Part 4. Behaviour and welfare

Stress influencing production and welfare in farmed mink

J. Malmkvist
Aarhus University, Department of Animal Science, Research Centre Foulum, Blichers Allé 20, 8830 Tjele, Denmark; jens.malmkvist@agrsci.dk

Abstract

Results linking stress, production and welfare in farmed mink, with special focus on the hypothalamic-pituitary-adrenal (HPA)-axis, are presented. Interpretation of stress responses are discussed, combining context, neuroendocrine and behavioural indicators. Results from the development and validation of a novel non-invasive sampling of faecal cortisol metabolites (FCM) are presented. Both genes and environment affect the state of stress in farmed mink, and an overview of the major experimental findings in mink are given. This includes results from (1) selection for and against fearfulness with effects on the HPA-axis and the serotonin system, (2) studies of parturition/early kit mortality, and (3) cage enrichment studies affecting stress responses. Finally, recent results exploring the link between stress and abnormal behaviour are presented, investigating the HPA-axis response of stereotypic animals and whether fur-chewing/stereotypic behaviour is associated with hippocampal neurogenesis in mink. Perspectives and suggested future research aiming to improve production and welfare in farmed mink are given.

Keywords: behaviour, hypothalamic-pituitary-adrenal (HPA)-axis, *Neovison vison, Mustela vison*, stress.

Introduction

The autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenocortical (HPA) axis are activated when animals anticipate or are faced with stressors. Measuring HPA activity is a standard approach to the study of stress and welfare in farm animals, usually integrated with data from behavioural observation, production, and pathology records (Morméde *et al.*, 2007). In addition to be involved in the stress response, hormones from the HPA-axis play a role in normal body functions, illustrated by increased concentration in circulation before waking-up in the late stages of sleep; a situation not usually considered as stress.

The main hormone of HPA-axis in mink is cortisol secreted from the adrenal cortex, under control of the pituitary hormone ACTH (adrenocorticotropic hormone) from the anterior pituitary gland. Cortisol exerts a negative feedback on the axis at several levels, including within the brain. The feed-back ensures that plasma cortisol usually decline after the acute response. Chronic stress, certain diseases, and aging impair this feed-back action in a range of mammals (Sapolsky, 2002). In general, factors like predictability, control, and possibility of behavioural outlet have been found to modify the HPA-axis response in a range of animals when faced with stressors (e.g. classical papers by Weiss, 1968, 1971); these factors modulate the animals' perception of stress. Also the behavioural reactivity of the animals, shaped by the interplay between genes and environment, affects the HPA-axis response. Stress can arise both as a consequence of adverse external stimulation and as a consequence of internal causal factors driving the animal to attempt

to carry out species-specific behaviour, without having the right possibility or sufficient feedback (Jensen and Toates, 1997).

Detrimental effects of prolonged stress responses

Although responses towards acute stressors are adaptive in nature, prolonged or repeatedly exposure to stress and stress hormones can have negative consequences, due to e.g. mobilization of energy at the cost of reserves, suppression of digestion, growth, reproduction, immunity and the inflammatory response (reviewed in Sapolsky, 2002). Additionally, high concentrations of glucocorticoids impair both memory and learning skills as shown in several mammalian species, and the link between stress and these functional deficits is believed to relate to changes in the hippocampal formation (Lupien *et al.*, 1998; McEwen and Sapolsky, 1995). One major finding across mammals is that stress or stress hormones can reduce the neuron number (Sapolsky *et al.*, 1985; McEwen, 1999; Jayatissa *et al.*, 2008) and decrease neurogenesis which occurs in the dentate gyrus of the hippocampal formation (Gould *et al.*, 1997; Sapolsky, 2004; Morris, 2007). Additionally, the hippocampus is involved as the central part of the feed-back control of the HPA-axis, with a high concentration of receptors for the corticoid hormones (McEwen, 1999). The negative effect on the hippocampus is one of the destructive effects of chronic stress.

