environmental samples. One hundred fourteen environmental samples collected from seven farms were used for this. The samples were grouped into nine different categories according to the type of sample and degree of proximity to the animals. The categories were as follows: “Cages”, “Soil/Manure”, “Catching gloves”, “Slaughter box”, “Facilities”, “Effluents”, “Periphery”, “Street” and “Visitors”. The last two categories represent people and elements that can move between the rest of the categories and even to other farms. To assess which was the most appropriate DNA extraction method for these types of samples, two different methods were used in parallel, the first being specific for soil samples (method A) and the second being more general and suitable for a wide variety of samples (method B). Altogether, a total of 97 positive samples (85.1%) were obtained, with 75 out of 114 samples being positive by both methods, while four samples only showed positive results by method A and 18 samples only by method B. Thus, method A detected fewer positive samples than method B (69.3% and 81.6% respectively). Regarding the viral load detected in the samples, a greater contamination was obtained for the first categories (in direct contact with animals or closer to them) than in the last ones (further away from the animals), with the differences between them being significant in some cases according to the extraction method used. To assess the degree of agreement between both methods to classify all samples as positive or negative, a kappa value of 0.49 was obtained, which corresponds to a “moderate” level of agreement. Furthermore, the McNemar test found statistically significant differences between the classifications obtained by each method. Finally, taking into account the 75 positive samples by both extraction methods, the number of copies detected was significantly higher for method B than for method A. These discrepancies show the need to combine both extraction methods for this kind of samples such as it has been done in this study, thus allowing the best possible classification and quantification. However, as an alternative, a general extraction method such as protocol B on the complete set of samples could be used first, followed by a more specific method such as protocol A to re-

analyze those negative samples in the first round. Regarding the different categories, AMDV DNA was detected in samples from all of them, regardless of the DNA extraction method used. This shows a high spread of AMDV in the environment of positive farms. As expected, the mean number of copies of AMDV decreased with both protocols from the first to the last category as these categories move away from animals. Furthermore, 100% of the samples in the first two categories were positive, while the percentage of detection in the latter categories was lower, although also very high. These findings, together with the significant differences between the

viral loads recovered between the first and last categories, seem to indicate the existence of a gradient of contamination by AMDV that decreases from the elements/areas closest to the animals to the most distant points. In this way, this study provides unpublished and very useful knowledge on the distribution of AMDV in different elements and areas of infected farms under certain epidemiological conditions. This enriches the knowledge about the epidemiology by providing objective data in support or revision of what, until now, have been paradigms assumed on the base of interpretation of epidemiological data and studies done by experimental infection of susceptible animals.



The **third study** of this thesis dealt with the contamination of personal protective equipment (PPE) during visits to infected farms, as well as the degree of protection offered by two different types of PPE. This study was carried out in two parts, a preliminary approach in 2015 and a more complete trial in 2017 on two different farms of the same owner, endemically infected since 2012. Both trials utilized the protection of two types of PPE (certified against infectious

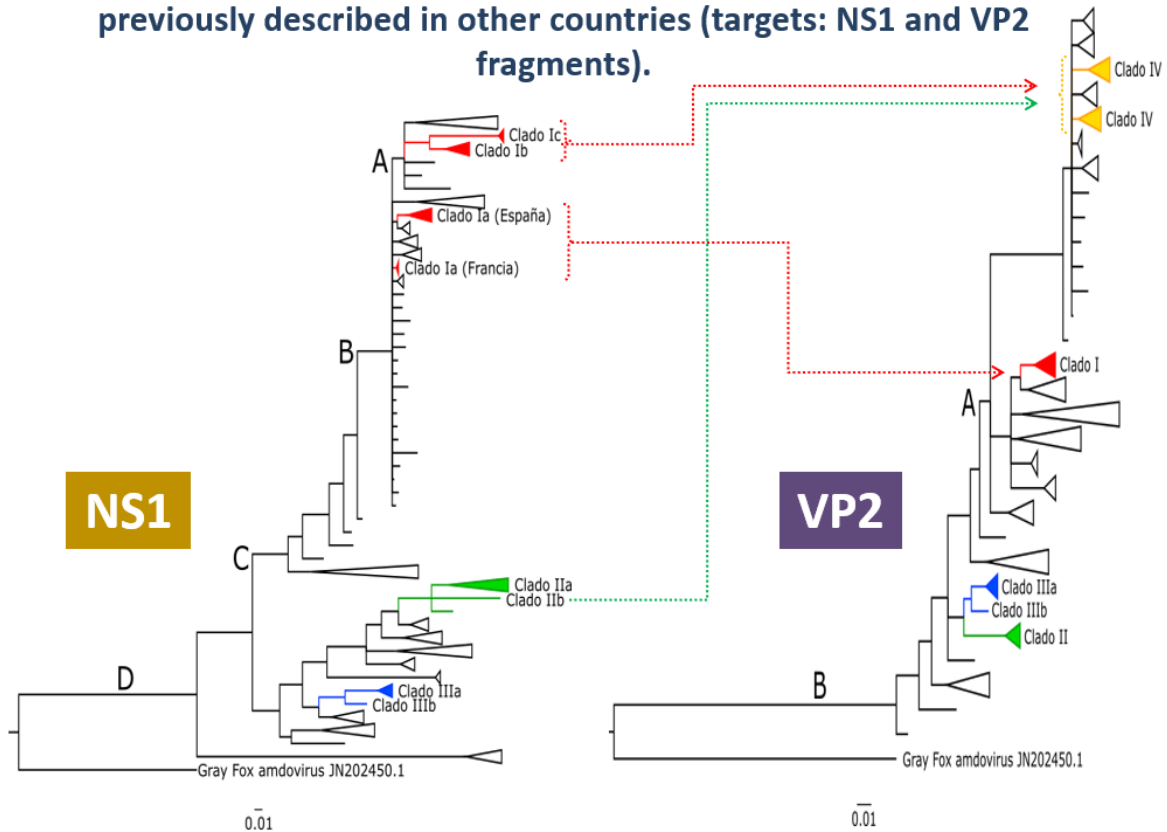
agents vs. non-certified), with different team members using different combinations of these PPE during visits to farms of 30-60 minutes in duration. The following procedure was used: samples were collected from the different PPE and also from various surfaces of the farms by the sampling method used in the previous studies and were subsequently analyzed by qPCR using the serial analysis scheme proposed in study two. In both parts of the study, all samples taken before the visit were negative. After the visit,

