



Environmentally enriching American mink (*Neovison vison*) increases lymphoid organ weight and skeletal symmetry, and reveals differences between two sub-types of stereotypic behaviour



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ABSTRACT

Enrichment studies for wild carnivores (e.g., in zoos) are often short-term, use enrichments of unknown motivational significance, and focus on glucocorticoids and stereotypic behaviour (SB), ignoring other stress-relevant variables. Our study assessed the broad behavioural and physiological effects of enriching American mink—a model carnivore—with preferred stimuli long-term, and investigated the welfare implications of individual differences in SB. We raised 64 male-female pairs with or without enrichment. At 7 months, pairs were split and mink individually housed (adults being solitary), first by being temporarily moved to identical non-enriched cages (permitting observation blind to rearing condition). Two weeks later, one mink per original pair (half female, half male) was returned to his/her rearing cage for re-observation, sample collection for faecal cortisol metabolite (FCM) analysis, and additional research for 1.5 years before being humanely killed. Stress-sensitive variables were then measured *post-mortem*. Enriched-raised mink in their rearing conditions excreted less FCM ($F_{1,29} = 8.33, p = 0.003$), and performed less SB than non-enriched mink. Two SB sub-types occurred: (1) 'loco' stereotypies: locomotor, whole body and head stereotypies (e.g., pacing, nodding), previously shown to correlate with recurrent perseveration; and (2) repetitive scrabbling with the forepaws. Enriched housing reduced both (at 7 months: loco stereotypies: $F_{1,60} = 25.3, p < 0.0001$; scrabbling: $F_{1,60} = 24.0, p < 0.001$; effects still trends 1.5 years later). However, the sub-types responded differently to the current availability and/or usage of enrichment. Thus enrichment-use (which was stable) tended to negatively correlate with scrabbling but not loco stereotypies. Furthermore, after the relocation to identical non-enriched cages, loco stereotypies remained lower in enriched-reared than non-enriched-reared mink ($F_{1,58} = 31.33, p < 0.0001$), but scrabbling rapidly increased (such that within two weeks, enriched- and non-enriched-reared mink were indistinguishable). *Post-mortem*, enriched-reared mink showed less skeletal fluctuating asymmetry ($F_{1,42} = 2.87, p = 0.048$) and had heavier lymphoid organs (thymus: $F_{1,41} = 3.43, p = 0.035$; spleen: $F_{1,45} = 13.11, p = 0.010$). However, *within* treatment groups, neither these measures nor FCM covaried with SB. In conclusion, long-term housing with preferred enrichments not only reduced SB and FCM, but also induced anatomical changes consistent with better cell-mediated immunity and reduced developmental stress. In addition, these results should refine the use of SB and its sub-types in welfare assessment, since scrabbling seemed to reflect the prevailing presence/absence and utilisation of enrichment, while motor, whole body and head SBs appeared to reflect more stable, long-term effects of differential rearing; and furthermore, within each housing type, individual differences in SB appeared to reflect response styles rather than differential welfare.

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1. Introduction

Environmental enrichment refers to the provision of stimuli to improve animals' welfare in captivity (Newberry, 1995; Shepherdson, 1998). Enriched environments are thus generally preferred by animals: they voluntarily interact with them, and may even work to access them, indicating high motivation (e.g., American mink *Neovison vison*, Mason et al., 2001; Hansen and Jensen, 2006; pigs *Sus scrofa*, Elmore et al., 2011). Enrichments also influence an array of stress-sensitive physiological and behavioural variables. For example, in diverse wild and domesticated species, environmental enrichment decreases the performance of stereotypic behaviour (reviewed by Swaisgood and Shepherdson, 2005); and reduces activity of the hypothalamic pituitary adrenal (HPA) axis, decreasing adrenal gland weight (Beattie et al., 2000; Abou-Ismaïl and Mahboub, 2011) and lowering glucocorticoid levels (Carlstead et al., 1993; Belz et al., 2003). In laboratory rodents, two lymphoid organs essential for cell-mediated immunity are also often heavier in enriched animals: the thymus (Van Loo et al., 2004) and spleen (Abou-Ismaïl, 2011), both of which involute with chronic stress (e.g., Mitchell et al., 2006; Corbin et al., 2008). Finally, in laboratory rats and farmed rabbits, environmental enrichment can reduce levels of fluctuating asymmetry (Sorensen et al., 2005; Tuytens et al., 2005): an anatomical measure of developmental instability (Polak, 2003) that is associated with early stress (Møller and Swaddle, 1997), and therefore used by females as part of mate choice in many species (e.g., Møller, 1993; Waitt and Little, 2006).

Our research had two main aims. The first was to gather for the first time basic information on all the behavioural, anatomical and physiological effects of environmental enrichment described above in a model carnivore, the American mink. Although captive wild carnivores have attracted many enrichment studies (Shepherdson et al., 1998; Swaisgood and Shepherdson, 2006), their scope has been rather limited, typically focusing on stereotypic behaviour or cortisol output, and often being rather short-term and using putative enrichments of unknown motivational significance to the animals (e.g., Poessel et al., 2011). Perhaps as a result, enrichment only halves the performance of stereotypic behaviour in such studies, instead of abolishing/preventing it (Shyne, 2006; Swaisgood and Shepherdson, 2006), suggesting it was inadequate or offered too late in development (Mason et al., 2007). American mink represent the single species of carnivore attracting the most enrichment and stereotypic behaviour research, because they are widely farmed for their fur (e.g., Hansen and Jeppesen, 2001; Mason et al., 2001; Mononen et al., 2008; Axelsson et al., 2009; Meagher et al., 2013). However, no mink study has yet explored the long-term effects of preferred enrichments on variables sensitive to developmental stress (e.g., fluctuating asymmetry) or aspects of stress physiology relevant to immune function, even though these could have implications for health and productivity on fur farms. More detailed research could also help promote the more effective use of enrichment to enhance breeding and health in carnivores in zoos and captive breeding centres (especially other mustelids, such as European mink *Mustela lutreola*, and black-footed ferrets *Mustela putorius*). Therefore we predicted that being raised and housed long-term with preferred enrichments would not only reduce cortisol output and adrenal weight, and reduce or even prevent stereotypic behaviour, but also reduce fluctuating asymmetry, and increase spleen and thymus weights.