Use of HPA-axis function to evaluate stress

Care should be taken when comparing animals of different genotypes and across studies in an attempt to evaluate consequences of e.g. different management and housing systems. However, within studies HPA-axis function has successfully been used as an indicator of stress, when sampled carefully. Baseline levels of glucocorticoid hormones have been sampled in blood, urine, and faeces in mink (an overview in Table 1). In addition, enlargement of adrenals – weight relative to individual body or brain size – has been taken as indicative of prolonged stress in some studies. Challenge tests are occasionally included to further evaluate the HPA-axis function, by following the change in cortisol concentration after standardised handling/immobilisation or after stimulation by ACTH injection. These HPA-challenges are used in order to detect effects – after chronic or repeated exposure to stressors – which potentially may change the set-point of the HPA-axis, making the animal less sensitive to secrete cortisol. There is no simple correlation between any single physiological/behavioural indicator and perceived stress. Farmed mink are seasonal breeders, with the photoperiod as the main regulator of reproduction, fur moulting, and hormonal rhythms. Therefore seasonality in the secretion of glucocortioids is observed, and the concentration may differ between sexes (summarized in Mormede *et al.*, 2007; also during gestation/around parturition in females: Malmkvist and Palme, 2008). Besides the influence of natural photoperiods on the hormonal levels, the normal farm routine with changing feeding strategies during the reproductive cycle may contribute to the variation. Feed restriction may for example increase both the baseline cortisol and the activity of mink (Bildsøe *et al.*, 1991).

Sampling of HPA-axis responses

The majority of blood cortisol reports in mink (Table 1) are on females, with the mean concentration from 25 to 95 nmol/L in plasma. These values cover a range of sampling procedures, such as nail

Table 1. HPA-function used to evaluate stress in relation to production and welfare in mink, examples from the literature. The listed indicators are typically linked to behavioural observations/other physiological measures. The table is not exhaustive.

Indicator	Focus variable/treatment	Reference
Weight of adrenals	Cage environment	Hansen 1988
	Housing system	Hanninen *et al.,* 2008a;b
	Fuel-oil feed contamination	Mohr *et al.,* 2008, 2010
Size of adrenals	Stereotyping mink	Mason 1992
Morphology of adrenals	Fuel-oil feed contamination	Mohr *et al.,* 2008
Cortisol in plasma	Infection, immobilisation	Jeppesen 1988
	Feed restriction, immobilisation in high and low stereotyping females	Bildsøe *et al.,* 1991
	Cage environment, immobilisation	Hansen and Damgaard 1991a
	Social environment	Hansen and Damgaard 1991b
	Early handling	Hansen 1993
	Selection for tame or fearful behaviour	Damgaard and Hansen 1996; Malmkvist and Hansen 2001
	Selection for inactivity or stereotypic activity	Zanella *et al.,* 1998
	Cage and social environment in two colour types	Pedersen and Jeppesen 2001
	Time of moving bitches from litter	Sørensen *et al.,* 2001
	Serotonin receptor agonist to mink selected tame or fearful behaviour	Malmkvist *et al.,* 2003
	Stereotypies and access to running wheel	Hansen and Damgaard 2009
Cortisol in serum	Pesticides in feed	Beard and Rawlings 1998
	Housing system, ACTH challenge	Hanninen *et al.,* 2008a;b
	Fuel-oil feed contamination, ACTH challenge	Mohr *et al.,* 2008, 2010
Cortisol in urine	PCB in food to pregnant females	Madej *et al.,* 1992
	Temperature, water availability in lactating mink	Tauson 1998
	Deprivation of food and resources in cage environment	Mason *et al.,* 2001
Cortisol in faeces	Offspring from high and low stereotyping parents	Tauchi *et al.,* 1999
Cortisol metabolites in faeces (FCM)	ACTH challenge, handling	Malmkvist *et al.,* 2011
	Cage size and environment	Hansen *et al.,* 2007
	Low and high stereotyping breeding lines	Svendsen *et al.,* 2007
	Low and high stereotypic mink	Malmkvist *et al.,* 2011
	Nest building possibilities, parturition	Malmkvist and Palme 2008
	Type of nesting materials, gestation-parturition	Lund and Malmkvist 2009
	Fuel-oil contamination	Mohr *et al.,* 2010
	Mink with/without abnormal behaviour	Malmkvist *et al.,* 2012
Cortisol binding protein -transcortin	Behavioural selection	Gulevich *et al.,* 2000
ACTH in plasma	Serotonin receptor agonist to mink selected for tame or fearful behaviour	Malmkvist *et al.,* 2003

