Effects of Environmental Enrichment on Stereotypic Behaviour and Reproductive Success in American Mink *Neovison vison*

by

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ABSTRACT

Effects of environmental enrichment on the stereotypic behaviours and reproductive success of the American mink *Neovison vison*

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The captive breeding of carnivores can be problematic for reasons that can be attributed to the chronic stress suffered in captivity. In addition, captive carnivores typically display high levels of stereotypic behaviour. These issues raise animal welfare concerns and also challenge the cost-effectiveness of *ex-situ* conservation breeding programs. This thesis is an investigation of the mechanisms by which environmental enrichment might improve reproductive success and reduce the performance of stereotypic behaviours in carnivores using a model species, the American mink (*Neovison vison*). This question was addressed in a research fur farm by studying the long-term effects of being raised in enriched *versus* non-enriched environments. Two hypotheses were tested: i) environmental enrichment increases individual reproductive success by reducing stress and/or normalising brain function; and, ii) stereotypic animals have compromised reproduction due to brain alterations induced by the impoverished captive environment that result in overall impaired behavioural control. Enriched animals showed physiological, anatomical and behavioural signs of improved welfare. In addition, enrichment improved male copulatory success by decreasing males' levels of stereotypic behaviour and repetitive tendencies. However, enrichment had no effect on female pre- or post-copulatory behaviour (with nest building perhaps being an exception). Other findings emerged from this study. Stereotypic animals were no worse or better off than non-stereotypic animals in either the enriched or non-enriched environments. This finding supports the hypothesis that within a given population, the performance of stereotypic behaviour reflects differences in response styles rather than differences in welfare. Moreover, different types of stereotypic behaviours seemed to have different causal basis, suggesting that when using stereotypic behaviours as welfare indices, different forms should be assessed separately. In conclusion, enrichment improved the welfare of mink as well as the reproductive performance of males by inducing changes in behavioural control. This has important implications for the millions of captive animals displaying stereotypic behaviours.

para mi aitatxo y para mi hermanica del alma

para mi amatxo

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"There is no subject so old that something new cannot be said about it"

- Fyodor Dostoyevsky (1821-1881)

Chapter I. The welfare of wild mammals in zoos and conservation breeding centers: the good, the bad and the ugly.

In this chapter, I examine the welfare of wild mammals kept in institutions that breed animals with the specific purpose of reintroducing them into the wild (i.e. conservation breeding centers) as well as those kept in zoological institutions[1]. I first briefly overview the history of keeping wild mammals in captivity and the paradigmatic change that zoos have undergone to participate in conservation efforts. I then highlight successful cases of species thriving in these institutions (i.e. "the good"), cases in which the animals' welfare is compromised (i.e. "the bad"), and cases where the evidence is less clear or where the display of certain behaviours is unappealing to humans (i.e. "the ugly"). I discuss the use of environmental enrichment as a tool to address welfare concerns. Finally, I explain why throughout the rest of the thesis I focus specifically on the problems of captive wild carnivores, why I chose to work with American mink *Neovison vison*, and the specific hypotheses I aimed to test.

As of 2010, over 270,000 individual wild mammals (either caught from the wild or their captive-bred offspring) were estimated to be kept in captivity (Conde et al., 2011) in at least 850 zoological institutions and conservation breeding centers (Müller et al., 2011) that spread over more than 80 countries (IUDZG, 1993; Müller et al., 2011). These institutions hold

[1]Some wild or at least semi-wild mammals are also farmed (e.g. American mink, red/arctic foxes, and raccoon dogs for fur; tigers and bears for Chinese traditional medicine products; civets for musk; Asian elephants for logging; elk for food) or used in research (e.g. several rodent, primate and cetacean species); however, since animal welfare and behavioural normalcy, although important for some of them, are not the primary concern for these enterprises, the welfare status of mammals in said places will not be the focus of this review.

diverse taxa including Carnivora, Artiodactyla, Perissodactyla, Cetacea, Proboscidea, and Primates. People's fascination with wild, 'exotic' mammals is not new: the first account of a zoological collection dates back 4300 years (Hancocks, 2010). Wild mammals were kept by the upper social classes as a status symbol until c. 200 years ago, when collections were opened to the public and zoos undertook a more scientific/educational role, defining the concept of the modern zoo (Hancocks, 2001; IUDZG, 1993). In the last decades of the 20th century, in response to an increase in the number of species at risk in the wild (Griffith et al., 1989; Soulé et al., 1986), *ex situ* propagation with the ultimate goal of re-establishing or supplementing wild populations became, and still is, a common technique to promote the conservation of species (Griffith et al., 1989; Mathews et al., 2005; Moehrenschlager and Macdonald, 2003; Philippart, 1995; Wolf et al., 1996). Over twenty years ago it was stated that *"Within two or three decades it might be necessary to devote most of the space in zoos to the captive breeding of endangered species"* because it was expected that by now *"... most of the species currently held in zoos will be shifted [...] from the non-endangered to the endangered category"* (Soulé et al., 1986). Although that scenario has not been reached, the number of endangered species kept in zoos is growing from an estimated low of 14% in the early nineties (Jenkins 1992 as cited in Rahbek, 1993) to a present value of 25% (Conde et al., 2011), and many conservation breeding centres specialising in one or a few endangered species have appeared (e.g. the National Black-footed Ferret Conservation Centre in the USA).

The ultimate goal of captive breeding for conservation is reintroduction into the wild (Ebenhard, 1995; IUDZG, 1993; Philippart, 1995); therefore, zoos and conservation breeding centers need to be able to keep and breed species while preserving the species'

natural phenotypes, including their natural behavioural repertoires (e.g. Kirkwood, 2003). However, preserving natural phenotypes is challenging in captivity due to the contrast between most captive settings and the wild environment (e.g. a typical zoo polar bear *Ursus maritimus* enclosure represents one millionth of their minimum home range in the wild, Clubb and Mason, 2003). Phenotypical changes - including loss of behaviours such as predator avoidance or fearfulness - can occur within one generation due to experience of new environmental conditions, and also through multiple generations as a result of adaptation by natural selection (reviewed in Mason et al., 2013). Captivity also promotes the appearance of abnormal behaviours (e.g. Snyder et al., 1996; also see below). The performance of abnormal behaviours in many captive individuals, and the presence of breeding problems that can be attributed to captivity-induced stress (see below), raise animal welfare concerns about the individuals involved in these programs (concerns that also apply to individuals kept in zoos for display purposes only, e.g. Miller, 2011), and also question their cost-effectiveness (Braithwaite and Salvanes, 2005; Mathews et al., 2005). Therefore, animal welfare - ensuring the complete mental and physical health of an individual so that it adapts to its artificial captive environment without suffering (Duncan and Dawkins, 1983) - has become a priority for zoos and conservation breeding centers (e.g. Hutchins, 2003; IUDZG, 1993; Maple, 2003; Maple, 2014; Wickins-Drazilova, 2006).

A growing need for research on zoo animal welfare has been recently acknowledged (Walker et al., 2014). Animal welfare assessments often focus on indicators of chronic stress - the prolonged exposure to any factor that involves sustained activation of the Hypothalamus-Pituitary-Adrenal (HPA) axis and/or the sympathetic nervous system, leading to lasting

negative effects on reproduction, immune function and/or cognitive abilities (see Chapters 2-5). Although the focus is shifting to measuring the psychological well-being of the animal, independent of HPA activation (e.g. Mendl and Paul, 2004), most animal welfare research carried out at zoos and breeding centres measures endocrine parameters indicative of HPA activation (e.g. corticosteroids), health-related variables (e.g. the prevalence and incidence of disease), and life-history traits such as breeding success and relative life expectancy (e.g. Müller et al., 2011) as measures of animal welfare (Goulart et al., 2009; Hill and Broom, 2009; Hosey et al., 2009). These studies also typically record abnormal behaviours (e.g. stereotypic behaviours; see below), even though the relationship between them and welfare is not straightforward (see below). Thanks to such research, sources of stress in captivity have been found to be many and varied, and include climate, enclosure design, and forced contact with humans (reviewed in Morgan and Tromborg, 2007). Indeed, most of the evidence about the aversive effects of captive environments on welfare comes from within-species comparisons of enclosure types that differ in these regards (e.g. cage sizes, Cassinello and Pieters, 2000; use of environmental enrichment, Swaisgood et al., 2001; also see below; effect of human proximity, e.g. Davis et al., 2005). In practice, this translates into considerable within-species variation in the welfare of captive wild mammals depending on the relative magnitude of each potential stressor in each zoological institution or breeding centre. In the following sections I use examples from different species to illustrate instances in which the welfare of the animals can be considered good, bad or ugly.

Animal welfare in captive wild mammals: the good

Overall, captive wild mammals often enjoy good levels of welfare, as can be expected in an environment where sources of wild mortality are removed, veterinary care is provided and nutrition tended to. For example, compared to free-ranging individuals, captive-born Iberian lynx *Lynx pardinus* show decreased antibody titers against toxoplasmosis (Garcia-Bocanegra et al., 2010), and captive bottlenose dolphins *Tursiops truncatus* develop oral and/or genital tumors at a later age than free-ranging individuals (Rehtanz et al., 2010). Also, mammalian adult lifespans are typically improved, with individuals in captivity living longer than individuals in the wild (Lynch et al., 2010; Müller et al., 2011). Examples of this include some charismatic endangered species such as black-footed ferrets *Mustela nigripes* and giant pandas *Ailuropoda melanoleuca*. There is also an overall improved life-long reproductive success in some species (Mason, 2010), as evidenced by increased fecundity (e.g. golden lion tamarins *Leontopithecus rosalia*, De Vleeschouwer et al., 2003), and lower percentages of perinatal mortality (e.g. Santa Catalina Island foxes *Urocyon littoralis*, Clifford et al., 2007; Vancouver Island marmots *Marmota vancouverensis*, Bryant, 2005) in captive compared to free-living wild animals.

Animal welfare in captive wild mammals: the bad

Despite these examples of good welfare in captivity, the breeding of some wild mammals in zoos and conservation centres continues to face problems that are related to captivity-induced chronic stress. The captive individuals of some species show evidence of sustained activation of the HPA compared to wild counterparts (e.g. cheetah *Acinonyx jubatus*, Terio et al., 2004); and some captive environments can also elevate morbidity and mortality, and depress reproduction, as outlined below.

Compromised health and reduced lifespan

There is evidence of stress-related health issues in captivity. For example, captive cheetahs show a higher incidence of gastric ulceration than do wild cheetahs (Munson et al., 2005). Captive Florida manatees *Manatus latirostris* are also more likely to suffer from papilloma virus infections than free-ranging counterparts, suggesting a stress-driven immuno-depressive state in captivity (Dona et al., 2011). Finally, African elephants *Loxodonta africana* and Asian elephants *Elephas maximus* experience health problems in captivity (e.g. obesity, lameness, diabetes and viral infections; Clubb and Mason, 2002; Richman et al., 1999), problems that may explain why captive adult females from both species die earlier than females who are protected in their range countries (Clubb et al., 2008).

Compromised reproduction

Chronic stress has also been suggested to cause certain forms of reproductive failure in captivity. For example, a high percentage of fertile males of several species fail to mate (e.g. black-footed ferret males, Wolf et al., 2000; cotton top tamarins *Saguinus oedipus,* Ginther et al., 2001; several felids, Mellen, 1991; Swanson et al., 2003). In females, levels of acyclicity are higher than expected under captive conditions in several species (e.g. African and Asian elephants, Brown et al., 2004; several small felids, Mellen, 1991; black rhinoceroses *Diceros bicornis* and white rhinoceroses *Ceratotherium simum*, Carlstead and Brown, 2005; Morrow et al., 2009; cheetahs, Wielebnowski et al., 2002; lion-tailed macaques *Macaca silenus*, Sharma et al., 2006; Santa Catalina Island foxes, Clifford et al., 2007). Captivity-induced stress has often

been invoked as an explanation. In captive southern white rhinoceroses, for example, females in small enclosures vocalised more (a sign of stress in other species, e.g. sheep, Carbajal and Orihuela, 2001) than other captive individuals kept in more spacious enclosures than their wild counterparts (Metrione et al., 2007), with lack of space in captivity correlating with reduced breeding success in another rhinoceros species (black rhinoceroses, Carlstead et al., 1999a; Carlstead et al., 1999b). Similarly, captive-born African and Asian elephant females have lower fecundity and shortened reproductive lifespans than those observed in naturally managed populations, for reasons consistent with stress and obesity induced by captivity (Clubb et al., 2009).

Maternal stress in captivity is also suggested to underlie the high rates of infant mortality observed in some species, which may surpass those found in wild populations. This is the case for at least seven species of captive wild mammals: Asian elephants (Clubb et al., 2008), lions *Panthera leo* (Clubb and Mason, 2007), chimpanzees *Pan troglodytes* (Courtenay and Santow, 1989) and other old-world primates (Debyser, 1995), black-and-white ruffed lemurs *Varecia variegata* (Schwitzer and Kaumanns, 2009), mongoose lemurs *Eulemur mongoz* (Perry et al., 1992); red wolves *Canis rufus* (Lockyear et al., 2009); and Indian rhinoceroses *Rhinoceros unicornis* (Zschokke and Baur, 2002). Indeed, in at least three mammalian orders (Artiodactyla, Carnivora and Primates), juvenile mortality is relatively higher in captive *versus* wild populations (Lynch et al., 2010).

Animal welfare in captive wild mammals: the ugly

Captive environments can promote a series of phenotypical changes that deviate from the natural norm and are therefore deemed undesirable by zoo managers and the public, even though empirical evidence suggests that these captive phenotypes might not always inform about the performers' welfare.

Anatomical and physiological changes

Compared to wild-born adults, captive-born wild mammals of some species have smaller skeletal pieces (e.g. shortened limb bones of black-footed ferrets, Wisely et al., 2005) and smaller brains (e.g. lions *Panthera leo* and tigers *Panthera tigris*, Yamaguchi et al., 2009; American mink, Kruska and Schreiber, 1999). However, these changes likely reflect adaptation to captivity due to loss of costly traits no longer needed in such environments (Mason et al., 2013) rather than reflect negative welfare states. Obesity is also common among elephants (e.g. Hatt and Clauss, 2006) and primates (e.g. lemurs, Goodchild and Schwitzer, 2008). Although it might not reflect current welfare states, it might negatively impact the animals' future welfare by increasing the risk of health issues and shortening lifespans (Clubb et al., 2009; Kuhar et al., 2013).

Behavioural changes

Some behaviours lost or modified in captive environments include predator avoidance (Griffin et al., 2000) and fearfulness (Carlstead et al., 1999b). While these behaviours might be adaptive *ex situ* and either neutral or positive (e.g. reduced fearfulness associated with lower endocrine stress responses, Carlstead and Brown, 2005) in terms of welfare, they

might compromise welfare if the individuals are to be reintroduced (e.g. Moehrenschlager and Macdonald, 2003). Additionally, wild captive mammals have been reported to engage in behaviours that, although they are seen in the wild, are much more prevalent in captivity. These behaviours are also unlikely to reflect welfare problems, yet are not desirable from a display point of view. One of such behaviours is the masturbation observed in several species (e.g. elephants, lions, monkeys, dolphins and porcupines, Morris, 1969), which at least in some primates (agile *Hylobates agilis* and Müllers gibbons *Hylobates muelleri*) might reflect improper social housing conditions (Cheyne, 2006). Another one is begging behaviour from humans, which might result from unintentional training by visitors' occasional feeding (Cook and Hosey, 1995). This could be welfare-neutral, or indicate a welfare problem if it reflects underlying hunger (Gothard, 2007), but either way is typically not wanted by zoo personnel. Other undesirable behaviours include coprophagy (e.g. gorillas *Gorilla gorilla*, Akers and Schildkraut, 1985; Przewalski's horses *Equus ferus*, Boyd, 1991) and high levels of intra-specific aggression (e.g. European mink *Mustela lutreola*, Habbe and Peters, 2007; black rhinoceroses, Carlstead et al., 1999a). In captivity, both coprophagy and aggression can be triggered by stressful events (e.g. Boyce et al., 1998; Faraldo and Taylor, 2003). However, coprophagy might also have diet-related functional consequences (e.g. Cassella et al., 2012; Krief et al., 2004; Sakamaki, 2010), while high levels of intra-specific aggression can also stem from deficient early experiences and/or brain changes (e.g. Mason 1968 as cited in Novak et al., 2006) that might not reflect current suffering. Thus, it is unclear whether these indicate poor welfare, although aggression can obviously cause it. However, they are both undesirable: they can make displays unappealing and animals hard to manage.

The most common among the "ugly" behaviours observed in captivity is the performance of stereotypic behaviours. These are abnormal repetitive behaviours (e.g. pacing, swaying, tongue rolling, regurgitation/reingestion, self-plucking and self-injurious behaviours) thought to be caused by frustration (defined as the inability to fulfill strong motivations, usually accompanied by HPA activation, e.g. Mason and Burn, 2011), attempts to cope (where coping refers to a behaviour that is performed due to its beneficial effects on reducing a negative state, e.g. Würbel et al., 2006) or neurological dysfunctions that result in altered motor control (Mason, 2006). Stereotypic behaviours are performed by tens or even hundreds of million individual animals in captivity (Mason and Latham, 2004) but they are never seen in the wild. In zoos and conservation breeding centres, across the species prone to such behaviours (cf. Clubb and Mason 2003), the proportion of individuals that display them varies from over 40% (elephants, both African and Asian pooled) to 80% and over (e.g. many carnivores, black rhinoceroses, giraffes *Giraffa camelopardalis* and okapis *Okapia johnstoni*; reviewed in Mason et al., 2007).

Stereotypic behaviours are typically regarded as a sign of poor welfare when comparing different populations or treatment groups because factors that independently decrease welfare also increase the performance of stereotypic behaviours (Mason and Latham, 2004). However, Mason and Latham (2004) found that, within a similarly treated population (i.e. at the individual level), the welfare (measured independently using physiological variables and/or preference tests) of stereotypic animals was no worse or even better than that of non-stereotypic individuals. There are three possible explanations for this apparent paradox. First, some stereotypic behaviours might be "scars" of past aversive conditions that, for

example, could have caused lasting alterations in how behaviour is controlled by the brain (e.g. Garner and Mason, 2002; Vickery and Mason, 2005); this therefore renders them neutral about the current emotional state of the animal (Mason and Latham, 2004). For example, stereotypic behaviours induced by early social deprivation and/or impoverished housing can persist into adulthood, long after social conditions have been relatively normalized or environmental conditions enriched (e.g. Lutz et al., 2003; Meyer-Holzapfel, 1968). Second, they can result from coping attempts and therefore might help improve individuals' current welfare states (Mason and Latham, 2004). For example, stereotypic horses have lower heart rates when responding to stressors than their non-stereotypic counterparts (Minero et al., 1999). Third, there are alternative responses to stereotypic behaviours that may indicate poorer welfare still, such as fear-induced hiding (e.g. Dalm et al., 2009), or other causes of high levels of inactivity, such as pain (e.g. Wiseman et al., 2001), sickness (Hart, 1988) and/or depression (e.g. in humans, Gold and Chrousos, 2002). For example, in the same laboratory setting, wild-caught South African striped mice *Rhabdomys pumilio* were less stereotypic and instead more inactive than their captive-born counterparts; they were also more fearful, with higher corticosteroid levels (Jones et al., 2011). Similarly, young rhesus macaques that were more gentle and fearful were less likely to develop stereotypic behaviours than their more active, curious counterparts (Gottlieb et al., 2013). Stereotypic behaviours thus broadly indicate sub-optimal conditions, but within those conditions, individuals with spontaneously high levels might not have the poorest welfare: just be disturbing and unappealing to human viewers.

Even though the welfare implications of some of these behaviours might not be clear at the individual level, there are still four reasons why zoos and breeding centers try to prevent and/or eliminate the performance of all these abnormal behaviours: i) as a rule, they warn of husbandry conditions leading to sub-optimal welfare at the group level; ii) they are viewed as undesirable by the general public (Miller, 2012); iii) from a reintroduction perspective, they are not part of the natural behavioural repertoire of the species, and might even indicate a loss of the behavioural flexibility needed in the wild (Vickery and Mason, 2003); iv) if they indicate brain dysfunction, stereotypic behaviours might have, or correlate with, other deleterious effects on, for example, courtship or maternal care (Chapters 3-5).

Typically the approach to tackle them, and also some of the indicators of stress reviewed above, has been to add biologically relevant stimuli to the captive environment to improve welfare, a technique known as environmental enrichment (Newberry, 1995; Shepherdson, 1998a).

Environmental enrichment

Environmental enrichment has positive effects on animals' brains, stress physiology and behaviour (see Chapters 2-5), although most of these benefits have never been studied in captive wild mammals. To illustrate, in some laboratory and farm animals, enrichment increases brain cortical thickness (Greenough et al., 1985) and neurotrophin expression (e.g. Ickes et al., 2000), and enhances learning and memory (e.g. Beattie et al., 2000; Schrijver et al., 2004). In terms of stress physiology, it typically reduces the activation of the HPA axis in a

wide range of species, including wild mammals (e.g. Boinski et al., 1999; Moreira et al., 2007); and in laboratory species, it improves immune-related parameters (e.g. Van Loo et al., 2004). Also, enrichment generally decreases the performance of stereotypic behaviours, including those displayed by animals in zoos and breeding centres (e.g. Shyne, 2006; Swaisgood and Shepherdson, 2006).

There are several non-mutually exclusive hypotheses to account for how enrichment produces this wide range of positive effects: it can reduce fearfulness levels (assessed via adrenocortical and behavioural responses) by, for example, providing places to hide (Carlstead et al., 1993); allow the performance of highly motivated behaviours, thereby reducing frustration (Mason et al., 2001; Wiedenmayer, 1997); induce neuronal growth and increase glucocorticoid receptors in brain areas that control emotional responses (e.g. amygdala, Nikolaev et al., 2002); provide control over the environment, thus reducing stress (Owen et al., 2005); and promote exercise that can promote neurogenesis as well as reduce stress (Hansen et al., 2010; Hansen and Jensen, 2006; Malisch et al., 2008). Due to its overall positive impact on welfare, environmental enrichment has been hypothesised as a means to address reproductive problems (Carlstead and Shepherdson, 1994) and deficiencies in parental behaviour (Shepherdson, 1998a) in captive wild mammals, although this hypothesis has not been empirically tested in captive wild mammals (see Chapters 3 and 4).

Scope of the thesis

In this thesis, I investigated the links between breeding success, stereotypic behaviours and housing conditions in captive wild carnivores through a long-term study of the effects of environmental enrichment on brain, stress physiology and behaviour in American mink (*Neovison vison*). Specifically, I investigated whether:

I. environmental enrichment increases individual reproductive success by reducing stress and/or normalising brain function; and,
II. stereotypic animals have compromised reproduction due to brain alterations induced by the impoverished captive environments that result in impaired behavioural control

To test these hypotheses, I examined how being raised in captive environments differing in complexity (i.e. enriched *vs.* non-enriched) affected mink in terms of the performance of stereotypic behaviours, aspects of behavioural control and their associated forebrain changes, stress responsiveness, and breeding success[2]. These hypotheses make the predictions that mink raised in enriched environments will have increased brain activity and behavioural flexibility; as well as increased welfare as evidenced by positive changes in stress-sensitive traits; and as a result better reproductive performance and offspring number and quality.

I focused on carnivores for the three reasons. First, the popular appeal of carnivore species helps raise awareness and financial support for conservation efforts (Ferrer and Negro, 2004;

[2]All research procedures carried out towards the completion of this thesis were approved by both the University of Guelph Animal Care Committee (AUP #07R033) and the Michigan State Institutional Animal Care and Use Committee (AUF #04/07-041-00).

Gittleman, 2001; Jule et al., 2008; Sergio et al., 2006). Second, over 35% of the Carnivora species are in one of the IUCN categories requiring conservation action (i.e. vulnerable, near threatened, endangered or critically endangered, IUCN, 2013), making them the focus of many captive breeding and reintroduction programs (Jule et al., 2008): programs that could be crucial to prevent the extinction of species with dramatically low numbers in the wild (e.g. Iberian lynx), but which are also expensive (e.g. red wolf: $4.5 million over five years, Wayne and Gittleman, 1995; giant panda: $1 million per animal, Goodman, 2006; black-footed ferret: $40,000 per animal, Hess, 1996). Finally, carnivores are the focus of many enrichment programs implemented in zoos and conservation breeding centres (Shepherdson, 1998b), partly due to the high prevalence of stereotypic behaviours in the taxon (e.g. Clubb and Mason, 2007; see above); but also because they display all of the welfare issues discussed so far (see Chapters 2-5 for more details). A phylogenetic tree and conservation status of each carnivoran family can be found on Figure 1 and Table 1.

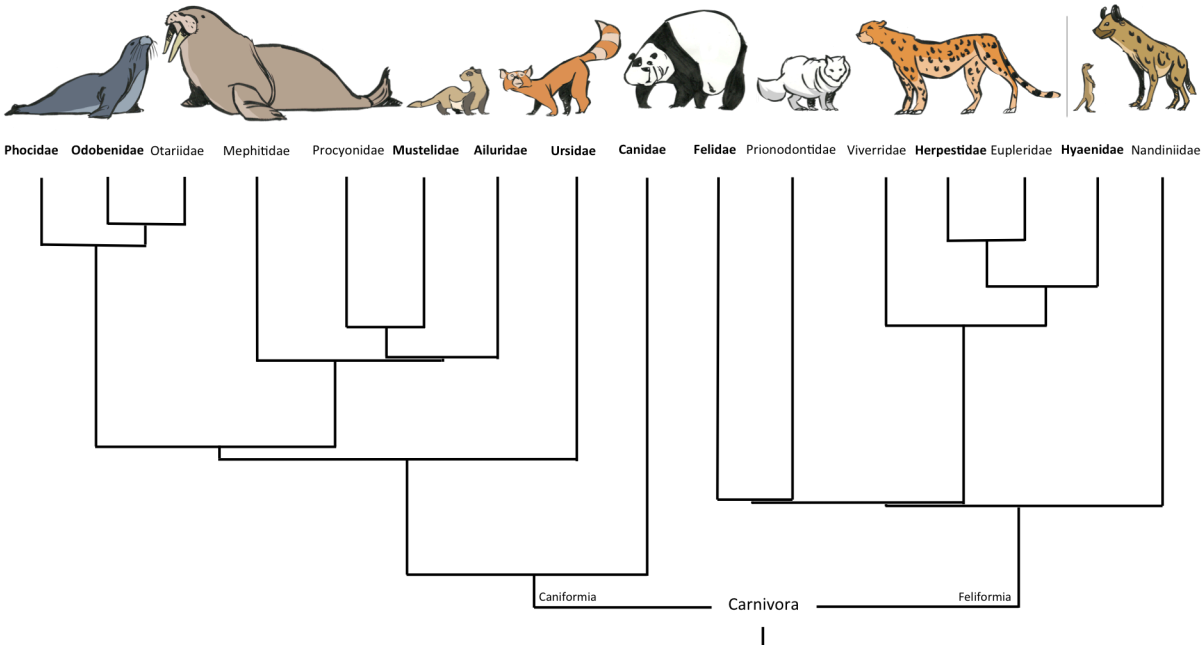


Figure 1. Phylogeny for the families within Carnivora. Modified from Nyakatura and Bininda-Emonds 2012. Families in bold are illustrated, from left to right: Mediterranean monk seal (Phocidae: *Monachus monachus*), walrus (Odobenidae: *Odobenus rosmarus*), black-footed ferret (Mustelidae: *Mustela nigripes*), red panda (Ailuridae: *Ailurus fulgens*), giant panda (Ursidae: *Ailuropoda melanoleuca*), Arctic fox (Canidae: *Alopex lagopus*), cheetah (Felidae: *Acinonyx jubatus*), meerkat (Herpestidae: *Suricata suricatta*), spotted hyaena (Hyaenidae: *Crocuta crocuta*). Illustrations by Íñigo Izal Azcárate.

Table 1. Percentage of living species within each Carnivora family falling in each of the International Union for the Conservation of Nature criteria for protection (2013). Species for each family follow those identified in Nyakatura and Bininda-Emonds 2012 (only species were counted, i.e. any subspecies found were disregarded). CR=critically endangered; EN=endangered; VU=vulnerable; NT=near threatened; LC=least concern; DD=data deficient.

	CR	**EN**	**VU**	**NT**	**LC**	**DD**	*total # of species*
Phocidae	11.11	5.55	5.55	0	66.67	11.11	*18*
Odobenidae	0	0	0	0	0	100	*1*
Otariidae	0	20	13.11	20	46.67	0	*15*
Mephitidae	0	0	8.33	0	91.67	0	*4*
Procyonidae	7.14	0	0	0	71.43	21.43	*8*
Mustelidae	1.75	10.53	8.77	5.26	63.16	10.53	*44*
Ailuridae	0	0	100	0	0	0	*1*
Ursidae	0	12.5	62.5	0	25	0	*8*
Canidae	5.88	8.82	0	11.76	73.53	0	*23*
Felidae	2.86	17.14	22.86	25.71	31.43	0	*30*
Prionodontidae	0	0	0	0	0	100	*2*
Viverridae	3.03	3.03	27.27	6.06	51.51	9.09	*28*
Herpestidae	0	0	6.06	3.03	81.81	9.09	*20*
Eupleridae	0	12.5	37.5	37.5	12.5	0	*6*
Hyaenidae	0	0	0	50	50	0	*4*
Nandiniidae	0	0	0	0	100	0	*1*

I conducted my research with a non-endangered carnivore model, the American mink, for the following reasons. First, captive breeding of two highly endangered species of mustelid - the black-footed ferret and the European mink – as reviewed, faces challenges including, poor male performance and high infant mortality (e.g. Kiik et al., 2013; Wolf et al., 2000; see also Chapters 3 and 4) and could thus benefit from basic research on the subject on a closely related species (Fig. 2). Second, the reproductive biology and development of American mink makes them good candidates for studying some fundamental questions not extensively addressed in carnivores yet (e.g. mate choice, maternal behaviour; see Chapters 3 and 5 for details). Third, the American mink is a particularly good model to study the effects of environmental enrichment and stereotypic behaviour on captive breeding problems. It represents the single species of carnivore for which most enrichment and stereotypic behaviour research has been conducted (Table 2, Chapter 2); the development, prevalence and other characteristics of the stereotypic behaviours of the species and the enrichments[3] that alleviate them and/or that mink value are well documented. Finally, looking beyond captive wild mammals in zoos and breeding centres, mink are farmed for their fur in very large numbers across the globe (over 50 million were produced worldwide in 2010, Fur Commission Commission, 2012), which makes them economically valuable and important in their own right due to the potential welfare implications for fur farming.

[3]Throughout the thesis, the terms 'enrichment' (referring to the husbandry technique as a whole) and 'enrichments' (referring to the specific husbandry modifications made) will be used interchangeably.

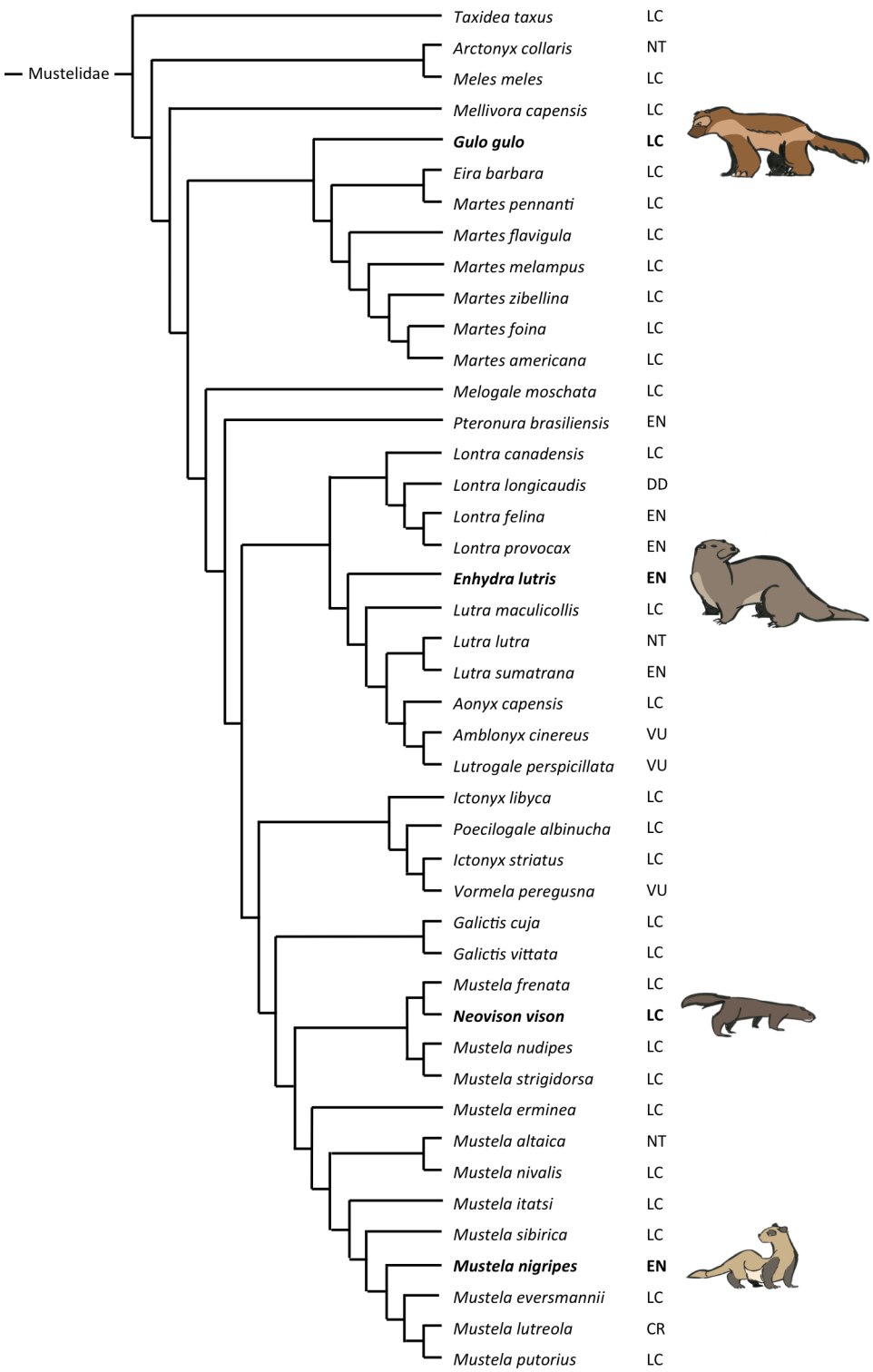


Figure 2. Phylogenetic tree of the Mustelidae (modified from Nyakatura and Bininda-Emonds 2012) with their conservation status as per the IUCN Red List 2013 (CR: critically endangered; EN: endangered; VU: vulnerable; NT: near threatened; LC: least concern; DD: data deficient). Species in bold are illustrated (from top to bottom: wolverine *Gulo gulo*, sea otter *Enhydra lutris*, American mink *Neovison vison*, black-footed ferret *Mustela nigripes*). Illustrations by Íñigo Izal Azcárate.

Table 2. Number of papers published on five different carnivores species to date as registered in Thomson Reuters Web of Science℠. Species other than mink were chosen because they typically stereotype in zoos and they are of known interest to zoo researchers and public. Three different searches were performed in which the species scientific name was entered as a topic (e.g. "*Mustela vison*") and then i) "environmental enrichment" was added as a topic; ii) stereotyp* was added as a topic; or iii) both "environmental enrichment" and stereotyp* were entered as topics. For the American mink, these searches were performed twice to account for both scientific names used in the literature (*Mustela vison* and *Neovison vison*).

	American mink	Tiger	Polar bear	Giant panda	Cheetah
	Mustela vison + Neovison vison	*Panthera tigris*	*Ursus maritimus*	*Ailuropoda melanoleuca*	*Acinonyx jubatus*
Environmental enrichment	26	14	11	14	10
Stereotypic behaviours	73	22	18	15	9
Environmental enrichment + Stereotypic behaviours	20	7	9	6	4

The outcomes of this research will help understand the problems seen in captive carnivores' reproduction, behaviour, morbidity, and responses to reintroduction (see Chapter 6). Ultimately it could potentially help determine which captive phenotypes are best suited to captivity and how to address specific adverse effects of captivity, so that the animals can enjoy good welfare in both captivity and also the wild if reintroduced.

The following chapter is in preparation for submission to Applied Animal Behaviour Science as:

Díez-León, M, Bursian, S, Galicia, D, Napolitano, N, Palme, R, Mason, G. Environmentally enriching American mink (Neovison vison) increases lymphoid organ weight and skeletal symmetry, and reveals differences between two types of stereotypic behaviour.

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

Abstract

Enrichment studies abound for wild zoo carnivores, but typically only measure effects on corticosteroids and stereotypic behaviour (SB), ignoring other stress-relevant variables (e.g. immune parameters). Furthermore, they are often short-term, and use enrichments of unknown motivational significance. Using a model carnivore, the American mink, we raised 64 male-female pairs long-term in non-enriched cages, or in enriched cages provided with preferred stimuli. At 7 months old, pairs were split and mink were individually-housed (adult mink are solitary), and temporarily moved to identical standardised cages (small, non-enriched cages) to allow behavioural assessment blind to treatment. After 2 weeks, one mink per pair was returned to its rearing cage (the other continuing to be observed in standardised cages for a further 2 weeks), for re-observation, collection of faecal samples for corticosteroid metabolite analysis, and additional research for another 1.5 years. They were then killed, and various stress-sensitive variables measured *post-mortem*. Two types of SB occurred: locomotor stereotypies, e.g. pacing, previously shown to correlate with recurrent perseveration; and repetitive scrabbling with the forepaws, unrelated to perseveration. As expected, enriched-raised mink in their rearing conditions performed less SB than non-enriched mink, and excreted fewer corticosteroid metabolites. The two subtypes of SB differed in how they were affected by the current environment. In enriched mink, enrichment use was stable and tended to negatively correlate with scrabbling, but was

unrelated to locomotor stereotypies. In standardised cages, enriched-rearing protected animals from performing high levels of locomotor stereotypy, but not from scrabbling, which increased such that enriched- and non-enriched-reared mink became indistinguishable. Finally, *post-mortem* data showed that enriched-reared mink had less skeletal fluctuating asymmetry and heavier lymphoid organs (thymus and spleen: important in cell-mediated immunity). None of these measures, nor corticosteroid metabolites, covaried with either form of SB. Thus, mink housed long-term with highly preferred enrichments, as well as expected reductions in SB and corticosteroid metabolites, showed changes consistent with better immunity and reduced developmental stress. Moreover, within a given housing type, individual differences in SB seemed to reflect response styles rather than differential welfare. SBs were also confirmed as heterogeneous: locomotor stereotypies were less sensitive than scrabbling to current housing and enrichment use, perhaps consistent with aetiology involving long-term brain changes.

Introduction

Environmental enrichment refers to the provision of stimuli required for animals' physiological and psychological welfare in captivity (Newberry, 1995; Shepherdson, 1998a). Effective enrichments improve an array of stress-sensitive physiological and behavioural variables. For example, in diverse wild and domesticated species, environmental enrichment decreases the performance of stereotypic behaviours (reviewed in Swaisgood and Shepherdson, 2005). Enriched environments are also generally preferred: animals voluntarily interact with them, and may even work to access them, indicating high motivation (e.g. American mink *Neovison vison*, Hansen and Jensen, 2006; Mason et al., 2001; pigs *Sus scrofa*, Elmore et al., 2011). In a wide array of species environmental enrichment has also been found to reduce activity of the hypothalamic pituitary adrenal (HPA) axis, decreasing adrenal gland weight (Abou-Ismail and Mahboub, 2011; Beattie et al., 2000) and lowering corticosteroid levels (Belz et al., 2003; Carlstead et al., 1993). In laboratory rodents, enrichment has also been associated with weight increases in two lymphoid organs essential for cell-mediated immunity: the thymus (Van Loo et al., 2004) and spleen (Abou-Ismail, 2011), both of which involute with chronic stress (e.g. Corbin et al., 2008; Mitchell et al., 2006). Finally, in laboratory rats and farmed rabbits, environmental enrichment can reduce levels of fluctuating asymmetry (Sorensen et al., 2005; Tuyttens et al., 2005). This is an anatomical measure of developmental instability (Polak, 2003) associated with early stress (Møller and Swaddle, 1997), and 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 aim was to gather for the first time basic information on all these behavioural, anatomical and physiological effects of environmental enrichment in a model carnivore, the American mink. Although captive wild carnivores have attracted many enrichment studies (Shepherdson, 1998b; Swaisgood and Shepherdson, 2006), their scope has been rather limited: focused on stereotypic behaviour or cortisol output, often rather short-term, and often using enrichments of unknown motivational significance to the animals (e.g. Poessel et al., 2011). Also, as a rule, in such studies, enrichment only halves the performance of stereotypic behaviour instead of abolishing/preventing it (Shyne, 2006; Swaisgood and Shepherdson, 2006), suggesting that the provided enrichments were inadequate or were offered too late in development (Mason et al., 2007). Likewise, although American mink represent the single species of carnivore attracting the most enrichment and stereotypic behaviour research (e.g. Axelsson et al., 2009; Hansen and Jeppesen, 2001; Mason et al., 2001; Mononen et al., 2008; see also Table 2 in Chapter 1), these studies have typically been as limited as those of other wild carnivores. None had explored the long-term effects on mink 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 (over 50 million farmed mink pelts were produced worldwide in 2010; Fur Commission, 2010). They could also help promote the more effective use of enrichment to enhance breeding and health in wild carnivores (especially other mustelids, such as European mink *Mustela lutreola*, and black-footed ferrets *Mustela putorius*) in zoos and captive breeding centres. Our specific predictions were that being raised with preferred enrichments would reduce cortisol output and adrenal weight; reduce or even prevent

stereotypic behavior, and also reduce fluctuating asymmetry, and increase spleen and thymus weights.

As well as investigating effects at the group level, our second main aim was to use mink 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 identical non-enriched housing conditions, where stereotypic behaviour is common, individuals typically vary greatly and consistently in how much of it they display. For example, some fur-farmed mink spend the majority 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 *Rhabdomis 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 poor environments. For example, stereotypic mink and African striped mice produce more offspring than less stereotypic, identically-housed peers (Jeppesen et al., 2004; Jones et al., 2010), while bouts of oral stereotypic behaviour are correlated with decreases in heart rate in 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 behaviours either help captive animals to cope with sub-optimal conditions (e.g. Wiepkema et al., 1987), or that non-stereotypic animals in such conditions are instead showing extreme inactivity due to

additional welfare problems, such as pain, sickness, fear-induced hiding, or depression-induced apathy (Mason, 2006; Meagher, 2011).

Individual variation in behaviour is also seen in enriched conditions. Although stereotypic behaviours are far less common here, certain individuals may still display them -- even if raised from infancy with enrichment (which has been hypothesised to have a neuroprotective effect against their development: Lewis et al., 2006; Powell et al., 2000). This has been observed in several rodent enrichment studies (e.g. Bechard et al., 2011; Jones et al., 2011; Latham and Mason, 2010; Ödberg, 1987; Turner et al., 2003), as well as in 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. Dallaire et al., 2012; Hansen et al., 2007; see also Chapter 3 / Díez-León et al., 2013). These findings suggest that for some individuals, the enrichments provided are insufficient at reducing the psychological stress 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, identically-housed individuals presented with identical enrichments show stable individual differences in the degrees to which they interact with them (Line et al., 1991; Lumeij and Hommers, 2008; Vickery, 2003; Walker and Mason, 2011), and in the

parrot study (Lumeij and Hommers 2008), feather-plucking individuals who voluntarily used enrichments the most, showed the greatest reductions in this stereotypic behaviour.

We therefore predicted that in non-enriched conditions, physiological and anatomical signs of stress would be lower in more stereotypic individuals. In enriched environments, in contrast, we predicted that highly stereotypic individuals would have poorer welfare according to physiological and anatomical indicators of stress than less-/non-stereotypic identically-housed conspecifics. Furthermore, we predicted that greater enrichment use would be linked to higher welfare, and less performance of stereotypic behaviour.

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 E, not collecting our first data until 5-6 months of differential exposure, and collecting the rest after one or two breeding seasons and up to 2 years of differential housing.

Material & Methods

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 individually housed 32 mink, distributed evenly by treatment and sex as shown in Figure 1. This was done to ensure proper experimental design for a later experiment (see Chapter 3 / Díez-León et al., 2013) as well as to avoid variation induced by proximity to the door (cf. Garner on mice [2011] and parrots [2003b]; and Gottlieb et al. [2013] on rhesus monkeys) and possible room effects.

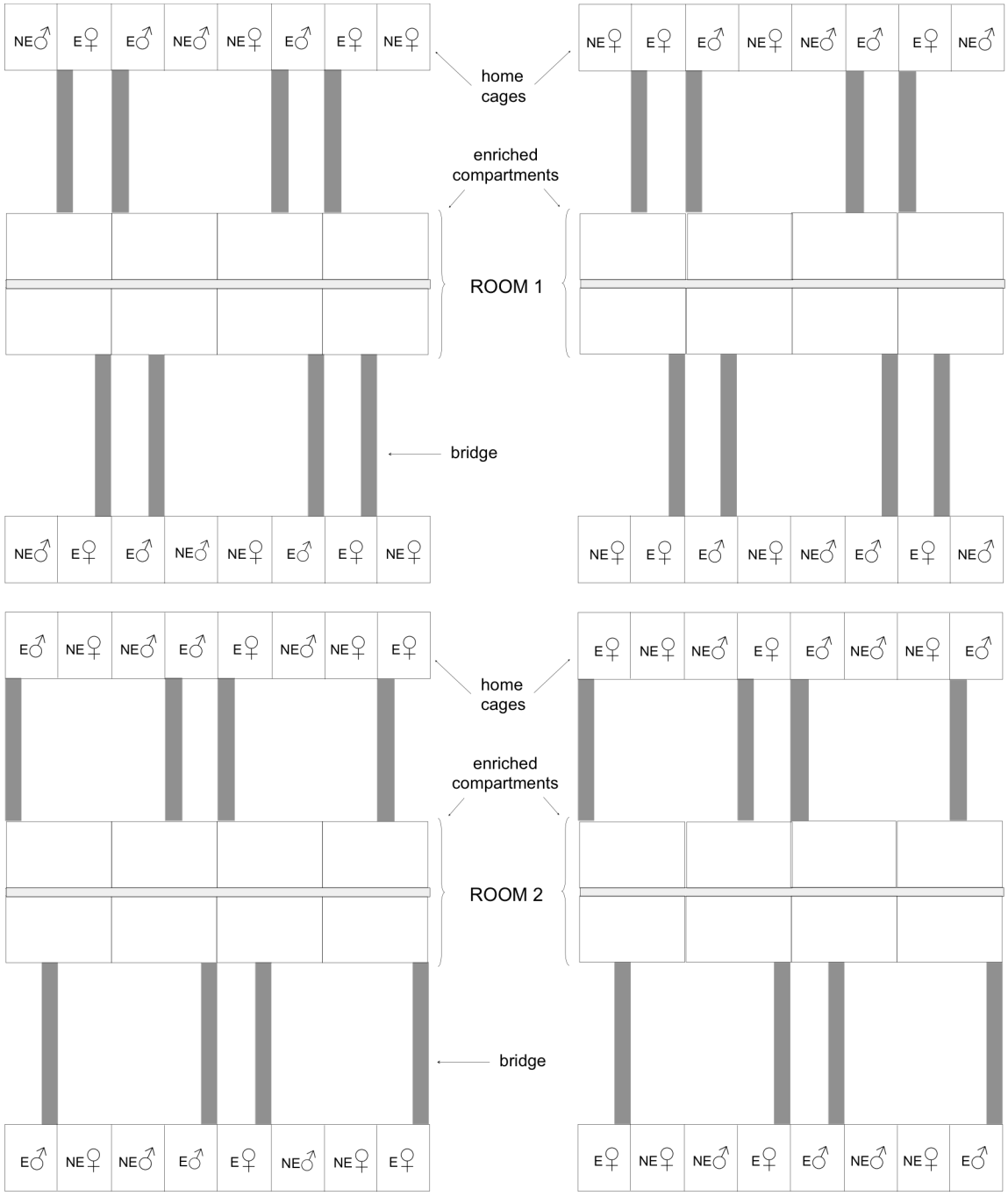


Figure 1. Distribution of experimental subjects according to treatment and sex. E=enriched-raised; NE=non-enriched-raised.

All mink had a wire mesh home cage measuring 61x76x46cm, provided with an attached plastic nest box (31x25x25cm; bedded with wood-shavings year round, which were replaced as needed). Water was available at all times via a nipple drinker and commercial mink food was provided approximately *ad libitum* on top of the cage once a day (at around 1200 hours). Solid blue high density plastic (polyethylene) partitions visually separated neighbouring cages. Non-enriched (NE) mink lived in unmodified home cages. Enriched (E) mink lived in cages modified by adding several structures based on the current knowledge of mink enrichment, as follows. Mink will work to access a water bath, preventing access to it will increase urinary cortisol levels (e.g. Korhonen and Niemelä, 2002; Mason et al., 2001), and swimming opportunities possibly decrease stereotypic behaviour (Mononen et al., 2008). Mink will also work to access alternate nesting places, novel objects (including toys), tunnels and platforms (Mason et al., 2001); and the provision of similar, simple enrichment items, like balls, chewing objects and elevated mesh tubes or shelves, also decreases stereotypic behaviour (Buob et al., 2013; Hansen et al., 2007) and faecal cortisol metabolite levels (Hansen et al., 2007). Therefore, we added at the back of the NE cage, a wire mesh stack (measuring 56x15x121cm) containing a series of sloping ramps for the mink to climb, leading to a 13x244x13cm overhead tunnel. This in turn was connected to an identical stack at the other end, which mink could descend via sloping ramps to access a separate E compartment. Each E compartment was double the size of the home cage (plastic partitions between them again visually separating neighbours), and provided with an extra wooden nest box (24Wx28Hx22L cm); a hard plastic "hammock" suspended from the cage ceiling by chains; and several objects that the mink could chase/chew, such as tennis balls, bungee balls and wiffle balls. In addition, a new toy (e.g. frisbees, maracas, etc.) was provided each month. All enrichment items were similar and replaced when needed. Enrichment items were always

new and free from other mink odours. At the front of the E compartment, mink could also access a water channel (c. 13Wx20Hx120L cm) provided with pumped and filtered circulating water 10-12 cm deep in which to wade and immerse themselves (see Fig. 2). Other experiments confirmed that the enriched environments used here greatly decreased stereotypic behaviours (Campbell et al., 2013; Dallaire et al., 2011; 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 cages as they would to reach food (Dallaire et al., 2012).

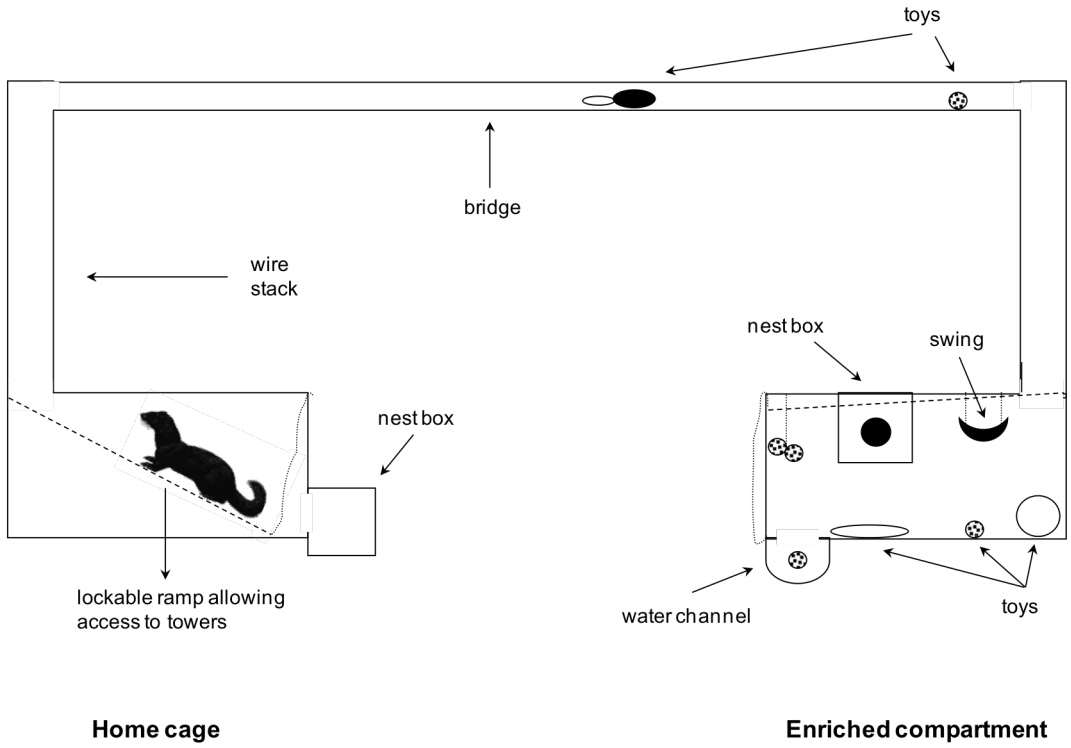


Figure 2. Diagram of the enriched treatment condition, showing the home cage and the connected enriched compartment. Two movable ramps could be raised (as shown in the enriched compartment in the figure) or lowered (as shown in the home cage in the figure) by a chain mechanism, in order to control access to the enriched compartment if needed.

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. 3 for an experimental timeline). The mother and the littermates were removed when our subjects were between 7-8 weeks old and each subject was housed with an unrelated opposite sex cagemate until seven months of age. This was done to enhance welfare, since pair-housing rather than isolating young mink improves their welfare (SCAHAW, 2001), while at the same time ensuring that all subjects had the same treatment and proper socialization for a later experiment (see Chapter 3 / Díez-León et al., 2013). Companion kits always came from the same housing treatment as the subject kits. At seven months, when mink reach 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. 3).

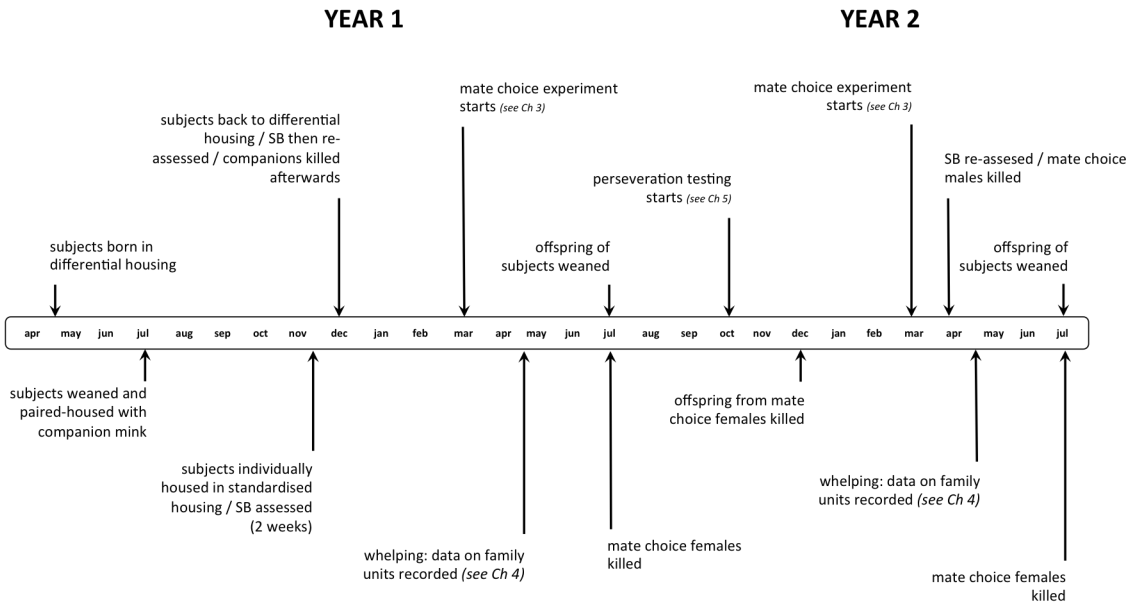


Figure 3. Experimental timeline for all experiments comprising the thesis.

Behavioural data collection

Behavioural variables (see Table 1 for the ethogram used) were first assessed when the mink were approximately seven months old – the age at which mink first start to show stereotypic behaviours consistently (Jeppesen et al., 2000; Mason, 1993). 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 25x76x38cm with a drop-in plastic nest box of 30x25x25cm) and housed singly for observation.

Based on previous studies (Meagher et al., 2009), we gave the animals five days to acclimatise to the new conditions before observations began. After five days, observations were performed every half an hour from 0800 until 1200 for eight days. This time of day was chosen to capture the pre-feeding period, when most stereotypic behaviours occur (Bildsøe et al., 1990; de Jonge and Carlstead, 1987; Mason, 1993). To minimise disturbance (cf. Clubb, 2001; Mason, 1993; Vinke et al., 2002), the observer 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 cage to be observed to allow the mink to habituate to her presence (e.g. Clubb, 2001; Pedersen et al., 2004). This type of direct observation has been validated for mink and shown to have minimal impact on the behaviour recorded (Mason, 1993; Svendsen et al., 2005). Observations combined scan and focal sampling (Martin and Bateson, 2007). 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 behaviours. Dallaire et al. (2011) divided them *a priori* into two classes: 'abnormal repetitive locomotion (ARL)' and 'scrabbling'. ARL includes pacing as well as whole body movements like repeated rearing in one spot (all typically reported in behavioural studies of caged mink; e.g. Bildsøe et al., 1990; Hansen et al.,

2007; Jeppesen et al., 2004; Malmkvist et al., 2011). Scrabbling describes a site-specific behaviour (i.e. not commonly reported in other populations, see Hansen and Jeppesen, 2000; Hansen and Jensen, 2006; Vinke et al., 2006) resembling the 'scratching' reported in some experiments as goal-directed attempts to enter areas to which access is denied (Hansen and Jeppesen, 2000). Dallaire et al. (2011) confirmed that these two classes of behaviour are distinct: under test, ARL correlated with recurrent perseveration (the inappropiate repetition of behavioural responses, e.g. Ridley, 1994), but scrabbling did not. Since recurrent perseveration has been suggested to implicate dorsal striatal ('motor loop') dysfunction (Garner, 2006; see also Chapter 5), following Garner (2006) and Mason (2006), here we term these locomotor and whole body behaviours 'locomotor stereotypies' (see Table 1).

Table 1. Ethogram used.

Behaviour		Description
stereotypic behaviours	locomotor stereotypies	repetitive body movements or locomotive route-tracing, performed as three of more consecutive repetitions (two or fewer repetitions were termed "borderline stereotypic behaviour", and were not counted as locomotor stereotypies but instead included in overall activity)
	scrabbling	scrabbling, normally at the corners 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 is scored as a locomotory 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 w/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)

Two weeks after these behavioural observations were completed in the standardised environments, the 64 subject mink were returned to their rearing environments (see Fig. 3), while the companion mink remained in the standardised environments until killed for their pelts ('pelted'). Subjects were given another five day period to re-acclimatise to their rearing cages and their behaviours were 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 compartment *vs.* the E compartment

(including the towers and the bridge); and ii) the amount of time spent interacting with the different enrichments (see 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.

At the end of the experiment, 48 mink remained – 16 females had been killed after the first year's reproductive trials (see Fig. 3; also Chapter 3 / Díez-León et al., 2013) – for which the differential rearing had lasted nearly two years. We re-assessed their stereotypic behaviours (to obtain a measure close to the time of death for use in analyses of physiological and anatomical stress-sensitive variables) and their use of enrichment for eight days right after the mating season following 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 (including enrichment use) was first checked by carrying out split-half analyses (Martin and Bateson, 2007), where data are randomly divided in two halves and the degree of correlation between those halves assessed. All were very significant ($p<0.001$), indicating strong internal reliability of the data. Stereotypic behaviours were calculated and analysed both as a % of observations (over total observations) and as % of activity (over total active behaviours, including stereotypic behaviour, see Table 1) (Campbell et al., 2013; Garner, 1999). Controlling for individual levels of activity is likely the more welfare relevant measure, and, furthermore, Campbell et al. (2013) found stereotypic behaviour expressed as a % of activity to correlate most closely with recurrent perseveration. However, measuring stereotypic behaviours as % of total observations is still most common

in the literature, and more helpful for understanding how animals budget their time. When results vary, they 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 using % 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 cages and animals allocating their time to different areas by chance would therefore spend more time there without this indicating true preference.

Data collection on physiological and anatomical stress-sensitive variables

At the end of the last breeding season and prior to pelting, adult male faeces were collected over five consecutive days (24h samples) and stored in a -20ºC freezer. Faecal samples were then thawed, mixed (i.e. all day samples for each male were pooled), homogenized and processed following the protocol of Palme and Möstl (1997). Samples were assayed using a mink-validated 11ß-hydroxyetiocholanolone enzyme immune assay (Malmkvist et al., 2011) by Dr. Rupert Palme's lab (University of Vienna).

Pelting of subjects occurred at three different times (Fig. 3). CO_2 is very aversive to mink (Cooper et al., 1998). Therefore, to make killing more humane, mink were anaesthetised first. Mink were trapped in carrying cages, which were placed individually in a transparent plastic container (approx. capacity 53l). This container had a hole in the lid into which we inserted a hose from a CO_2 cylinder. Mink were anaesthetised by squirting 10ml of

isoflurane into the container. This rendered mink unconscious in less than 60 s, with no obvious signs of suffering such as attempts to escape, vocalizations, hyperventilation or the release of 'skunk-like' stress odours. Then the container was filled with CO_2 for 3 minutes. Once dead, each mink was weighed, measured and pelted. Body weight and body size (recorded from the tip of the nose to the base of the tail) were quantified to be used as controls in analyses pertaining to organ weights.

Thymus, spleen and adrenals were removed by gross dissection (blind to treatment) as in previous enrichment studies (e.g. Abou-Ismail, 2011). Fat and connective tissue were dissected away, and each organ was then weighed to the nearest 0.0001 g (as was the precision of the scale used). Following weighing each organ was then either stored in formaldehyde (adrenals) or immediately frozen in liquid nitrogen (thymus and spleen) and stored in a -80ºC freezer.

Mandibles were also removed in order to assess fluctuating asymmetry (FA). They were selected for this purpose since they are a well studied structure in terms of development (e.g. Badyaev et al., 2000; Duarte et al., 2000; Klingenberg et al., 2003; Sans-Fuentes et al., 2009; see Fig. 4), and unlikely to be affected directly by differential stereotypic behaviour. After manual removal of the tissue around the mandibles (tissues were softened first by boiling the lower part of the mandible in a water and common detergent solution for 3-4h following overnight soaking in a similar solution), biometrical analyses of the labial side of the mandibles followed those of Galicia Paredes (2006). Briefly, pictures were taken of each side of each mandible and landmarks located using the software TpsDig (v. 2.0, Rohlf, 2004).

We processed mandibles by sides rather than by individuals (i.e. all the left mandibles were processed first, then all the right ones), to remove possibilities of subjective bias: this method ensured that the second set of landmarks for each individual was placed blind to the location of the first set of landmarks. The process was repeated twice to assess measurement error. Measurement error was smaller than levels of FA and therefore deemed negligible (Palmer and Strobeck, 2003). Both sets were averaged and mandibular FA was then calculated using Procrustes analyses to generate a landmark based index ("FA18", Palmer and Strobeck, 2003) which quantified the shape differences between sides.

Thus, in total, for both female and male mink, we had behavioural data (locomotor stereotypies, scrabbling, normal activity, inactivity and enrichment use) and physiological and anatomical data on stress-sensitive variables (adrenal weight, thymus weight, spleen weight and FA), as well as data on body length and weight. In the case of corticosteroid output, we only had data for males because at the time when these data were collected (at the end of the second breeding season) females were potentially gestating.

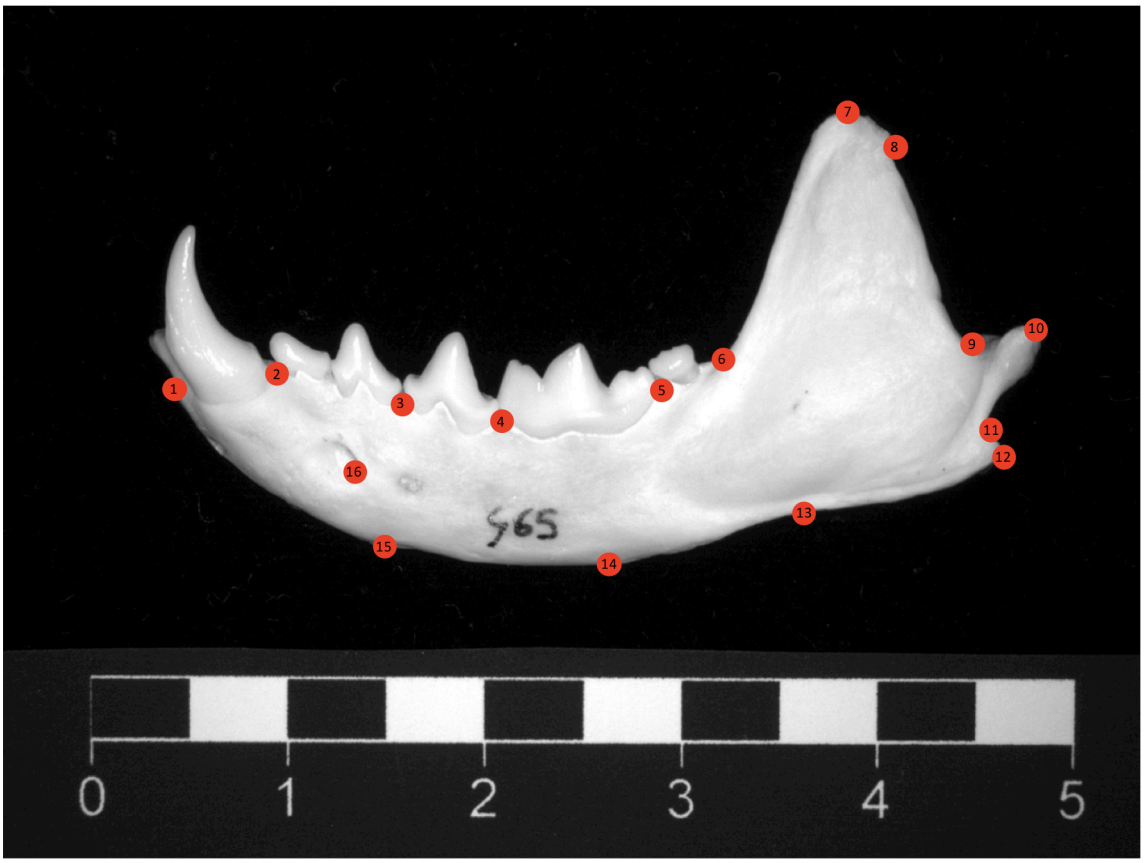


Figure 4. Location of landmarks on the labial side of the mandible.

Statistical analyses

For analyses involving prevalence 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 (SAS Institute, Inc., 2012). All interactions were included and 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). 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.

We investigated the impact of rearing condition on behaviour and physiology with GLMs in which either behavioural or physiological variables were the dependent variables, and rearing condition, sex and their interaction added as blocking factors. To assess whether individual behaviour was consistent between locations (i.e. between standardised and rearing conditions), behaviour in standardised cages was the dependent variable while behaviour in the rearing condition, rearing condition and sex were independent variables. In models investigating the relationships between behavioural measures, physiology and enrichment use, stereotypic behaviour or the various stress-sensitive variables were each set as dependent variables, and enrichment use was an independent variable, with sex as a blocking factor. 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 cortisol levels, because levels of physical activity can affect baseline output of this hormone (Hansen et al., 2010; Malisch et al., 2008), 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. halved when in the predicted direction) since we had clear *a priori* predictions (Levine and Banas, 2003; Rice and Gaines, 1994). 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 one-tailed, since we predicted positive relationships among HPA measures (adrenal weight and fecal cortisol metabolites levels), and among immune status indicators (thymus and spleen); negative co-variation between HPA and immune variables; and positive co-variation between fluctuating asymmetry and HPA variables, but negative co-variation between fluctuating asymmetry and immune variables (see Satterlee et al., 2008; Sealander and Bickerstaff, 1967; Walzer et al., 1984). Analyses pertaining to inactivity and the opportunistic observation of companion mink were two-tailed. We used "false discovery rate" procedures (FDR; Benjamini and Hochberg, 1995) to reduce risks of Type I error.

Results

Enrichment effects on behaviour

Overall, stereotypic behaviour was lower in enriched environments. When assessed in standardised cages at seven months of age, E mink spent less of their active time performing locomotor stereotypies than mink reared in NE housing, with no main or interactive effect of sex ($F_{1,60}$=23.66, p<0.001; Fig. 5a). However, E mink in these standardised cages did not differ in their scrabbling levels from NE mink. Treatment differences in locomotor stereotypy were also clear when subjects were assessed in their rearing cages (3 wks. later: $F_{1,60}$=25.27, p<0.0001; Fig. 5b; 1.5 yrs. later: $F_{1,44}$=4.40, p=0.020, although this last result was not significant after FDR corrections; Fig. 5c). E animals now also scrabbled less than NE animals (3 wks. later: $F_{1,60}$=24.89, p<0.0001; Fig. 5b; 1.5 yrs. later: $F_{1,44}$=4.60, p=0.018; again this last result was not significant after FDR corrections; Fig. 5c). The reduced levels of stereotypic behaviour in E housing reflected in part reduced prevalence (Table 2), along with reduced levels of the behaviour in those individuals displaying it (Table 3).

Repeated measures analyses comparing subjects' behaviour in the standardised cages and after return to their rearing environments, showed that locomotor stereotypies were not significantly different in the two locations, regardless of whether animals were E or NE, but scrabbling was significantly elevated in the standardised environments compared to rearing environment for E animals ($F_{1,30}$=125.15, p<0.0001; not significantly different for NE).

Data opportunistically collected from the companion animals after being in standardised cages for 5 weeks revealed that even at this time, treatment effects could still be detected on locomotor stereotypies, E animals displaying less than NE ($F_{1,58}$=31.33, p<0.0001); and now

treatment effects were evident for scrabbling, with previously E animals displaying more than NE ($F_{1,58}$=6.95, p=0.018), although this result was not significant after FDR corrections.

Table 2. Prevalence of stereotypic behaviours (% of animals performing them). Significant results in bold. E: enriched-reared. NE: non-enriched-reared.

	Standardised cages			**Rearing environments**					
				3 wks. later			1.5 yrs. later		
	E	NE	*n*	E	NE	*n*	E	NE	*n*
Locomotor stereotypies	**25**[1]	**75**	*64*	**22**[2]	**75**	*64*	29	58	*48*
Scrabbling	97	94	*64*	66	90	*64*	42	67	*48*

[1] $\chi 2$=4.50, df.=1,p=0.034 (not significant after FDR corrections)
[2] $\chi 2$=5.40, df.=1,p=0.01 (not significant after FDR corrections)

Table 3. Differences in levels of performance between enriched and non-enriched on the sub-set of mink displaying stereotypic behaviours. a) in standardised cages; b) in their rearing environments. Standard errors are given for least-square means. E=enriched-reared; NE=non-enriched reared. Significant results are in bold. Degrees of freedom vary between locomotor stereotypies and scrabbling analyses due to a larger number of mink performing the latter than the former.

a)

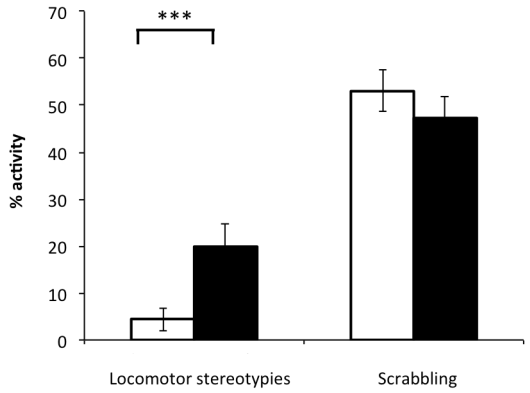
		n	Mean±SE	Statistic	P
Locomotor stereotypies	E	16	72.79±5.05	$F_{1,28}$=1.56	0.161
	NE	48	80.15±3.01		
Scrabbling	E	62	49.04±4.33	$F_{1,57}$=0.43	0.514
	NE	60	44.98±4.41		

b)

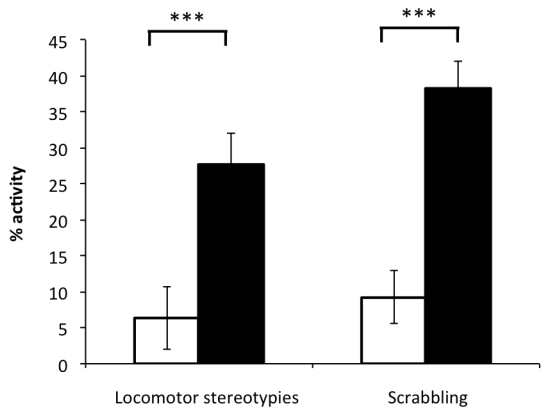
		3 wks.				**1.5 yrs**		
		n	*Mean±SE*	*Statistic*	*P*	*n*	*Mean±SE*	*Statistic*	*P*
Locomotor stereotypies	E	14	53.14±8.09	$F_{1,27}$=1.73	0.09	14	**68.97±7.92**	**$F_{1,17}$=8.26**	**0.01**[§]
	NE	48	65.22±4.32			28	**97.00±6.33**		
Scrabbling	E	42	**32.20±3.38**	**$F_{1,47}$=20.29**	**<0.0001**	20	28.52±7.36	$F_{1,22}$=2.27	0.072
	NE	58	**52.38±2.93**			32	41.97±5.02		

[§]not significant after FDR corrections.

a)



b)



c)

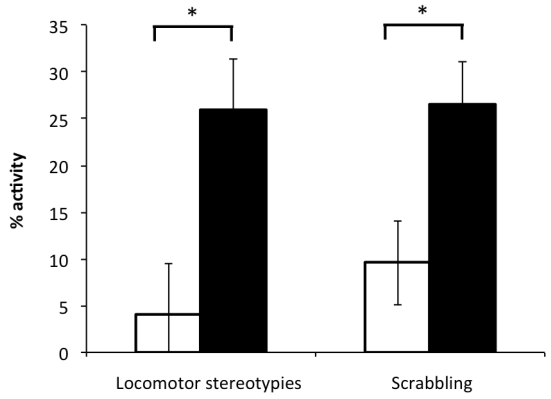


Figure 5. Amount of stereotypic behaviour exhibited by mink in: a) the standardised condition (i.e. smaller, barren cages to which all the animals were transferred to at seven months of age); b) the rearing condition 3 wks. later; c) the rearing condition 1.5 yrs. later (not significant after FDR corrections). Means represent raw least square means. White bars = enriched-rearing; black bars = non-enriched-rearing. Sexes are pooled as there was no main or interactive effect of sex. '*' = $p<0.05$; '***' = $p<0.001$.

In the standardised cages, E mink were more inactive than NE mink ($F_{1,60}$=3.158, p=0.04). Inactivity in the rearing environments three weeks after rehousing was affected by a housing type by sex interaction ($F_{1,60}$=4.46, p=0.039). *Post hoc* analyses revealed that E males were more inactive than NE males ($F_{1,30}$=4.24, p=0.048), while E and NE females did not differ. However, 1.5 years later, all E mink, regardless of sex, were more inactive than NE mink ($F_{1,44}$=6.39, p=0.015). Also, repeated measures analyses revealed that inactivity was significantly greater in the standardised environments compared to rearing environment, for E females only ($F_{1,30}$=10.01, p=0.004). However, all significant results regarding inactivity became non significant after FDR corrections.

In terms of using the E compartment, mink spent on average 20% of their active time interacting with enrichments; and both males and females spent most of their time in the E compartment/tunnel, with the exception of overall time at the end of the experiment (see Table 4).

Table 4. Time spent in the enriched compartment (EC) *versus* the home cage (HC) corrected by floor size (% time/m^2). Significant results in bold.

		3 wks.			1.5 yrs.		
		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**		

[§]not significant after FDR corrections.

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 (assessed only in males) (see Table 5). The effect of E on cortisol was still significant after statistically controlling for total activity ($F_{1,27}$=4.67, p=0.05). The positive effect of E on thymus weight was only evident after five outliers were removed (9% of the sample for this test).

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.

	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
Fecal glucocorticoid metabolites (ng/g)	**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**

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

Locomotor 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 expected relationships emerged between our measures, revealed just two significant

correlations (see Table 6): adrenal weight positively predicted faecal glucocorticoid metabolites (FCM) output ($F_{1,25}$=2.85, p=0.05); and spleen weight negatively covaried with FCMs, but only in the non-enriched group ($F_{1,9}$=4.33, p=0.05). However, these correlations became non significant after FDR corrections.

Table 6. Inter-relationships between stress-sensitive variables. FA = fluctuating asymmetry; FCM = faecal corticosteroid metabolites. Significant results in bold.

	FA	**FCM**	**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			$F_{1,25}$=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					

[§]not significant after FDR corrections.

Correlates of individual differences in enrichment-use

Turning to the subset of mink in E housing, there was no relationship between time spent in the E compartment on locomotor 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 ($F_{1,28}=2.78$, $p=0.054$; non-significant after FDR corrections). However, this effect disappeared after 1.5 years. Time spent interacting with enrichments was unrelated to either locomotor 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. Correlates of enrichment-use were thus rather few, even though split half analyses (see Methods) had revealed that individual differences in enrichment-use were stable ($p<0.001$ for all measures).

Discussion

Our first aim had been to see whether carefully chosen and implemented enrichments could have the types of effect on carnivores that have been documented in domesticated rodents and lagomorphs: lymphoid organ increases and reduced fluctuating asymmetry. In this we were successful. 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, but did reveal some interesting interactions between enrichment use and both types of stereotypic behaviours.

As expected, our enriched conditions reduced stereotypic behaviour: locomotor stereotypies and scrabbling were more than halved by E rearing, and living with enrichments significantly reduced both the number of animals displaying stereotypic behaviour and the time that stereotypic individuals devoted to this activity. The magnitude of this effect was thus considerably greater than the typical zoo study (where the average reduction in performance is 50%, Shyne, 2006; Swaisgood and Shepherdson, 2006) and most mink studies (where stereotypic behaviours are reduced by 50% or less, Buob et al., 2013; Dallaire et al., 2012; but cf. Hansen et al., 2007, who found larger reductions likely due to early enrichment rearing). Furthermore, screening our subjects in standardized conditions revealed that enriched-rearing conditions had long-lasting effects on locomotor stereotypies, protecting mink from displaying them even after five weeks in these small, non-enriched cages (although the same was not true for scrabbling: treatment effects on this behaviour vanished within two weeks of being moved to standardised cages). Given the magnitude of these effects, we suggest that studies in other carnivores that failed to find similar effects of enrichment on stereotypic behaviours may not have used sufficiently motivating enrichments. Alternatively, enrichments might not have been provided early enough/for a long enough time. The other expected finding was a reduction in FCM output, as also found by Hansen and colleagues (2007) and Meagher and colleagues (2013) for enriched mink, and Carlstead and colleagues (1993) for other enriched species of wild carnivore. Our data showed for the first time in carnivores, and as previously shown for lab mice (e.g. Groenink et al., 2002), that levels of these metabolites positively covary with adrenal weight (a result significant until FDR corrections) -- although this did not translate into a treatment effect on adrenal weight. The treatment effect also survived correcting for activity levels: important because physical activity can increase HPA activity (Hansen et al., 2010; Malisch et al., 2008), and our

enriched mink were significantly more inactive than NE animals making inactivity a potential confound. Overall, again given our successful results, we suggest that studies in other carnivores that failed to find effects of enrichment on corticosteroid metabolites (e.g. Poessel and colleagues' [2011] study of black footed ferrets) may have used enrichment of low motivational value or perhaps not given them early enough/for a long enough time.

Our results also revealed effects of enrichment that had not been previously demonstrated for this species or any other carnivore. We predicted that environmentally enriched mink would have heavier lymphoid organs and greater skeletal symmetry, and these predictions were supported. Larger spleens likely indicate larger red and white splenic pulps (Corbin et al., 2008): changes that could be investigated histologically in future work, and, if confirmed, would suggest greater lymphocyte production and enhanced capacities to initiate cell-mediated immune responses (e.g. Cesta, 2006). In our mink, at least in the NE animals, smaller spleens were also predicted by elevated FCMs, consistent with previous studies (Walzer et al., 1984). The larger thymic masses of E mink again are likely to indicate enhanced immune responses, due to greater amounts of thymopoyetic tissue and therefore higher capacity to produce T-cell precursors (or thymocytes) (Haynes et al., 2000; Taub and Longo, 2005; Wyllie, 1980); however, our thymus results should be taken with caution due to the possibility of measurement error induced by difficult dissection. An additional new finding was that enriched mink were more morphologically symmetrical. Greater FA in challenging environments or under inbreeding has been recorded before in wild carnivores (Edwards et al., 2013; Korablev et al., 2013; Pertoldi et al., 2000), 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. Such effects likely would not have been detectable had enrichment only been provided in adulthood.

Our second aim was to test the welfare correlates of stereotypic behaviour. We had predicted these 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; and highly stereotypic animals in enriched environments having poorer welfare, and showing less enrichment-use, than less stereotypic enriched conspecifics. We found little evidence to support either of these predictions. Within the non-enriched mink, there were thus no apparent difference 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; Meagher et al., 2012b, for similar arguments). In addition, within the enriched mink, relatively more stereotypic subjects showed no physiological or anatomical signs of finding their enrichments inadequate, again failing to replicate patterns suggested by Mason and Latham's (2004) meta-analysis. Nor did these animals 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. Furthermore, mink with the least enrichment-usage showed no physiological or anatomical evidence of elevated stress. 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. This suggests that our sample sizes were too low and/or these data deficient in quality (e.g. too spread intermittently over too long a time period; crude weights used instead of histological analyses of thymus and spleen morphology, etc.).

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 only clear finding from this part 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: a topic considered further in our "Future research" section. We suspect that to better resolve the welfare significance of individual differences in stereotypic behaviour in barren and enriched conditions, this study needs to be replicated on a larger scale, improving the number of replicates and the quality of data collected on HPA activity, fluctuating asymmetry, and lymphoid organ structure and function.

Our results also suggest new avenues for future work. One is to investigate the health and reproductive implications of the enrichment effects found here. The effects on spleen and thymus weight suggest that enrichment could enhance minks' abilities to resist infection, a hypothesis that could be tested using controlled immune challenges (e.g. Heller and Jeppesen, 1985). Furthermore, since enhanced immune systems can improve animals' attractiveness in mate choice scenarios (e.g. von Schantz et al., 1999), and since asymmetry in human faces and sexually selected traits in male birds reduces individuals' attractiveness as potential mates

(e.g. Waitt and Little, 2006), the enrichment effects on both lymphoid organs and morphological symmetry together suggest that enriched-reared mink could have enhanced mating success (see Chapter 3 / Díez-León et al., 2013). Such potential benefits could also be explored in wild carnivores in breeding centres and zoos. A second avenue could investigate the welfare significance of inactivity more thoroughly. We found that enriched mink spent more time sleeping and resting than NE mink; yet inactivity also tended to increase when enriched mink were deprived of enrichments and placed in small standardized cages. Meagher and colleagues (2013) found similar effects in other study populations of mink, where their more detailed records of forms of inactivity further showed that enrichment removal induced increases in specific types of inactivity that might reflect anxiety (hiding in the nest box) and boredom-like states (lying awake). This suggests that different forms of inactivity reliably indicate increases or decreases in well-being: a possibly useful tool for other welfare studies.

A third avenue could explore why animals show such strong individual differences in enrichment-use. One possibility is that individual variation in boldness plays a role (cf. mouse studies, Walker and Mason, 2011); another, that individual differences in motivations to perform certain specific activities like wading, chewing, resting in tunnels, etc. (cf. Mononen and colleagues [2008] for seemingly heritable individual differences in water use by mink; also Dallaire and colleagues [2012] for consistent individual differences in enrichment use in our same set up) are instead important. Fourth and finally, our data add to growing evidence that stereotypic behaviours are not homogeneous (Garner et al., 2011; Mason, 2006; Mason, 1991; Pomerantz et al., 2012a; Pomerantz et al., 2012b), and show that more

work is needed to understand how and why they differ. Recurrent perseveration had already been shown in mink to co-vary with locomotor stereotypies but not with scrabbling, suggesting that not all forms of mink stereotypic behaviour reflect behavioural disinhibition (Campbell et al., 2013; Dallaire et al., 2011). Pomerantz and colleagues (2012a) also recently found similar effects in primates, where some types of stereotypic behaviours (e.g. head twirling, hair plucking) positively co-varied with perseveration but others (e.g. pacing) did not. To this, our data add that when all animals were moved to small non-enriched cages, only locomotor stereotypies were reduced by a past history of enrichment, suggesting that only they reflect lasting, intrinsic effects of environmental enrichment on behavioural control (Powell et al., 2000). Scrabbling, in contrast was not, suggesting that it reflects instead the animal's more immediate response to its current environment: a suggestion backed by our possible findings of links between scrabbling and low current enrichment use. We therefore hypothesise that scrabbling is driven by readily reversible states of current frustration, but that locomotor stereotypies reflect longer-lasting intrinsic changes in minks' brains (cf. Mason, 2006) -- an idea that could be tested 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 and Lewis, 2003; Turner et al., 2003; Turner et al., 2002; also see Chapter 5).

For all these future research ideas, we highly recommend continuing to use the American mink as a subject: its diurnal, solitary, bold nature makes it easy to study; and as we reviewed in the Introduction, it is both a good model for other wild carnivores, especially mustelids, and a worthy subject in its own right thanks to the very large populations housed on fur

farms around the globe. From our study, we can conclude that environmentally enriching mink increases lymphoid organ weight and skeletal symmetry, and reveals differences between two types of stereotypic behaviour.

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*The modified sections in the text have been highlighted as follows: **modified text**; methodological sections that are similar to those presented in Chapter 2 have been highlighted in light grey so the reader can skip them.*

Chapter III. Environmentally enriched male mink gain more copulations than stereotypic, barren-reared competitors.

Abstract

Wild carnivores in zoos, conservation breeding centres and farms commonly live in relatively small, unstimulating enclosures. Under these captive conditions, in a range of species including giant pandas, black-footed ferrets and European mink, male reproductive abilities are often poor. Such problems have long been hypothesized to be caused by these animals' housing conditions. We show for the first time that rearing under welfare-improving (i.e. highly valued and stress-reducing) environmental enrichments enhances male carnivores' copulatory performance: in mate choice competitions, enriched male American mink (*Neovison vison*) mated more often than non-enriched males. We screened for several potential mediators of this effect. The first was physiological stress and its impact on reproductive physiology; the second, stress-mediated changes in morphology and variables related to immunocompetence that could influence male attractiveness; the third, behavioural changes likely to affect social competence, particularly autistic-like excessive routine and repetition ('perseveration') as is reflected in the stereotypies common in captive animals. Consistent with physiological stress, excreted steroid metabolites revealed that non-enriched males had higher cortisol levels and lower androgen levels than enriched conspecifics. Their *os penises* (bacula) also tended to be less developed. Consistent with reduced attractiveness, non-enriched males were lighter, with comparatively small spleens and a trend to greater fluctuating asymmetry. Consistent with impaired social competence, non-enriched males performed more stereotypic behaviour (e.g. pacing) in their homecages.

Of all these effects, the only significant predictor of copulation number was stereotypy (a trend suggesting that low bodyweights may also be influential): highly stereotypic males gained the fewest copulations. The neurophysiological changes underlying stereotypy thus handicap males sexually. We hypothesise that such males are abnormally perseverative when interacting with females. Investigating similar problems in other taxa would be worthwhile, since many vertebrates, wild and domestic, live in conditions that cause stereotypic behaviour and/or impair neurological development.

Introduction

Breeding problems are common in certain captive wild species, including several endangered Carnivora. To illustrate, in zoos and captive breeding centres many captive male European mink (*Mustela lutreola*, Kiik et al., 2013), black-footed ferrets (*Mustela nigripes*, Santymire et al., 2007), giant pandas (*Ailuropoda melanoleuca*, Peng et al., 2007; Zhang et al., 2004) and some felids (Mellen, 1991; Morato et al., 2001) fail to sire offspring; captive females may be acyclic (e.g. black-footed cat *Felis nigripes* and sand cat *Felis margarita*, Herrick et al., 2010); and infant mortality can also be excessive (e.g. in black-footed ferrets, Biggins and Godbey, 2003; Bronson et al., 2007; giant pandas, Zhu et al., 2001; African wild dogs *Lycaon pictus*, Ginsberg, 1994; and other carnivores, Clubb and Mason, 2007). Several authors have attributed this compromised reproductive performance to sub-optimal housing or husbandry (Bronson et al., 2007; Carlstead and Shepherdson, 1994; Clubb and Mason, 2007; Howard and Allen, 2007; Lindburg et al., 2001; Morais et al., 2002; Poessel et al., 2011; Roberts, 1989; Wielebnowski et al., 2002). For wild carnivores in zoos and breeding centres, the differences between natural and manmade environments can certainly be stark: the sizes of polar bear enclosures, for instance, have averaged approximately one millionth the area of even the smallest home ranges in the wild (Clubb and Mason, 2003). Furthermore, stereotypic behaviours (abnormal repetitive movements such as pacing, e.g. Mason and Rushen, 2006), are common in this taxon under these conditions: in behavioural studies of captive wild carnivores, 82% of individuals displayed some type of stereotypic behaviour (Mason et al., 2007). Caused by motivational frustration and/or changes in brain function (Mason, 2006), these typically indicate inappropriate husbandry (Mason and Latham, 2004). To date, researchers attributing reproductive problems to wild animals' housing conditions have emphasised the likely adverse effects of stress on reproductive physiology. This idea is

plausible because a number of studies, primarily of laboratory and farmed species, have shown that a range of aversive stimuli can act to reduce males' androgen levels, testis size, sperm production and effective spermatogenesis (Blanchard et al., 1995; Hardy et al., 2002; Lombardo and Thorpe, 2009; Suarez et al., 1996), and also compromise females' receptivity, neonate birthweights, litter sizes, and lactation (Braastad, 1998; Collier et al., 1982; Dodzi and Muchenje, 2011; Goetz et al., 2008; Sheriff et al., 2009; Wegner et al., 1976; Wegner and Stott, 1971). These effects have been shown to reflect inhibition of the hypothalamic-pituitary-gonadal (HPG) endocrine axis by hypothalamic-pituitary-adrenal (HPA) hormones (Torpy and Chrousos, 1996; Wingfield and Sapolsky, 2003). Furthermore, some HPG-dependent variables can be compromised in impoverished housing conditions (e.g. reduced testis size in Siberian hamsters *Phodopus sungorus*, Workman et al., 2010). Thus if captive conditions cause chronic HPA activation, reproductive problems might well ensue.

We further hypothesise two additional processes by which sub-optimal housing might lead to poor breeding in captive mammals. These ideas have been suggested respectively by behavioural ecology research on a wide range of species (including humans), and by behavioural neuroscience research (primarily on laboratory rodents). Our first novel hypothesis is that poor captive rearing environments act as "developmental stressors", altering the ontogeny of young males in such a way as to diminish their attractiveness to females as adults. To illustrate, larger mates are often preferred by females than smaller mates (Poole, 1989; Roed et al., 2007); yet developmental stress can compromise growth rates (Blanchard et al., 1995; Sapolsky, 1998), and captive conditions are known to render some males smaller than their free-living conspecifics (e.g. in black-footed ferrets, Wisely et

al., 2002; Wisely et al., 2005); jaguars *Panthera onca*, Morato et al., 2001). Morphological symmetry is another attribute that is generally preferred by females choosing mates (Møller, 1993; Waitt and Little, 2006). Deviations from symmetry (assessed as "fluctuating asymmetry", Ludwig, 1932 cited in Van Valen, 1962) can be induced by stress during early ontogenic stages (Møller and Swaddle, 1997; Spencer and MacDougall-Shackleton, 2011), and symmetry is also potentially compromised by development in barren cages (Sorensen et al., 2005; Tuyttens et al., 2005). Finally, immunocompetence and attributes that correlate with this are also important in mate choice (Thornhill and Gangestad, 1999), and yet impaired by both known stressors (Mitchell et al., 2006) and by impoverished housing (Abou-Ismail and Mahboub, 2011; Van Loo et al., 2004; Workman et al., 2010). Males raised in barren environments therefore seem at risk of developing into physically unattractive adults, but this idea has never been experimentally tested. Our second novel hypothesis is that poor developmental environments impair brain function, so as to render courtship, mating and/or parental behaviours less effective. In male songbirds, to illustrate, the neural substrates for attractive singing require low stress rearing conditions (Nowicki and Searcy, 2005; Spencer and MacDougall-Shackleton, 2011), while in mammals, barren rearing environments have two well-studied effects on brain and behaviour that are likely to compromise interactions with potential mates and/or dependent offspring. First, barren environments retard hippocampal and cerebral development (and even overall brain weight, Latorre, 1968; