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ORIGINAL ARTICLE

Age-dependent baseline values of faecal cortisol metabolites in the American mink (*Neovison vison*) under semi-natural housing conditions

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Summary

The welfare of an animal is ensured if it is able to fully satisfy its essential species-typical needs in all functional aspects of behaviour. In mink, stereotypies and apathy, internal and/or external injuries as well as increased susceptibility to disease have been known to occur as a result of chronic stress. The non-invasive method of analysing faecal cortisol metabolites (FCM) allows conclusions to be drawn about the stress level in the respective housing system. The objective of this study is to find out how the cortisol metabolites content in the faecal changes with increasing age of the mink under semi-natural housing conditions. Thus, 40 American mink (*Neovison vison*) were housed in two outdoor enclosures imitating natural conditions. Throughout the entire study (13th to 32nd week of life), faecal samples were collected to measure cortisol metabolites. No differences in FCM concentrations between the two outdoor enclosures were found. In the young mink lower, less fluctuating FCM levels were found than in older animals. After the first faecal collection in the 13th/14th week of life, the level of metabolites decreased slightly ($p = 0.032$; 17th/18th week). From the 22nd/23rd week onwards until the 30th/31st week, shortly before the animals were pelted, continuously increasing concentrations were then measured. Increasing FCM levels with advancing age of the animals are probably attributable to the onset of sexual maturity and/or the respective season. This has to be taken into account in future studies using this method for assessing welfare and when comparing different mink housing systems.

Keywords American mink, welfare, faecal cortisol metabolites

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Introduction

For a long time, there were no mandatory requirements for mink farming in Germany. Irrespective of the fact that in the wild, mink are semi-aquatic animals, they have hitherto been kept without any access to water and in an extremely confined space on farms. It is not least for this reason that mink farming had often been the subject of public protests in the past. However, since the 3rd Amendment of the Ordinance on the protection of animals kept for farming purposes (TierSchNutztV, 2006, with a transitional period of up to 10 years for existing farms), which includes fur animals and thus for the first time lays down specific requirements for the housing of mink in Germany. Mink farming has been in a state of transition. According to the ordinance from December 2016 onwards, the animals must, among other things, be provided with a water-filled swimming basin with a minimum surface of 1 m^2 and a water depth of 30 cm. Mink farms as are still in existence today, where the animals are kept exclusively in small standard-sized cages (L: 90 cm; W: 30 cm; H: 45 cm) with wire flooring, have been violating the existing law since December 2011. Since that time, 1 m^2 per mink and additionally a basal ground of at least 3 m^2 per housing system is mandatory by law.

The welfare of an animal is ensured if it is able to fully satisfy its essential species-typical needs in all functional aspects of behaviour. The farming of

animals can be considered as species-appropriate if it does not overtax the adaptability of the individuals. Overtaxed adaptability manifests itself in behavioural disorders, in chronic stress, in morphological damage and in chronic somatic dysfunctions (Stauffacher, 1992). In mink, stereotypies and apathy, internal and/or external injuries as well as increased susceptibility to disease have been known to occur as a result of stress (Wiepkema and Koolhaas, 1995). Environmental stimuli that lead to an imbalance of homoeostasis are called 'stressors', and the corresponding defence reactions of organisms are called 'stress response' (Dehnhard et al., 2001). To better assess the stress levels of farmed mink, haematological parameters as well as clinical chemistry can be used as indicators (Daamgard and Hansen, 1996). As cortisol, as one of the main 'stress hormones', is an indicator of the hypothalamic-pituitary-adrenal (HPA) axis in mink (Mormède et al., 2007), the measurement of cortisol levels is of major importance (Clausen et al., 1999). In animals, cortisol can be measured in blood and also its metabolites in faeces; however, taking blood samples from mink is impossible without catching or fixating the animals in traps first (Möstl and Palme, 2002). This procedure would place additional stress on the animals, and as a consequence, the results may be compromised (Hansen et al., 2007). Alternatively, faecal cortisol metabolites (FCM) are used to evaluate stress responses in the respective housing system (Möstl and Palme, 2002; Palme, 2012). This type of sample collection can be performed easily and without any stress for the animals.