clipping (Hansen and Damgaard, 1991a; Pedersen and Jeppesen, 2001), vein puncture (Malmkvist *et al.*, 2003) and catherization (Børsting and Damgaard, 1992) across seasons in different breeds. A duration of 2-2.5 min from the first capture until sample obtained is generally regarded as the upper time limit for detecting baseline values. Otherwise, the baseline concentration of cortisol hormones in the blood is not easily obtained as the capture of one animal may arouse and activate the secretion in other not yet sampled animals housed nearby (Pedersen, 1996), and the handling and blood sampling itself act as a stressor eliciting secretion of corticosteroid hormones into circulation. Therefore, examination of HPA-axis hormones in other biological excreta, such as urine and faeces sampled non-invasively, has been introduced. Sampling for saliva cortisol has to my knowledge not been used in mink. Repeated blood sampling by insertion of permanent catheters is possible (Bonneford *et al.*, 1988; Børsting and Damgaard, 1992), but this method is not stress-free (involves surgery and some movement restrictions) and infeasible when larger groups are to be sampled.

In particular, the measurement of faecal 11,17-dioxoandrostanes, a group of cortisol metabolites, has been proven useful to evaluate adrenocortical activity. In mink, these cortisol metabolites in faeces increase after injection of adrenocorticotropic hormone (ACTH) (Malmkvist *et al.*, 2004). Other studies have evaluated HPA-axis responses in mink based on non-invasive measurement of urine cortisol (e.g. Bildsøe *et al.*, 1991; Madej *et al.*, 1992; Mason *et al.*, 2001).

Mammalian species differ in the main excretory route of glucocorticoids; e.g. being predominately faeces for cats, and urine for dogs. The excretory route has only recently been studied in mink (Malmkvist *et al.*, 2011). Following an injection of radioactive cortisol, faeces were found to be the predominating excretory route in mink, with the ratio of cortisol in faeces:urine being about 5:1. The time lag until maximum concentration of cortisol was 3-7 hours in faeces and 2-5 hours in urine after a single dose injection (Figure 1).

Both faeces and urine integrate circulating cortisol levels over time, but are several steps away from the active hormone in the blood, which may interfere with validity/reliability. The cortisol to creatine ratio can be used to control for the amount of urine voided. Fresh faeces samples are more easily obtained than urine in the farm situation; besides faeces is the predominant excretion route for cortisol in mink. The concentration of cortisol itself is very low compared to that for cortisol metabolites (FCM), which are more detectable (Malmkvist *et al.*, 2011). FCM has mostly been used when mink are housed singly, and to my knowledge the effect of pooling faeces within/between animals has not been studied in detail. The calculation of concentration per unit wet weight versus dry weight of collected sample should be investigated in order to improve reliability. The interaction with steroid hormones (e.g. the sex hormone testosterone, sharing the precursor 17-a-hydroxyprogesterone with cortisol) on FCM should be studied to increase validity. Diurnal as well as seasonal effects should be taken into account when the sampling procedure is prepared. Knowledge of these and other sampling effects is to some extent described in the available literature using FCM. Adrenal evaluation may be an indicator of prolonged/severe stress, but is an invasive post mortem measure. This indicator is suspected to be insensitive to transient, milder types of stress. At pelting adrenal weight could with some effort be used, in case of post-mortem examination. The size of adrenals is dependent on sex/size of the animal, so the relative weight to e.g. body and brain size may be considered.