Our second main aim was to investigate why individuals vary in their reactions to both enriched and non-enriched housing conditions, and the welfare implications of such variation. These individual differences are most clearly manifested, and best studied, in stereotypic behaviour. In non-enriched housing conditions, individuals typically vary greatly and consistently in how much

of it they display. For example, some fur-farmed mink spend most of their active time performing this behaviour, while other, identically-housed, conspecifics show none at all (e.g., Hansen, 1993; Mason, 1993; Meagher et al., 2012b). Similar effects occur in other species (e.g., pigs, Terlow et al., 1991; African striped mice *Rhabdomys pumillio*, Jones et al., 2010). Paradoxically, given the links between stereotypic behaviour and poor environments, the more highly stereotypic individuals generally seem to have better welfare than less stereotypic conspecifics within these sub-optimal conditions. For example, highly stereotypic mink and African striped mice produce more offspring than less stereotypic, identically-caged peers (Jeppesen et al., 2004; Jones et al., 2010), and bouts of oral stereotypic behaviour correlate with decreased heart rate in both sows (Schouten et al., 2000) and horses *Equus caballus* (Minero et al., 1999). Meta-analyses suggest that such individual-level (i.e. within-population) patterns are typical (Mason and Latham, 2004). This has led to speculation that stereotypic behaviour either helps captive animals cope with sub-optimal conditions (e.g. Wiepkema et al., 1987), or that non-stereotypic animals in such conditions are instead very inactive due to additional welfare problems, such as pain, sickness, or depression-induced apathy (Mason, 2006; Meagher and Mason, 2012; Fureix et al. in press)

Similar individual variation in behaviour is also seen in enriched conditions. Stereotypic behaviour is far less common here, but certain individuals may still display it. This has been observed in several rodent enrichment studies (e.g., Ödberg, 1987; Turner et al., 2003; Latham and Mason, 2010; Bechard et al., 2011; Jones et al., 2011) as well as two parrot species (orange-winged Amazon parrots *Amazonica amazonica*, Meehan et al., 2004; African grey parrots *Psittacus erithacus*, Lumeij and Hommers, 2008), and mink (e.g., Hansen et al., 2007; Dallaire et al., 2012; Díez-León et al., 2013). These findings suggest that for some individuals, the enrichments provided are insufficient at reducing the psychological stress or central nervous system changes generally implicated in stereotypic behaviour. If true, this hypothesis predicts positive correlations between stereotypic behaviour and physiological signs of stress across enriched-housed animals. Furthermore, one potential explanation for individual differences in apparent enrichment-effectiveness is individual variation in enrichment-use. In mink (Dallaire et al., 2012), plus species as diverse as mice, bears (Asiatic black bears, *Ursus thibetanus*, and Malayan sun bears, *Ursus malayanus*), rhesus monkeys *Macaca mulatta*, and African grey parrots, individuals housed with identical enrichments show stable differences in the degree to which they interact with them (Line et al., 1991; Vickery, 2003; Lumeij and Hommers, 2008; Walker and Mason, 2011); and when feather-plucking parrots were given enrichments, the individuals who voluntarily used them the most, showed the greatest reductions in this stereotypic behaviour (Lumeij and Hommers, 2008).

We therefore predicted that in non-enriched conditions, physiological and anatomical signs of stress would be lower in more stereotypic individuals. In contrast, in enriched environments, we predicted that highly stereotypic individuals would show more physiological and anatomical indicators of stress than less-/non-stereotypic identically-housed conspecifics, and relatively low enrichment use.

To maximise our chances of success, we chose environmental enrichments shown in other studies to be motivating for mink. We also raised subjects from infancy with or without enrichment, not collecting our first data until young adulthood after 5–6 months of differential exposure, and collecting the rest after one or two breeding seasons and up to 2 years of differential housing. Data belonging

to the adult male subjects of this study have been reported elsewhere (Díez-León et al., 2013).

2. Material & methods

All housing conditions and experiments were approved by the University of Guelph's Animal Care Committee and the Michigan State University's Institutional Animal Care and Use Committee.

2.1. Treatments and animals

Data were collected at the Michigan State University Experimental Fur Farm, a research farm run as a semi-commercial operation. Mink were kept in two inter-connected indoor rooms with artificial light (four 150 watt bulbs per room; switched on and off automatically at natural sunrise and sundown) and where temperature and humidity were allowed to fluctuate with seasonal and daily rhythms. Each room held 32 individually-housed mink, distributed evenly by treatment and sex, such as to avoid possible room effects, and variation induced by proximity to the door (cf. Garner et al. on mice (2011) and parrots (2003); and Gottlieb et al. (2013) on rhesus monkeys) (for more details see Díez-León, 2014).

Housing was as described in several previous papers (Dallaire et al., 2011; Meagher and Mason, 2012; Campbell et al., 2013; Díez-León et al., 2013). In brief, all mink had a wire mesh home cage measuring 61 × 76 × 46 cm, provided with an attached plastic nest box (31 × 25 × 25 cm). Non-enriched (NE) mink lived in unmodified versions of these cages. Enriched (E) mink lived in cages modified by adding access to a large compartment containing resources chosen based on current knowledge of mink enrichment (see Díez-León et al., 2013). Other experiments on other cohorts of mink confirmed that the enriched environments used here greatly decreased stereotypic behaviour (Dallaire et al., 2011; Campbell et al., 2013; Meagher et al., 2013); and furthermore, when access was made costly by placing weights on an entry door, mink would work as hard to access the E compartments as they would to reach food (Dallaire et al., 2012).