contamination was detected in a total of 9/10 samples (90%) in 2015 and 25/36 samples (69.4%) in 2017. In general, the external protections of the body and footwear were contaminated in all cases, but the degree of contamination of the elements covered by them was variable, and in both cases this was consistent with the environmental contamination detected on the farms. The items protected by non-certified external coveralls were contaminated in most cases, while in the case of certified coveralls there was only internal item contamination in one case, probably due to accidental contamination at the time of coverall removal. The hair of the visitors protected by the hood did not show contamination, whereas it did so in all the visitors who did not use it. Regarding the protection of footwear, the external bootcovers were contaminated in all cases. In the case of the internal protections (shoe covers) under the non-certified bootcovers (S), contamination also occurred in all cases, observing a concentration of it with higher values than on the corresponding external bootcovers or on the environment of the farm. On the other hand, for the visitors who wore certified bootcovers, contamination was only detected in the internal protection in one of the cases, coinciding with the only certified bootcover that was perforated during the visit. Samples taken from farm surfaces to confirm environmental contamination (the cages and the sheds' floor), were positive in all cases but were between one and two log units higher in 2015 (cages and shed floor, respectively). Regarding the area outside the farm where samples were taken after visits, contamination was only detected in the preliminary test in 2015. To the best of our knowledge, this is the first investigation that has used qPCR to evaluate the protection of different PPE against AMDV under field conditions. The quantification of the viral DNA in the PPE was consistent with the level of surface contamination detected in the farms studied. This suggests an exposure to the virus during visits proportional to the environmental load even in the absence of direct contact with the animals. The use of certified PPE equipment is thus justified by this study; likewise, the use of the coverall's hood is justified, as well as elements to protect the footwear which were resistant to breakage and perforation. In this case, it is preferable to use rubber boots or another reusable footwear that are easily disinfected. Finally, the probable contamination of the street clothes of one of the visitors from group C at the end of the visit suggests the need to establish precise protocols on how to remove PPE, and also points out the risks of visiting a negative

farm after visiting a positive one, even if the appropriate protective equipment has been used, and therefore it is also necessary to establish protocols in this regard.

In the **fourth and last study** of this thesis, the molecular characterization of the isolated AMDV strains from all the infected farms in Spain during the period 2012-2020, as well as from a farm in France and two in Portugal, were carried out by means of amplification and sequencing of two fragments of the virus genome (NS1 and VP2 genes). In addition, this was followed by a phylogenetic study trying to establish the origin and routes of infection of the new outbreaks that had emerged in that period. In total, seventeen mink farms were included (fourteen from Spain, two from northwestern Portugal and one from southwestern France). All these farms had been identified as infected through the CIEP testing that is carried out within the annual control program. Two types of samples were used to carry out the experimental part: spleens obtained from necropsies of CIEP positive animals and environmental samples from different elements of the farms which had contact with CIEP positive animals. Thus, 43 samples were selected for partial amplification of the NS1 and VP2 genes by conventional PCR and the subsequent sequencing of the obtained amplicons. With the resulting sequences, five phylogenetic trees were constructed, three of them only with the sequences of this study (NS1 and VP2 separately and concatenated) and two more including other sequences previously described in the GenBank database of the National Center for Biotechnology Information. All the trees were constructed using the Bayesian approach of the Markov-Monte Carlo chain method, using in each case the nucleotide substitution model chosen by the Akaike Information Criterion (AIC). All included samples were successfully amplified and sequenced for both genes. The NS1 sequences showed 83.2-100% identity to each other, while the VP2 sequences ranged from 91-100% identity. In those cases where samples from the same farm but from different years were analyzed, the sequences obtained were practically identical for both genes regardless of the sampling year, except for three farms in which different strains were obtained. This is, as far as we know, the first descriptive study of AMDV strains isolated from mink farms in Spain, Portugal and south-western France. For this, the work relies not only on phylogenetics, but also makes use of the knowledge acquired in three previous studies, the serological history of the farms and epidemiological data particular to each one.