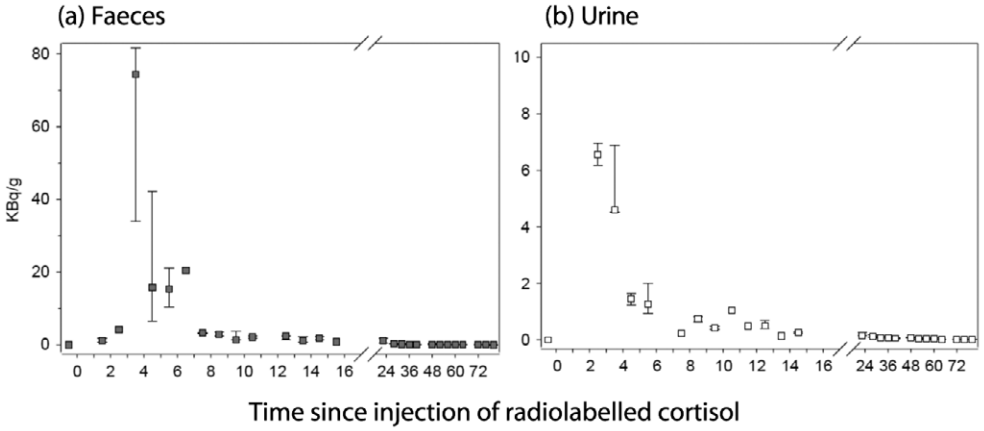

Figure 1. Time course of excretion of radioactive metabolites in (a) faeces (kBq/g) and (b) urine (kBq/ml) in mink females (n=8) after injection of ^{3}H-cortisol at 0 h. Data are given as hourly median with 25 and 75% quartiles for the first 16 h and per every 4th hour for the rest of the period. Note the different scaling of the y-axis for radioactivity, as faeces was the major route of excretion (83% vs. urine 17%). The proportion of injected radioactivity recovered was 84%. Modified from Malmkvist et al. *(2011).*

Stress in the production of mink

In this section, I have selected examples from studies into both genetic and environmental factors modulating stress responses, being of relevance for mink welfare and production.

Selection for behaviour

Selection for behaviour can influence the HPA-function in mink. Long-term selection based on reaction towards humans in the stick test have created lines differing markedly and consistently in their fearfulness (Malmkvist *et al.,* 2002) and in the HPA-axis response (e.g. secretion of cortisol) (Malmkvist and Hansen, 2001; Malmkvist *et al.*, 2003). Administration of an anxiolytic drug (Buspirone, a serotonin receptor agonist), neutralized the HPA-axis differences between fearful and explorative mink, illustrating the regulating role of the brain serotonin (5-HT) system (Figure 2). Consequently, the possibility to render mink more explorative, by increasing the relative amount of the essential serotonin precursor – the amino acid tryptophan – in mink feed has been investigated (Malmkvist *et al.*, 2011); the rationale was to reduce stress responses e.g. during periods of inevitable handling such as evaluation of fur and transport of mink. Following the selection for more tame/confident mink, other production relevant difference emerged, such as an increased mating willingness/earlier onset of oestrus (Klotchkov and Trapezov, 1991; Malmkvist *et al.,* 1997), and signs of quicker ontogeny (postnatal kit development) including an earlier onset of drinking (Malmkvist *et al.*, 2007a). In another behavioural selection experiment, mink females from a highly stereotypic breeding line had a higher cortisol baseline (Svendsen *et al.*, 2007).