Experimental subjects were 64 unrelated mink of the Black colour type, half female and half male, half born and raised in NE conditions and half born and raised in E conditions. Their mothers (all multiparous) had been moved to our cages from the farm stock late in gestation and the doors to the E compartments were opened once kits were mobile with open eyes (at five weeks of age; see Fig. 1 for an experimental timeline). Their mothers and littermates were removed when our subjects were between 7 and 8 weeks old, and each subject was housed with an unrelated opposite sex cagemate until seven months of age. This was to enhance welfare, since pair-housing rather than isolating young mink is recommended (SCAHAW, 2001), while also ensuring that all subjects had the same treatment and proper socialization for a later experiment (Díez-León et al., 2013). Companion kits always came from the same housing treatment as the subject kits. At seven months, when mink reached adult weight, they were separated into singletons as per normal farm practice, and subjects then remained differentially housed in either E or NE environments for another 17 months (details below, also Fig. 1).

2.2. Behavioural data collection

Behavioural variables (see Table 1 for ethogram) were first assessed when the mink were approximately seven months old—the age at which mink first start to consistently show stereotypic behaviour (Mason, 1993; Jeppesen et al., 2000). Collecting reliable data from paired animals is difficult, since mink can be hard to tell apart while moving. We also wanted to observe all our differentially-reared mink in a standardised environment to assess

whether housing effects could be detected when the observer was blind to treatment (cf. Powell et al., 2000). Therefore, all mink were temporarily moved to standardised cages on the farm (measuring 25 × 76 × 38 cm with a drop-in plastic nest box of 30 × 25 × 25 cm) and housed singly for observation.

Based on previous studies (Meagher et al., 2009), we gave animals five days to acclimate to the new conditions before observations began. Observations were then performed every half hour from 0800 until 1200 for eight days, a time of day chosen to capture the pre-feeding period when most stereotypic behaviour occurs (de Jonge and Carlstead, 1987; Bildsøe et al., 1990; Mason, 1993). To minimise disturbance (cf. Mason, 1993; Clubb, 2001; Vinke et al., 2002), the observer (MDL) recorded the behaviour of mink housed three rows away from the cage she was standing in front of, and stood still for 30s opposite to the subject to be observed to allow habituation to her presence (e.g., Clubb, 2001; Pedersen et al., 2004). This type of direct observation has been validated for mink, and has minimal impact on the behaviours recorded (Mason, 1992, 1993; Svendsen et al., 2005). Observations combined instantaneous and focal sampling (Martin and Bateson, 2007), with an inter-scan interval of 15–20 min. Mink in different cages were observed in the same order on each successive scan. Instantaneous sampling was used for consummatory, non-stereotypic behaviours such as drinking or sleeping. For other activities (those not obviously consummatory or that seemed to be stereotypic) focal sampling was used: mink were continuously observed for up to 20s or until the behaviour could be categorised, whichever occurred first (e.g., Mason, 1993; Pedersen et al., 2004).

Most mink showed several forms of stereotypic behaviour. Dallaire et al. (2011) divided the behaviours into two classes: 'locomotor stereotypies' and 'scrabbling'. 'Locomotor' was shorthand for 'locomotor, whole-body and head', a group that included pacing, route-tracing, whole-body movements like repeated rearing in one spot, and head movements like head-twirling (all typically reported in behavioural studies of caged mink; e.g., Bildsøe et al., 1990; Jeppesen et al., 2004; Hansen et al., 2007; Malmkvist et al., 2011). These behaviours, that here we abbreviate further to 'loco' stereotypies, correlate with recurrent perseveration (the inappropriate repetition of behavioural responses, e.g., Ridley, 1994) (Dallaire et al., 2011). Scrabbling, in contrast, describes an apparently site-specific behaviour (i.e. not commonly reported in other populations, see Hansen and Jeppesen, 2000, 2006; Vinke et al., 2006) wherein mink make scratching or digging movements with their forelegs at the floor or a cage wall. It resembles the 'scratching' reported in some experiments as goal-directed attempts to enter areas to which access is denied (Hansen and Jeppesen, 2000), and it does not covary with recurrent perseveration (Dallaire et al., 2011).

Two weeks after behavioural observations were completed in the standardised environments, the 64 subject mink were returned to their rearing environments (see Fig. 1), while the companion mink remained in the standardised environments until humanely killed for their pelts ('pelted'). Subjects were given another five days to re-acclimatise to their rearing cages and their behaviours subsequently re-assessed using the protocol described above for an additional eight days. Additionally, for E animals, we recorded enrichment use as (i) the amount of time (total and active) spent in the NE cage vs. the E compartment; and (ii) the amount of time spent interacting with the different enrichments (Table 1). We also opportunistically observed the companion mink, since they were left in the standardised cages for another two weeks (longer than anticipated) before being pelted.

A year and five months later, at the end of the experiment, 48 mink remained — 16 females having been humanely killed after the first year's reproductive trials (see Fig. 1; Díez-León et al., 2013) — for which differential rearing and housing had lasted one year and 11 months. We re-assessed their stereotypic behaviour (to obtain