What must be taken into account when measuring the cortisol metabolites in the faeces is that the status of the blood cortisol levels only becomes detectable in the faeces with some delay. This delay time depends mainly on the time of passage from the duodenum to the rectum and is therefore species-specific (Palme et al., 1996, 2005). In one study, the highest levels of cortisol metabolites in female mink were reached in the faeces after 4.2 h (Malmkvist et al., 2011). Whereas the measurement of cortisol in the blood reflects the current steroid concentration, the cortisol metabolites indicate the production rate, which includes the cumulative secretion and elimination of the hormones over a period of several hours (Palme et al., 2005). Compared to blood samples, faecal samples are therefore less influenced by episodic fluctuations or the pulsatile (circadian) hormone release and as a consequence provide more accurate information on the hormonal status of the animals than one individual blood sample (Touma and Palme, 2005; Palme, 2012).

To date, reference values for cortisol metabolites in the faeces of mink under semi-natural housing conditions are lacking. The studies published so far were conducted on commercial mink farms, where the animals were usually housed individually in wire cages [L: 90 cm; W: 30 cm; H: 45 cm (example given Hansen et al., 2007; Svendsen et al., 2007; Malmkvist and Palme, 2008)] or under special test conditions during which the mink were administered radioactively labelled cortisol (Malmkvist et al., 2011).

The objective of this study was to determine baseline FCM values in semi-naturally housed mink and to test whether age of the animals exerts an influence. Within the scope of continuation studies, it is then intended to compare these with the values pertaining to other housing systems, which may for instance comply with the provisions of the applicable ordinance on the protection of animals kept for farming purposes (TierSchNutztV, 2006).

Animals, materials and methods

A total of 40 American mink (*Neovison vison*) were housed in two outdoor enclosures (each approximately 300 m^2) imitating natural conditions. In each of the two identical enclosures, the mink were offered three different water basins, which differed in shape, depth and surface area. There were a rectangular 'swimming pool' (surface area about 20.5 m^2, depth 30 cm), a round 'pond' (surface area 4.9 m^2, depth 80 cm) and a running 'creek' (lengh about 10 m, depth 3–4 cm, which contained two pools/hollows along its length) available. The 'creek' established a connection between the 'swimming pool' and the 'pond'. All three water basins were linked by a pump, which allowed the running of the 'creek'. The enclosures were equipped with roofed nest boxes (35 × 35 × 30 cm, 20 nest boxes per enclosure with straw bedding), and the ground floor was covered with bark mulch. Both groups were put together randomly (group 1: 9 males, 11 females; group 2: 10 males, 10 females) and fed once a day at around 7.30 a.m. The animals were purchased at 9 weeks of age from a commercial mink farm after they were weaned from their mother, and moved to the outdoor enclosures in their 13th week of life. Throughout the entire study procedure, from placement inside the trial enclosure in the 13th week of life until the animals were pelted in their 32nd week of life, faecal samples were collected between 10 and 11 a.m. to measure cortisol metabolites, and health assessments including weighing of the animals as well as behavioural observations and microbiological examinations of water

were performed. For the faecal samples, 10 fresh piles of faeces were collected per day and per outdoor enclosure at five time points (collection time point 1: 13th/14th week; 2: 17th/18th week; 3: 22nd/23rd week; 4: 26th/27th week; 5: 30th/31st week) on 10–19 consecutive days and immediately frozen at −20 °C. Individual linking of the faecal samples to a certain animal or sex was not possible with this experimental set-up. Faecal samples were thawed, weighed and extracted with methanol (80 %) as described before (Malmkvist et al., 2011). Extracts were sent on dry ice to the Department of Biomedical Sciences (University of Veterinary Medicine, Vienna) where FCM were analysed by a group-specific 11ß-hydroxyaetiocholanolone enzyme immunoassay (EIA). Details of the EIA and its validation for mink faecal samples are given by Malmkvist et al. (2011). Except for the faecal samples, all other results will be presented elsewhere.