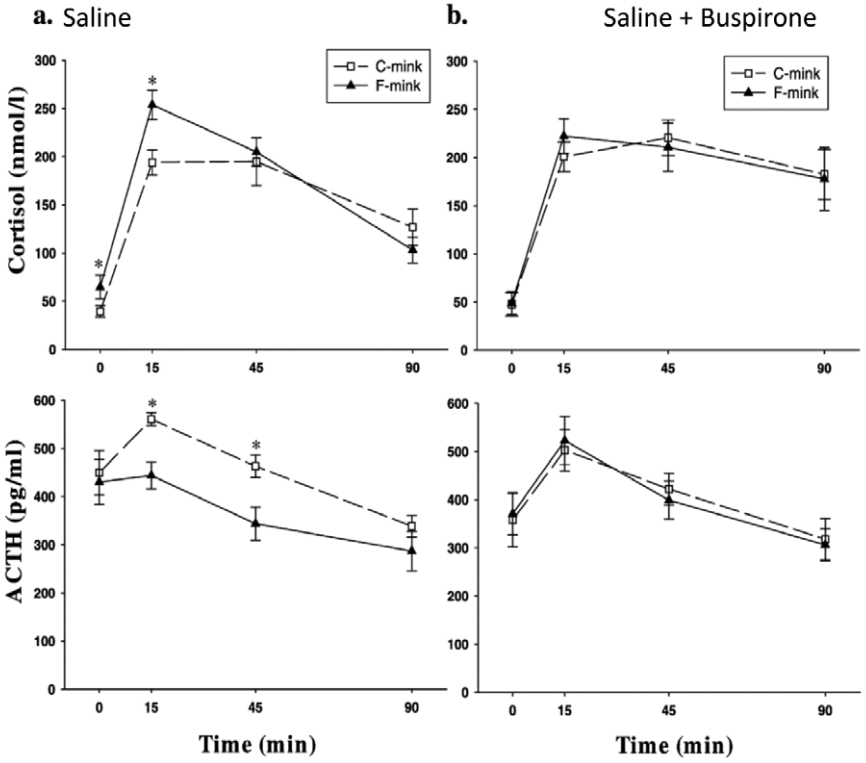

*Figure 2. Cortisol and ACTH concentration (mean±SE) in plasma vs. time after capture, in confident (C mink) and fearful mink (F mink) treated with (a) saline or (b) the anxiolytic serotonin (5-HT$_{1A}$) receptor agonist, Buspirone (1.25 mg/kg/day), for 28-29 days, administered continuously via osmotic minipumps placed s.c. in the neck. *Significant difference between the genetic lines. From Malmkvist* et al. *(2003).*

Cage enrichment

Changes in the cage environment can influence the stress responses markedly. Including tube platforms and chewing-ropes in cages resulted in significantly reduced abnormal behaviour (tail-chewing, stereotypies) and the baseline cortisol level (FCM). The mean concentration of FCM was approximately 25% lower in females kept in enriched compared to barren cages during the winter season. Thus, the cage additions reduced HPA-axis output significantly (P<0.01). In contrast, there was no effect whether females were housed in single or double cages (P=0.53) and no interaction between cage type (barren, enriched) and cage size (one, double) (P=0.28) (Hansen *et al.*, 2007). Hansen *et al.* (2007) concluded that increased cage complexity in form of occupational materials improve the welfare of the mink, whereas doubling the cage size without additional enrichment had little or no effect in relation to mink welfare.

Additionally, a nest box is important for the welfare of farmed mink as documented in studies of demand elasticity (longer stays more valued; Hansen and Jensen, 2001), HPA-axis responses (50%

higher plasma cortisol in mink without access to a nest box; Hansen and Damgaard, 1991), and behaviour (more stereotypic behaviour in mink without access to a nest box; Hansen *et al.*, 1994).