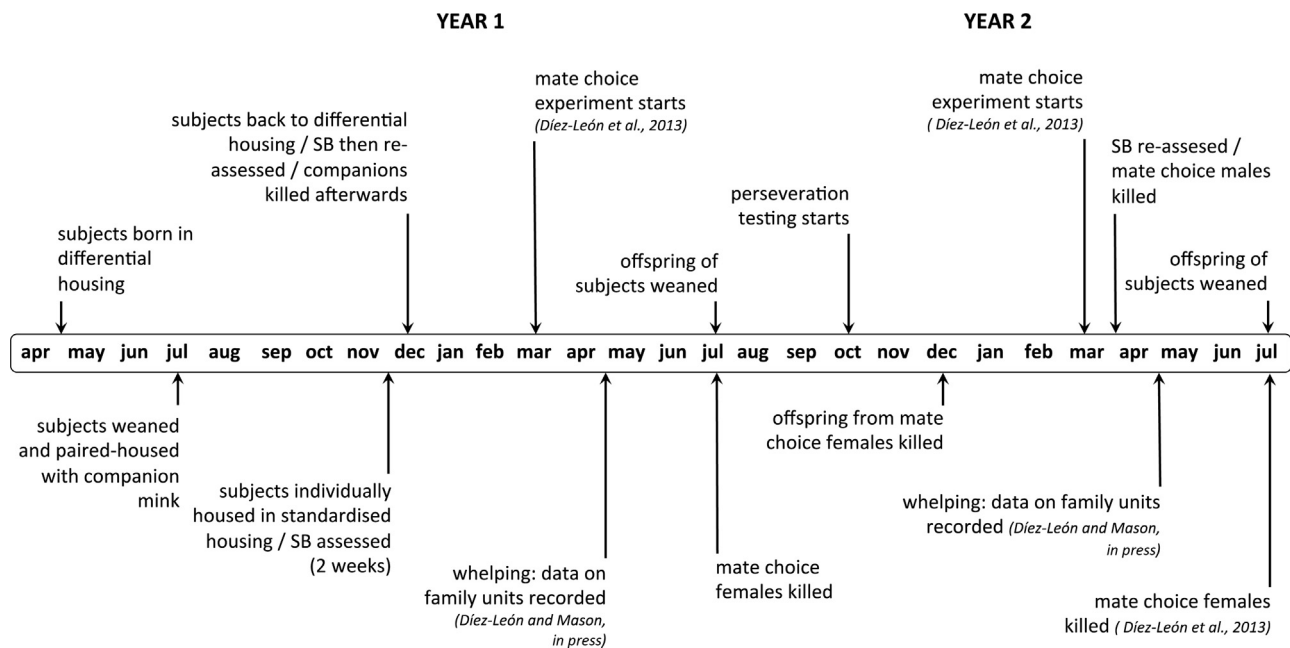


Fig. 1. Experimental timeline for this and related experiments.

Table 1
Ethogram used.

Behaviour	Description
Stereotypic behaviour	Loco stereotypies
	Repetitive whole body movements or locomotive route-tracing, performed as three or more consecutive repetitions (two or fewer repetitions were termed "borderline stereotypic behaviour", and were not counted as loco stereotypies but instead included in overall activity)
	Scrabbling
	Scrabbling or scratching at the corners and walls of the cage/nest box (if performed as part of a locomotive route-tracing bout—e.g. pacing + scrabbling + rearing at the corner—that was performed three times or more, then it was scored as a loco stereotypy)
Normal activity	Any other activity (e.g. climbing, eating, drinking, etc.).
Inactivity	Motionless behaviours (e.g. lying on the cage floor, sleeping, sitting)
For the subset of E-reared mink also: Interaction with enrichments	Contacting the enrichment objects (sniffing/manipulating items, head dipping or any other normal activity in the water channel, sleeping under/on top of frisbee/baskets)

measures close to the time of death, to compare with physiological and anatomical stress-sensitive variables) and their use of enrichment, for eight days right after the mating season. This followed a similar protocol to that described above, but with double the frequency of observations as allowed by the smaller sample size.

The reliability of behavioural data was always checked by carrying out split-half analyses (Martin and Bateson, 2007), where data within an observation period are divided into halves (e.g., odd and even days as used here) and the degree of correlation between those halves assessed. All were very significant ($p < 0.001$), indicating strong internal reliability. Stereotypic behaviour was calculated and analysed both as a proportion of observations and as proportion of total activity (see Table 1; also Garner, 1999; Campbell et al., 2013). Controlling for individual levels of activity is likely the more welfare relevant measure, and, furthermore, Campbell et al. (2013) found that stereotypic behaviour expressed as a % of activity correlated most closely with recurrent perseveration. However, measuring stereotypic behaviour as a % of total observations is still most common in the literature, and more intuitive for understanding how animals budget their time. When measuring

stereotypic behaviour as % of observations yield different results, these are discussed in the text; otherwise results are always provided as % of activity. For investigations involving time spent in the enriched compartment, analyses were corrected for the relative floor area of this region by calculating proportions of time spent per unit floor area (i.e., % of time spent in either the enriched compartment vs. the home cage divided by their respective floor areas) as the dependent variable, since the enriched compartment was larger than the home cage and animals allocating their time to different areas just by chance would therefore spend more time there without this indicating true preference (see Koistinen et al., 2008 for problems assessing preference using unequal floor sizes).

2.3. Data collection on physiological and anatomical stress-sensitive variables

The humane killing method and the protocol for data collection on physiological and anatomical stress-sensitive variables have been described elsewhere (Díez-León et al., 2013). Briefly, faeces were processed to assess levels of faecal cortisol metabo-

Table 2a
Stereotypic behaviour in enriched (E) and non-enriched (NE) subject mink only. (a) All mink. Significant results are in bold. Italicised results were no longer significant after FDR corrections (see Statistical Analyses).

	After move to standardised cages				After return to rearing environments										
		<i>n</i>	Mean ± SE	Statistic	<i>P</i>	3 weeks later				1.5 years later					
						<i>n</i>	Mean ± SE	Statistic	<i>P</i>	<i>n</i>	Mean ± SE	Statistic	<i>P</i>		
Loco stereotypies	E	32	4.38 ± 2.42	F_{1,60} = 23.7	<0.001	E	32	6.37 ± 4.38	F_{1,60} = 25.3	<0.0001	E	24	4.06 ± 5.47	<i>F_{1,44} = 4.40</i>	<i>0.020</i>
	NE	32	19.9 ± 4.76			NE	32	27.7 ± 4.38			NE	24	25.9 ± 5.47		
Scrabbling	E	32	53.1 ± 4.46	<i>F_{1,60} = 0.84</i>	0.181	E	32	9.2 ± 3.68	F_{1,60} = 24.9	<0.001	E	24	9.58 ± 4.5	<i>F_{1,44} = 4.60</i>	<i>0.018</i>
	NE	32	47.2 ± 4.77			NE	32	38.3 ± 3.68			NE	24	26.5 ± 4.5		

Table 2b
Stereotypic behaviour in enriched (E) and non-enriched (NE) subject mink only. (b) Sub-set of mink displaying stereotypic behaviours. Significant results are in bold. Italicised results were no longer significant after FDR corrections (see Statistical Analyses). Degrees of freedom vary between loco stereotypy and scrabbling analyses due to more mink performing the latter.