Statistical evaluation was performed using the programs SPSS Statistics 20 (IBM SPSS Statistics 20.Ink, IBM Deutschland GmbH, Ehningen, Germany) and Sigma Plot 11.0 (SigmaPlot 11.0.Ink, Systat Software GmbH, Erkrath, Germany). The biologically relevant difference was defined as 0.5 times the standard deviation (effect size 0.5). Differences with an error probability of $p < 0.05$ were considered to be significant. To compare the differences in mean values, the one-way ANOVA combined with Tamhane's *post hoc* test was used. Equivalence testing according to Schneider (1998) was employed to establish the equivalence of the two groups.

Results

Enclosure and group composition had no influence on the FCM levels of the mink (see Table 1). The equivalence of the mean values of the two groups was demonstrated by means of an equivalence test, which is why the groups were combined for the statistical evaluation.

In the young mink (13–18th week of life), cortisol metabolites were lower than in older animals and did not exhibit the same strong fluctuations. After the first faecal collection in the 13th/14th week of life, levels of metabolites decreased ($p = 0.032$) slightly (17th/18th week of life). From the 22nd/23rd week of life onwards until the 30th/31st week of life, shortly before the animals were pelted, continuously increasing concentrations were measured (see Fig. 1 and Table 1).

A large variation of FCM concentrations of the samples collected at the different time points was found. For instance, the lowest level of 1.7 ng/g was measured in the 4th faecal collection period (26th/27th week of life), while the highest was determined at 1150 ng/g in the 30th/31st week of life (see Table 1).

The mean faecal cortisol metabolite levels at the first and the second faecal collection time point were significantly lower than those at the other three collection time points (see Fig. 1).

Table 1 Overview of FCM levels (ng/g faeces; median, min, max) in mink subdivided into groups 1 and 2 as well as according to the age of the animals at the time of sampling (*n* = number of samples analysed)

Week of life	Group	*n*	Median	Min	Max
13th/14th	1	190	45	2.3	737
	2	190	50	4.6	462
	1 + 2	380	47	2.3	737
17th/18th	1	110	33	3.2	187
	2	110	46	3.3	240
	1 + 2	220	39	3.2	240
22nd/23rd	1	100	74	5.9	712
	2	100	87	16.5	456
	1 + 2	200	81	5.9	712
26th/27th	1	110	82	14.5	975
	2	110	78	1.7	744
	1 + 2	220	80	1.7	975
30th/31st	1	123	94	2.4	1150
	2	125	127	21.1	1061
	1 + 2	248	115	2.4	1150

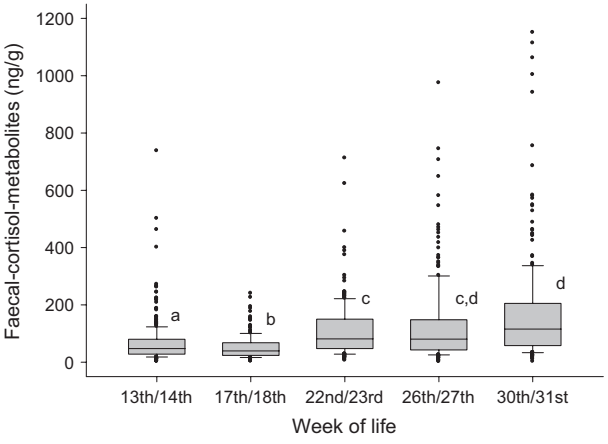

Fig. 1 Concentrations (ng/g) of faecal cortisol metabolites (FCM) in minks of both groups (together) at different weeks of life. Per collection time point, samples were collected on 10–19 consecutive days (10 samples/group each day; total *n* = 1267). Time points with different superscripts differed statistically significant (a to b: $p < 0.05$; all other combinations $p < 0.001$).

Discussion

As there are significant differences regarding metabolism and excretion of glucocorticoid metabolites, careful assessment is essential with every species and each sex (Touma and Palme, 2005). A study by Malmkvist et al. (2011) during which female American mink were injected with radioactively labelled cortisol found—based on the subsequent testing of urine and faecal samples of those animals—that in the female mink, cortisol is mainly excreted via the faeces (83%). The available literature regarding FCM concentrations (e.g. Hansen et al., 2007; Svendsen et al., 2007; Meagher et al., 2012) mostly deals with female mink, which is why there is only few data on male mink (e.g. Meagher et al., 2013). The trial groups tested in our study contained both male and female animals. However, since due to the study design it was not possible to link the faecal samples individually to an animal and sex, no definitive statement can be made if FCM concentrations are influenced by sex.