Parturition, maternal behaviour and early kit survival

As illustrated above, fulfilment of behavioural needs by adding relevant resources to the cage environment can reduce stress in mink. However, their stress responses can also vary with age and reproductive state; one example being during the time of parturition. Birth problems and prolonged parturition characterized mink females losing a high proportion of their litter (Malmkvist *et al.*, 2007b). Stressors may influence the labour controlling hormone oxytocin and prolong the delivery in other polytocous species, such as rats (*R. norvegicus*) and pigs (*S. scrofa*). For example, moving to a new environment during mid-parturition prolonged the birth process in rats (Leng *et al.*, 1987, 1988). Similarly, moving of sows to a farrowing crate during mid-parturition resulted in depressed oxytocin release through opioid inhibition and prolonged the birth process (Lawrence *et al.*, 1992). Stress can arise both as a consequence of adverse external stimulation and as a consequence of internal causal factors driving the animal to attempt to carry out species-specific behaviour, such as nest building, in case of no or insufficient feedback (Jensen and Toates, 1997). In one study, we compared different possibilities for female mink to perform nest building behaviour prior to and during the delivery season. Access to straw significantly reduced the variation in inter-birth intervals by 31-40% (P=0.044, during the delivery of in average 8.7-9.3 kits), indicating that access to straw for nest building is beneficial for the progress of parturition (Malmkvist and Palme, 2008).

Hypothermia is involved in early kit mortality, and may be the reason of the higher mortality and less growth in neonates raised by females with access to wood shavings only for nesting material. Thus, in spite of its worldwide use at mink farms, this nesting environment appears insufficient, which the higher basal cortisol level (faecal 11,17-dioxoandrostanes) of dams raising kits in this environment also indicate (at P=0.064). The nesting environment also affected the maternal behaviour of mink. Females with access to straw in combination with an artificial nest were more attentive and quicker to retrieve one of their 5-day-old progeny placed away from the safe nest. An artificial nest in combination with *ad libitum* access to straw significantly enhanced maternal kit-retrieval, and an artificial nest alone or in combination with straw tended to reduce maternal stress postpartum. For the kit vitality and survival, an artificial nest appeared as good as a nest of straw created by the dam, due to an improved in-nest climate postpartum. However, access to straw for nest building resulted in a less variable parturition, whereas the feedback from an artificial nest had no such effect (Malmkvist and Palme, 2008).

Abnormal behaviour and stress

The most common type of abnormal behaviour in farmed mink is stereotypies and fur-chewing, however, the link between abnormal and stress in mink has been debated. Although results linking individual stereotypic performance and welfare are inconsistent (cf. mink: Svendsen *et al.* (2007); review: Mason and Latham, 2004), a recent study reported that stereotypic behaviour is concurrent with higher baseline concentrations of faecal cortisol metabolites (FCM), and highly stereotypic mink react with more adrenocortical activity towards stressors than non-stereotypers (Malmkvist *et al.*, 2011) (Figure 3).