	After move to standardised cages				After return to rearing environments										
		<i>n</i>	Mean ± SE	Statistic	<i>P</i>	3 weeks later				1.5 years later					
						<i>n</i>	Mean ± SE	Statistic	<i>P</i>	<i>n</i>	Mean ± SE	Statistic	<i>P</i>		
Loco stereotypies	E	8	72.79 ± 5.05	<i>F_{1,28} = 1.56</i>	0.161	E	7	53.14 ± 8.09	<i>F_{1,27} = 1.73</i>	0.09	E	7	68.97 ± 7.92	<i>F_{1,17} = 8.26</i>	<i>0.01</i>
	NE	24	80.15 ± 3.01			NE	24	65.22 ± 4.32			NE	14	97.00 ± 6.33		
Scrabbling	E	31	49.04 ± 4.33	<i>F_{1,57} = 0.43</i>	0.514	E	21	32.20 ± 3.38	F_{1,47} = 20.29	<0.0001	E	10	28.52 ± 7.36	<i>F_{1,22} = 2.27</i>	<i>0.072</i>
	NE	30	44.98 ± 4.41			NE	29	52.38 ± 2.93			NE	16	41.97 ± 5.02		

lites (FCM) with a mink-validated 11β -hydroxyaetiocholanolone enzyme immunoassay (Malmkvist et al., 2011), mandibles were extracted for fluctuating asymmetry assessment, and adrenal glands and lymphoid organs (thymus and spleen) were weighed. Dissection, fat removal and weighing of organs each were performed by a different person (AN, SB and two other MSU staff members), all of them blind to the hypotheses under test. Similarly, data on FA were gathered by MDL in a way that blinded her from mandible treatment (see details in Díez-León, 2014), and DG performed statistical analyses, blind to treatment, to confirm that FA (and not other types of bilateral symmetry) was present in the dataset.

2.4. Statistical analyses

To compare prevalences of behaviour, we used chi-squared tests in GraphPad Software Inc. (2014). All other analyses were run using General Linear Models (GLMs) in JMP v.10 (JMP, 2012). Sex, treatment group and their interactions were always included as predictor variables. Sex effects and their interactions are not shown as they were always non-significant. Individual data points were considered outliers if they were greater than two times the standard deviation away from the group mean (c.f. Sokal and Rohlf, 1995), and discarded from analyses. Where models were non-orthogonal, we used the sequential (Type I) sum of squares (Grafen and Hails, 2002) and ran the model placing the factor of interest as the last main effect (Doncaster and Davey, 2007). Repeated measures analyses compared subjects' behaviour in the standardised cages and after returning to their rearing environments. Transformations were carried out when standardised residuals did not meet the assumptions for normality and/or homogeneity of variances.

In all analyses pertaining to organ weights, body length was used as a covariate (except for spleen weights, where body weight was used as a covariate as both measures were positively correlated). Weights from both adrenals per mink were averaged for analyses. In analyses pertaining to FCM levels, because levels of physical activity can affect baseline output of this hormone (Malisch et al., 2008; Hansen et al., 2010), we corrected for activity by running analyses in which total activity (normal activity + borderline stereotypic behaviour + stereotypic behaviour) was added as an independent variable to the model.

Analyses aimed at investigating overall treatment differences in welfare in the rearing conditions were performed one-tailed (i.e., p values were halved when in the predicted direction, but treated as non-significant if under 0.05 in the other direction [not something that ever occurred]). This was because we had clear *a priori* directional predictions (Rice and Gaines, 1994; Levine and Banas, 2003). This related to all variables described in the Introduction. Additionally, some opportunistic *post hoc* analyses on the co-variation of stress-sensitive variables (run to help us assess the quality of our own data) were also run one-tailed, since we predicted only positive relationships among HPA measures (adrenal weight and FCM levels), and among immune status indicators (thymus and spleen); only negative co-variations between HPA and immune variables; and only positive relationships between fluctuating asymmetry and HPA variables, but specifically negative co-variation between fluctuating asymmetry and immune variables (see Sealander and Bickerstaff, 1967; Walzer et al., 1984; Satterlee et al., 2008) (relationships other than these falsifying our hypothesis that these effects should co-vary in ways reflecting their stress-sensitivity). In contrast, exploratory analyses pertaining to the opportunistic extra observations of companion mink were two-tailed, since we had no clear predictions here about how their behaviour would change over time. To be conservative, as we were running multiple tests, and both this and using one-tailed tests can increase chances of Type I errors, we used "false discovery rate" procedures

(FDR; Benjamini and Hochberg, 1995) to reduce the thresholds of P required to conclude significance. Results that were significant (at $p < 0.05$) before FDR corrections, but which became non-significant after the new thresholds for significance are reported as trends in the Discussion.

3. Results

3.1. Enrichment effects on behaviour

Overall, stereotypic behaviour was lower in enriched-reared mink. When first assessed, in standardised cages at seven months of age, E mink spent less of their active time performing loco stereotypies than mink reared in NE housing, although in this environment they did not differ in scrabbling levels from NE mink (Table 2a). Treatment differences in loco stereotypy were also clear when subjects were assessed once back in their rearing cages (both 3 weeks later and 1.5 years later, although this last result was not significant after FDR corrections; Table 2a). Furthermore, once observed back in their rearing environments, E animals now also scrabbled less than NE animals (both 3 weeks later and 1.5 years later; although again this last result was not significant after FDR corrections; Table 2a). These reduced levels of stereotypic behaviour in E housing reflected different effects on the two subtypes of SB. Thus for those individual mink displaying scrabbling, being raised and housed with E reduced the levels of this behaviour, but the same was not true for those individual mink affected by loco SB (Table 2b): affected individuals were similarly stereotypic in both environments. The prevalence of loco stereotypy was, in contrast, significantly reduced in E mink: thus there were far fewer effected animals there; while the prevalence of scrabbling was not reduced in E-housed animals (see Table 3). Other behavioural data from these E animals showed that in terms of using the enriched compartment and the resources within it, E mink spent on average 20% of their active time interacting with enrichments, with both males and females typically spending most of their time in the E compartment/tunnel (see Table 4 for details).

Repeated measures analyses comparing subjects' behaviour in the standardised cages and after return to their rearing environments further showed that loco stereotypies were not significantly different in the two locations, but that in E animals only, scrabbling was significantly elevated in the standardised cages compared to rearing environments ($F_{1,30} = 125.15$, $p < 0.0001$): an increase that explained why the two rearing groups' scrabbling was indistinguishable after 5 weeks in that environment (Table 2a) as seemingly more mink scrabbled, and those that did also spent more time engaged in this behaviour (Table 2b and Table 3). Furthermore, data opportunistically collected from the companion animals after being in these standardised cages for 5 weeks revealed that even at this time, the same treatment effects could still be detected on loco stereotypies, with E animals still displaying less than NE ($F_{1,58} = 31.33$, $p < 0.0001$). However, treatment effects in the opposite direction had become evident for scrabbling, the previously E animals now displaying *more* of this behaviour than NE-raised mink ($F_{1,58} = 6.95$, $p = 0.018$) (although this result was not significant after FDR corrections).

3.2. Enrichment effects on physiological and anatomical stress-sensitive variables

As predicted, E mink had reduced levels of fluctuating asymmetry, heavier thymuses and spleens, and lower levels of excreted cortisol metabolites (the latter being assessed only in males) (see Table 5). The effect of E on FCM concentrations was still significant after statistically controlling for total activity (previously reported

Table 3

Prevalence of stereotypic behaviour (% of animals performing it). Significant effects were no longer significant after FDR corrections (see Statistical Analyses). E: enriched-reared; NE: non-enriched-reared; n = total number of mink per rearing group.

	After move to standardised cages			After return to rearing environments					
	E	NE	n	3 weeks later			1.5 years later		
				E	NE	n	E	NE	n
Loco stereotypies	25 ¹	75	32	22 ²	75	32	29	58	24
Scrabbling	97	94	32	66	90	32	42	67	24

¹ $\chi^2 = 4.50$, df. = 1, $p = 0.034$.

² $\chi^2 = 5.40$, df. = 1, $p = 0.01$.

Table 4

Time spent in the enriched compartment (EC) versus the home cage (HC) corrected by floor size (% time/m²). Significant results in bold. Italicised results were no longer significant after FDR corrections (see Statistical Analyses).

		3 weeks			1.5 years		
		Mean ± SE	Statistic	p	Mean ± SE	Statistic	p
Total time	EC	78.07 ± 3.99	F_{1,60} = 93.35	<0.0001	66.91 ± 7.52	F _{1,44} = 2.23	0.071
	HC	23.59 ± 3.99			51.04 ± 7.52		
Active time	EC	78.69 ± 3.78	F_{1,60} = 111.81	<0.0001	68.89 ± 6.37	F _{1,44} = 6.34	0.015
	HC	22.07 ± 3.78			46.18 ± 6.37		

Table 5

Enrichment effects on physiological and anatomical variables. Means represent least-squared means. Significant results in bold. E = enriched-reared; NE = non-enriched-reared. Tests corrected for body size when appropriate. Results for faecal glucocorticoid metabolites (FCM) have been previously reported in Díez-León et al. (2013).

		E		NE		Statistic	p
		Mean	SE	Mean	SE		
Adrenal weight (g)		0.067	0.013	0.087	0.011	F _{1,46} = 0.27	p = 0.302
FCM (ng/g) – males only		185	42.5	299	41.6	F _{1,29} = 8.33	p = 0.003
Fluctuating asymmetry		0.0358	0.0021	0.0410	0.0022	F _{1,42} = 2.87	p = 0.048
Thymus weight (g)		1.0198	0.0752	0.8774	0.0602	F _{1,41} = 3.43	p = 0.035
Spleen weight (g)		4.458	1.0327	3.424	0.8448	F _{1,45} = 13.11	p = 0.01

in Díez-León et al., 2013). The positive effect of E on thymus weight was only evident after five statistical outliers were removed (9% of the sample for this test).

3.3. Inter-relationships between physiological and anatomical stress-sensitive variables and behaviour

Loco stereotypies were unrelated to any physiological or anatomical measure of stress ($p > 0.10$ in all cases), regardless of housing condition. Scrabbling also did not co-vary with any other stress-sensitive variable ($p > 0.10$ in all cases). Analyses investigating inter-relationships between these various stress-sensitive variables, to see if any expected relationships emerged between our measures, revealed just two significant correlations (see Table 6), although both became non-significant after FDR corrections: adrenal weight positively predicted FCM output; and spleen weight negatively covaried with FCM, but only in the non-enriched group.

3.4. Correlates of individual differences in enrichment-use

Focusing on the subset of mink in E housing, there were no relationships between time spent in the E compartment on loco stereotypies ($p > 0.10$ for all analyses). For scrabbling, however, when considering the behaviour over total observations, there was a barely significant negative correlation with overall time spent in the E compartment ($R^2 = 0.12$, $F_{1,28} = 2.78$, $p = 0.054$; non-significant after FDR corrections). However, this trend disappeared after 1.5 years. Time spent actually interacting with enrichments was unre-

lated to either loco stereotypies or scrabbling. Finally, neither total nor active time spent in the E compartment, nor enrichment-use, related to any physiological or anatomical stress indicator.