**Trees constructed with the sequences from this study and those previously described in other countries (targets: NS1 and VP2 fragments).**



Based on the **NS1 phylogenetic tree**, the AMDV sequences obtained in this study were divided into three clades, representing an imperfect geographic grouping as well as regarding the infectivity and virulence inside each group. Clade I was subdivided into three subclades (a, b, and c), one comprising the strains from three farms near Santiago de Compostela and also the one from south-western France (Ia), and two comprising strains from farms in southwestern Galicia and north of Portugal (Ib and Ic). Clade II comprises three farms near Madrid and one in the southwest of Galicia, located in the geographical area of clades Ib and Ic. Clade III includes all the farms near A Coruña and one close to the farms in clade Ia. The **phylogenetic tree for VP2** were subdivided into four clades with a high degree of geographic clustering, but again more relaxed in terms of infectivity and virulence. In this new tree, Clade I only contains the sequences from three farms near Santiago de Compostela and the French sequences, while clade II only contains the three farms in the center of the country, and Clade III remains intact; however, a new clade (IV) appears grouping the sequences of all the farms in the southwest of Galicia and northwest of Portugal.

The **concatenated tree NS1 + VP2** is also divided into four clades forming groups identical to the phylogenetic tree VP2 with some differences in terminal branching. Regarding the **comparison with the global sequences** recovered from GenBank, the sequences of the NS1 gene belonging to clades II and III presented low homology and distant phylogenetic relationships with the previously described global sequences. Clade II is grouped with strains from Poland, Italy, Greece and Sweden, while in clade III the homology was almost identical to the four strains previously described from the farms of that clade, being the closest strains Swedish, Finnish and Chinese, in addition to the AMDV-K strain. Subclade Ia was the one with the highest degree of homology with regard to the global sequences and grouped together with Polish, Greek and Dutch strains. The subclades Ib and Ic formed a well-defined subgroup, with moderate homologies with strains from multiple countries, such as Greece, Poland, Denmark or the Netherlands. With respect to the VP2 gene, the number of global sequences available was lower than for the NS1 gene, reducing the resolution when comparing the Spanish strains with those from other countries. The se-

quences of clades II and III are again the most conserved, showing little relationship with the strains from other countries, but there is a certain relationship between both clades. Clade I, which corresponds to subclade Ia of the NS1 tree, was related to strains from Poland, Finland, Russia, Belarus and China. Finally, clade IV was mainly related to strains from Finland, Denmark, Russia, Belarus, Canada and the USA. In general, the phylogenetic analysis of the strains of this study showed a clear geographical grouping for the different clades, as has also been described by other authors. This grouping of strains is not surprising given that historically the relationships between farms have been mainly linked to each geographic group; furthermore, the high degree of epidemiological surveillance and the sanitary qualification system of Spanish farms since 2000 have largely prevented the movement of infected animals. The phylogenetic tree built with the NS1 gene fragment only allowed the differentiation of the main groups but did not show the same resolution as the VP2 tree and the concatenated NS1 + VP2 tree, where the clades better represent the geographical, historical and commercial circumstances of the farms. This higher resolution has been previously mentioned by other authors, pointing out that the analysis of longer fragments or even the entire genome allows for establishing phylogenetic relationships between different strains with higher resolution.

**Finally**, in this study the characteristics of the outbreak or endemic infection are discussed farm by farm, as well as the infectivity and inferred virulence for each strain. For many of the farms in this study, the phylogeny shows the origins and routes of infection with a much higher degree of resolution and objectivity than previously assumed solely based on epidemiological data. The phylogenetic study suggests that the origin of more than half of the outbreaks was in the reinfection from the reservoirs of the farm itself, while the exchange of farm material and the movement of people and animals was the probable origin of the other outbreaks. It should be noted that for one of these outbreaks, the indirect transmission from a shared worker with a positive farm was doubly proven by environmental detection and phylogeny. The most complex case, the farms from clade IV, presents some characteristics that open the door to speculate about the possible entry of the virus from the wild fauna of the area, which may possibly be clarified if sequences from the feral mink of that area become available. Finally, we also discuss the possi-

ble origin of some of the outbreaks in the international trade of farm material, which was very active in the 2012-2018 period.

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