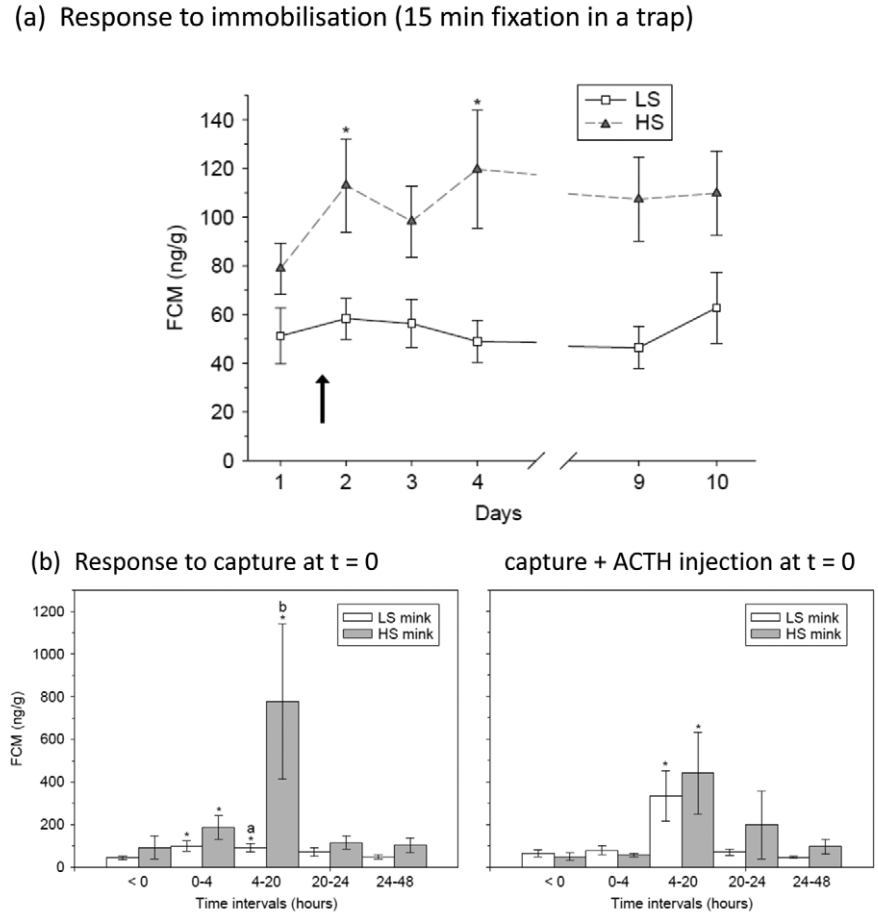

Figure 3. (a) FCMs (ng/g) in non-stereotypic (LS, n=71-80 per day) and high stereotypic (HS, n=69-76 per day) mink females sampled once daily at 14:00 to 18:00, given as mean±SEm. All mink were exposed to an immobilisation challenge for 15 min at Day 2 between 09:00 and 10:00 h (indicated by the arrow). The concentration of FCM was higher in HS than in LS mink (F-test, P*<0.001), also at Day 1 (pairwise post test P = 0.002). Significant differences from within-group baseline (Day1) are marked with an asterisk. (b) Response to handing without and with ACTH injection at time t = 0 given as mean±SEM concentration of FCM in low (LS, n=12) and high (HS, ns=12) stereotypic mink females. Stereotypic mink reacted more during 4-20 h (F-test,* P*=0.001) after handling, but not after ACTH injection (F-test,* P*=0.74). Concentrations significantly greater than baseline are marked with asterisk, difference between LS and HS mink is marked by the letter 'a'. Modified from Malmkvist* et al. *(2011).*

One major finding across mammals is that stress or stress hormones decrease neurogenesis which occurs in the dentate gyrus of the hippocampal formation (Gould *et al.*, 1997; Sapolsky, 2004; Morris, 2007). Adult neurogenesis – the generation of new neurons – has been demonstrated in the hippocampal formation in a range of mammals (e.g. rat, mouse, rabbit, guinea pig, cat, tree

shrew and in primates, reviewed in Balu and Lucki, 2009), and recently also in mink (Malmkvist *et al.*, 2012). Comparing groups of mink females with and without abnormal behaviour, self-inflicted fur-chewing and stereotypic behaviour, we fail to demonstrate significant difference in number of new hippocampal cells (P=0.18) using 12 adult mink females per group. Repeating this study with more experimental animals could be beneficial to increase statistical power. In contrast to our expectations, there was positive correlation between the amount of stereotypic behaviour performed and the number of new hippocampal cells formed (P=0.016). Thus, the study did do not support that stereotypies in mink is concurrent with reduced hippocampal neurogenesis, typically taken as a sign of chronic stress and increased activation of the HPA axis. On the contrary, cell proliferation increased with increasing performance of stereotypic behaviour, being of an active/locomotory nature in mink (Figure 4). Further studies are needed to conclude whether stereotypy is an adaptive response rather than only reflecting pathology in mink. Additionally, it would be relevant to further understand effects of activity level, in order to investigate whether the positive effect on hippocampal cell proliferation relate to increased motor activity rather than to reduced stress in mink. In my opinion, these studies are needed for adding bricks to the puzzle of understanding different type of activity and stereotypies in mink, and how these relates to stress and welfare of relevance under production conditions.

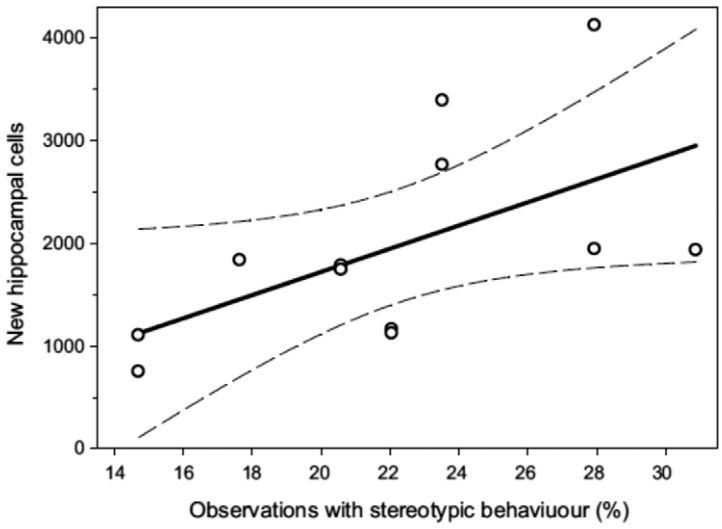

Figure 4. The number of new hippocampal cells (in the granule cell layer in the dentate gyrus) versus the amount of stereotypic behaviour in stereotypic mink (n=12). The line represents the linear regression, with 95% confidence intervals indicated by the broken lines. The number of new cells increase with the amount of stereotypic behaviour. From Malmkvist et al. *(2012).*

Conclusion and perspectives

Stress influence both welfare and production, and several studies have successfully used indicators of HPA-axis function to evaluate stress in farmed mink. Different sampling options are possible for determination of both baseline and response concentrations. The selected method should depend on the study hypothesis, remembering that handling and capture of the individual are acute stressors, eliciting secretion of HPA-axis hormones. When using the HPA function to evaluate stress one should particularly consider the influence of the fixed annual cycle of mink.

Recently, non-invasive FCM measurement reflecting circulating cortisol has been developed and validated for mink females. Faeces are the predominant excretory route of cortisol in mink, and nearly all cortisol present in faeces and urine are in metabolized forms. Consequently, assays measuring cortisol in mink urine may be less feasible to reflect the concentrations in circulation. Due to a relative high variation in non-invasive FCM, this method are suggested mainly in larger controlled experiments, e.g. when comparing different breeding lines, or different type of cage resources, with a sufficient number of animals per group. Further methodological developments are suggested, and include additional validation for FCM in males. Enlargement of adrenals could perhaps be developed into a measure of repeated/chronic stress, in case post-mortem examinations are considered; however, this indicator needs further investigation in order to increase validity.

We are in many cases interested in long-term effects of stress responses, due to its greater implications for both production and welfare. Therefore some focus in research should be devoted into understanding long-term effects, including e.g. dose-response relationships between stressors (including stress alleviation/fulfilment of needs) and the HPA-axis function in mink, and an improved understanding of how several indicators (e.g. abnormal behaviour, affective states, positive and negative effects on the brain) interact in mink. It would be naïve to rely on only one indicator as the ultimate stress measure; most study performed in mink include other indicators as well, and consider the influence of previous experiences, emotional states on perceived stress in mink. Future studies could address the border between adaptive and maladaptive stress responses. For example, what levels of prolonged stress/circulating cortisol is detrimental to the mink and their brain? Can we distinguish between detrimental vs. positive type of arousal/activity in mink? These may not be easy questions to answer, cf. studies of stereotypic behaviour. However, as the understanding of behavioural needs and welfare depend on reliable stress indicators, answering these questions are valuable for future progress in the production of farmed mink.

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