In the present study FCm Levels of mink in their 13–18th week of life were lower than those from week 22 onwards. After the first faecal collection in the 13th/14th week of life, the level of metabolites decreased slightly in the 17th/18th week of life. One reason for that may be that transfer to the outdoor enclosures and the new composition of the mink groups in the 13th week of life were associated with some stress for the animals. The subsequent decrease in the levels indicates that the animals were getting accustomed to their fellow animals and the housing system. The increase in FCM levels from week 22 onwards may be attributed to various causes. For one thing, the increasing age or the onset of sexual maturity of the animals at the collection time point in the 31st week of life, shortly before pelting, along with the frequently associated conflicts, may play a role. In addition, towards the end of the study, an increasing number of minor and severe tail injuries were found in some animals, which supports this assumption. No other injuries except few animals with minor skin lesions and one mink with a seroma at the chest could be found during the biweekly conducted health check of each individual mink. None of the observed minks during direct and video observation showed stereotypic behaviour (data not shown). Besides the mentioned injuries, season may be a decisive factor as well. The young mink were transferred to the outdoor enclosures at the age of 13 weeks in the summer month of August and pelted in December at the age of approximately 32 weeks. For instance, Huber et al. (2003) discovered in their study on red deer that FCM concentrations vary depending on the season with higher values during winter. Weingrill et al. (2004) also explained most of the variance of FCM levels with environmental factors. In their study with baboons, FCM measures were strongly correlated with seasonal differences such as daylight duration, temperature and the amount of time that baboons were resting. They measured higher cortisol levels during winter months and suggested that this could be related to shorter resting periods and to the cold minimum ambient temperatures at their study site. In addition, Corlatti et al. (2011) also described a clear seasonal pattern of glucocorticoid metabolites secretion in red deer (*Cervus elaphus*). They found higher levels in winter and lower levels in summer. In the course of the conducted study from August till the end of November, we also had seasonal influence on the FCM levels; therefore, this could also be a reason for the increase in the FCM concentrations towards the end of the study.

Apart from the season, faecal FCM concentrations also depend on the social composition of the group. Goymann et al. (2003) examined the faeces of hyenas and found out that the FCM concentration depends on the pack size: The animals that lived in larger packs and were consequently exposed to greater social stress exhibited higher FCM concentrations in the faeces than animals living in smaller groups. Since within the scope of the study 20 mink were kept in each outdoor enclosure, the group size, in addition to the increasing age of the animals, may be a contributing factor in the increase in FCM levels. Besides, a recent study in spiny mice (Fraňková et al., 2012) reported that differences in social settings among groups were an important source of FCM variations. Although the base area of one enclosure with 300 m^2 seems quite big compared to standard housing conditions of caged minks with average cage sizes of 90 × 30 × 45 cm (L × W × H)—in contrast to their natural habitat with territories ranging up to 4 km^2 (Wiepkema and de Jonge, 1997), the enclosure might still not be big enough to allow the minks to live out their natural behaviour, which could result in stress and end up in higher FCM concentrations. Weingrill et al. (2004) found in their study with 10 female chacma baboons, which lived at a nature reserve in South Africa, in a pack size of 40 to 50 individuals, no relationship between social rank and the rate of agonistic interactions with baseline FCM levels.

All authors of the mentioned literature in Table 2 worked with adult minks (at least 10 month of age and older); therefore, it is difficult to compare FCM concentrations of subadult minks used in this study to

Table 2 Faecal cortisol metabolites concentrations of mink in available literature

Author	FCM	Method	Sex	Housing conditions (L × W × H) in cm	Remarks to housing condition
Svendsen et al. (2007)*	71 ± 13.1 ng/g†	11β-Hydroxyaetiocholanolone-EIA	F	90 × 30 × 45	LSL
	150 ± 23.4 ng/g†				HSL
Malmkvist and Palme (2008)*	82 ± 13.0 ng/g†	11β-Hydroxyaetiocholanolone-EIA	F	90 × 30 × 45	NON
	58 ± 7.1 ng/g†				ART
	63 ± 7.2 ng/g†				STR
	54 ± 8.1 ng/g†				ART + STR
Malmkvist et al. (2011)	54 ± 9.1 ng/g†	11β-Hydroxyaetiocholanolone-EIA	F	91 × 3 × 45	LSL (before handling)
	71 ± 27.9 ng/g†				HSL (before handling)
	90 ± 19.3 ng/g†				LSL (4–20 h after capture)
	778 ± 364.7 ng/g†				HSL (4–20 h after capture)
Meagher et al. (2012)	69.0 (56.8–83.3)‡	11β-Hydroxyaetiocholanolone-EIA	F	Cage sizes varied between farms	Inactive females
	93.4 (76.6–113.8)‡			Minimum 61 × 19 × 46	Active females
Meagher et al. (2013)	234 ng/g§	11β-Hydroxyaetiocholanolone-EIA	F and M	75 × 60 × 45	
	160 ng/g§			75 × 60 × 45 + access to 2nd cage, 120 cm wide	

LSL, low stereotyping line; HSL, high stereotyping line; NON, restricted possibility of nest building; ART, restricted possibility of nest building, but an artificial plastic nest placed in the nest box; STR, full possibility of nest building using straw; ART + STR, full possibility of nest building using straw and an artificial plastic nest placed in the nest box; F, Female; M, Male.

*Because of the comparability data converted from original nmol/kg in ng/g.

†Mean ± SE.

‡Means are back transformed last square means, with 95% confidence intervals.

§Last square means.

adult minks under commercial housing conditions on the farms. Nevertheless, it could be shown that husbandry system as well as enrichment can have an influence on the FCM concentrations.

Hansen et al. (2007) for example showed that adult female mink kept in traditional wire cages had significantly higher concentrations of faecal corticoid metabolites than those kept in enriched wire cages ($p < 0.001$) and performed less stereotypies during the winter. Meagher et al. (2013) came to the same result that minks with no access to a second cage had higher FCM levels than minks with access to it.

Last but not least, also the diet an animal consumes can have an effect on the cortisol and metabolite levels excreted in faeces (Dantzer et al., 2011).

Because adequate protein supply (especially with sulphur-containing amino acids methionine and cystine) is required for the formation of the minks winter coat, a change in diet took place in our study, starting in October, which may also play a role in increasing the concentration of FCM.

The great variability of the concentrations measured (min.: 1.7 ng/g in the 26th/27th week; max.: 1150 ng/g in the 30th/31st week) illustrates the differences between the individual mink within the same housing system. As mentioned earlier, faecal samples could not be linked to the individual animals. However, as there were always 10 randomly collected fresh piles of faeces per outdoor enclosure, one can assume that the samples were mostly from different mink. Nevertheless, the possibility that by chance an increased number of faecal samples of highly stressed animals was collected cannot be excluded, which could also be an explanation for the increase in concentrations with advancing age.

At present, researchers around the world implement various techniques; results between studies are only comparable in their physiological outcome, but usually not in absolute metabolites concentrations (Möstl et al., 2005; Schwarzenberger, 2007).

As outlined previously, the adrenocortical activity may be influenced by many different factors; thus, all these aspects have to be taken into consideration when assessing the results (Touma and Palme, 2005).

The fact that compared to the 2nd faecal collection time point, the concentrations measured at the 1st faecal collection time point were higher suggests that in the mink, too, faecal cortisol metabolites indicate a higher stress level as the increase is probably due to the stress of settling in. To validate these results further, studies are required during which the animals are deliberately exposed to stressors around 4 h before faecal collection time points. This may reveal a direct

relationship between cortisol metabolites and the stress status.

For assessing the stress on the animals associated with the housing system, this non-invasive method of analysis by means of faeces holds a clear advantage over blood sampling as the animals are spared the stress of being trapped and anaesthetized for the purpose of blood sampling in accordance with animal welfare. This allows more meaningful conclusions to be drawn about the welfare of the animals within the respective housing systems.

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