4. Discussion

Our first aim had been to assess whether carefully chosen, long-implemented enrichments could have the types of effect on carnivores that had been documented in domesticated rodents and lagomorphs: better lymphoid organ development and reduced fluctuating asymmetry. In this respect, our predictions were met. Our second aim was to test hypotheses about individual differences in stereotypic behaviour and how they relate to welfare. Here, our predictions were largely not met, save for one weak trend suggesting that low enrichment use might predict one type of stereotypic behaviour.

As expected, our enriched conditions reduced stereotypic behaviour. At the group level both loco stereotypies and scrabbling were reduced by approximately 75%, and living with enrichments both reduced the number of animals displaying stereotypic behaviour and the time that stereotypic individuals devoted to these activities. The magnitude of this effect was thus greater than found in the typical zoo study or mink study, where the average reduction in performance of stereotypic behaviour is 50% or less (Shyne, 2006; Swaisgood and Shepherdson, 2006; Dallaire et al., 2012; Buob et al., 2013; but cf. Hansen et al., 2007). We suggest that studies in other carnivores failing to find similarly large effects of enrichment on stereotypic behaviour may not have used sufficiently motivating enrichments, or not have provided enrichments

Table 6
Inter-relationships between stress-sensitive variables. FA = fluctuating asymmetry; FCM = faecal glucocorticoid metabolites. Italicised results were no longer significant after FDR corrections (see Statistical Analyses). §Only in the non-enriched group (interaction term: $F_{1,20} = 8.64, p < 0.01$).

	FA	FCM (males only)	Adrenal weight	Thymus	Spleen
FA		$F_{1,26} = 0.12$ $p = 0.733$	$F_{1,25} = 0.66$ $p = 0.424$	$F_{1,21} = 0.02$ $p = 0.903$	$F_{1,25} = 0.14$ $p = 0.711$
FCM (males only)			$F_{1,25} = 2.85$ $p = 0.05^{*ve}$	$F_{1,21} = 0.76$ $p = 0.394$	$F_{1,9} = 4.33$ $p = 0.05^{-ve§}$
Adrenal weight				$F_{1,34} = 1.90$ $p = 0.177$	$F_{1,33} = 0.01$ $p = 0.934$
Thymus					$F_{1,33} = 1.22$ $p = 0.258$
Spleen					

early enough in development or for a long enough time. Screening our subjects in standardized conditions further revealed that enriched-rearing conditions had long-lasting ameliorating effects on loco stereotypies, protecting mink from displaying them even after five weeks in these small, non-enriched cages. The same was not true for scrabbling: treatment (i.e., rearing environment) effects on this behaviour vanished within two weeks of being moved to standardised cages because scrabbling so markedly increased in the E mink, and there was even evidence that scrabbling continued to increase in E-raised mink over the following two weeks, such that E mink came to display it more than NE mink.

As also expected, enrichment reduced FCM output, as previously found by Hansen et al. (2007) and Meagher et al. (2013) for enriched mink, and Carlstead et al. (1993) for enriched leopard cats *Felis bengalensis*. This treatment effect remained significant after correcting for activity levels, which is an important consideration because physical activity can increase HPA activity (Malisch et al., 2008; Hansen et al., 2010), and our enriched mink were significantly more inactive than NE animals making inactivity a potential confound. Our data also showed for the first time in carnivores that levels of these metabolites tended to covary with adrenal weight (as previously shown for lab mice, e.g. Groenink et al., 2002; Gurfein et al., 2014)—although this did not translate into a detectable treatment effect on adrenal weight. Given our results, we suggest that studies in other carnivores that do not find effects of enrichment on FCM (e.g., Poessel et al. (2011)'s study of black footed ferrets) may have used enrichment of low motivational value or perhaps not given them early enough or for a long enough time.

As we had hoped, our data also revealed effects of enrichment that had not been previously investigated for mink or any other carnivore. Our predictions that environmentally enriched mink would have heavier lymphoid organs, along with greater skeletal symmetry, were supported. Larger spleens likely indicate larger red and white splenic pulps (Corbin et al., 2008): changes that should be investigated histologically in future work. If confirmed, this would suggest greater lymphocyte production and enhanced capacities to initiate cell-mediated immune responses (e.g., Cesta, 2006). Furthermore, at least in the NE animals, smaller spleens tended to be predicted by elevated FCM, consistent with previous studies showing the same effect in laboratory rodents (Gurfein et al., 2014). The larger thymic masses of E mink may likewise indicate enhanced immune responses, greater amounts of thymopoietic tissue elevating capacities to produce T-cell precursors (thymocytes) (Wyllie, 1980; Haynes et al., 2000; Taub and Longo, 2005; Gurfein et al., 2014). Our additional new finding was that enriched mink were more morphologically symmetrical. Greater FA in challenging environments, as well as under inbreeding, has been recorded in wild carnivores (Pertoldi et al., 2000; Edwards et al., 2013; Korabiev et al., 2013), but this result is the first of its kind for a captive carnivore. It suggests that our enriched cages provided a lower-stress environment for development—effects that probably would

not have been detectable had enrichment only been provided in adulthood. These findings further suggest that greater resistance to infection and attractiveness as mates could be possible benefits of truly effective carnivore enrichments: potentially fascinating and useful topics for future work.

Our second overall aim was to test the welfare correlates of individual differences of stereotypic behaviour. We had predicted these correlates would differ between enriched and non-enriched environments, with highly stereotypic animals in non-enriched environment having better welfare than less stereotypic, identically-housed conspecifics; but highly stereotypic animals in enriched environments having poorer welfare, and showing less enrichment-use, than less stereotypic enriched conspecifics. With one possible exception, we found no evidence to support either of these predictions. Within the non-enriched mink, there were no apparent differences in welfare between high and low stereotypers, failing to replicate the patterns suggested by Mason and Latham's (2004) meta-analysis. This could indicate that, instead of reflecting housing-induced differences in individual welfare, varying levels of stereotypic behaviour in mink reflect different individual response styles to sub-optimal conditions (see e.g., Meagher et al., 2012a,b and Fureix et al. in press for similar arguments), perhaps akin to the 'active' and 'passive' response styles well studied in pigs and other species (e.g., Koolhaas et al., 1999). Furthermore, within the enriched mink, the more stereotypic subjects showed no physiological or anatomical signs of finding their enrichments inadequate, nor did they use their enrichments less, with the possible exception of high scrabblers: enriched mink displaying the most scrabbling showed a weak tendency to spend the least time in their enriched compartments (an effect that now needs replicating). Finally, mink with the least enrichment-usage showed no physiological or anatomical evidence of elevated stress. Correlates of enrichment-use were thus rather minimal, even though split half analyses (see Section 2) had revealed that individual differences in enrichment use were very stable ($p < 0.001$ for all measures). However, Type II errors could have occurred here. This possibility is suggested by the unexpected absence of relationships between spleen and thymus weights, between thymus weight and HPA variables, and between fluctuating asymmetry and all other indices of chronic stress, which suggests that our methodology could have been improved upon by, for example, collecting all data closer together in time, and using histological analyses of thymus and spleen morphology.

Overall, we thus could not conclusively identify which types of individual thrived most in enriched conditions, nor identify which individuals had the poorest welfare in non-enriched cages. The clearest finding from the second strand of our study was that individuals showed strong, consistent differences in their enrichment use, in terms of both time spent in that compartment and interaction with the enrichments. Future research could usefully explore why animals show such strong individual differences in enrich-

ment use, with one possibility being that individual variation in boldness plays a role (cf. mouse studies, Walker and Mason, 2011), and another, that individual differences in motivation to perform certain specific activities such as wading, chewing, and resting in tunnels are instead important (cf. Mononen et al. (2008) for seemingly heritable individual differences in water use by mink; also Dallaire et al. (2012) for consistent individual differences in enrichment use in our same set up).

While we ascertained relatively little about the welfare significance of individual variation in stereotypic time-budgets, our results add to growing evidence that stereotypic behaviour is heterogeneous (Mason, 1991, 2006; Mason and Turner, 1993; Garner et al., 2011; Pomerantz et al., 2012a,b). Recurrent perseveration, which is suggested to implicate dorsal striatal ('motor loop') dysfunction (Garner, 2006; Lewis et al., 2006), had already been shown in mink to co-vary with loco stereotypies but not with scrabbling, suggesting that not all forms of mink stereotypic behaviour reflect this form of behavioural disinhibition (Dallaire et al., 2011; Campbell et al., 2013). Pomerantz et al. (2012a) also recently reported similar effects in primates, where some types of stereotypic behaviour (e.g., head twirling, hair plucking) positively co-varied with perseveration but others (e.g., pacing) did not. In addition, mink with loco stereotypies show low interest in exploring stimuli, while no such relationship is found for scrabbling mink (Meagher and Mason, 2012). Furthermore, loco stereotypies predict reduced copulatory success in male mink, while scrabbling does not (Díez-León, 2014). To this, our data adds that the proportion of mink displaying loco stereotypies was higher in NE-raised animals (though affected animals had similar time-budgets); but when differentially-reared animals were moved to small non-enriched cages, the number of mink displaying this type of SB did not increase, nor did affected individuals become more stereotypic. Thus across the population, loco stereotypies were stably reduced by a past history of enrichment, suggesting that they reflect lasting, intrinsic developmental effects on behavioural control (cf. Powell et al., 2000). Scrabbling, in contrast, displayed a different pattern, seeming more labile. E and NE mink were similarly likely to scrabble within their rearing conditions, but NE scrabblers spent more time in this behaviour. When moved to standardized environments nearly every mink revealed themselves as capable of this behaviour, regardless of rearing condition. Furthermore, the scrabbling levels of E mink tended to rapidly increase there to first become indistinguishable from, and then tending to be even higher than, those of NE-raised mink. Together this suggests that this sub-type of SB instead reflects animals' more immediate responses to perceived deficits in their current environment: a suggestion backed by our tentative findings of links between scrabbling and low current enrichment use. We therefore hypothesise that scrabbling is driven by reversible states of current frustration, while loco stereotypies instead reflect longer-lasting intrinsic changes in minks' brains (cf. Mason, 2006)—ideas that could be tested in the future by examining the physiology and anatomy of brains from differentially-raised mink with different forms of stereotypic behaviour (c.f. work on deer mice, Turner et al., 2002, 2003; Turner and Lewis, 2003).

5. Conclusion

Our study showed for the first time that long-term rearing and housing of a model carnivore, the American mink, with highly preferred enrichments induced expected reductions in performance of stereotypic behaviour and levels of excreted glucocorticoid metabolites. Furthermore, it led to anatomical changes consistent with better immunity and reduced developmental stress. It also confirmed that the degree to which enrichments are used seems to be a stable trait that differs between individuals. In addition,

different subtypes of stereotypic behaviour were revealed to differ in their relative sensitivities to developmental versus prevailing housing conditions, suggesting different aetiologies. However, within a given housing type, individual differences in stereotypic behaviour seemed to reflect behavioural response styles rather than differential welfare. To investigate the implications of these findings, we recommend continuing to use the American mink as a subject. Its diurnal, solitary, bold nature makes it easy to study; and it is both a good model for other wild carnivores, especially mustelids, and a worthy subject in its own right thanks to the large populations housed on fur farms around the globe (over 50 million farmed mink pelts were produced worldwide in 2010; Fur Commission, 2012).

Conflict of interest

The authors declare no conflict of interest.

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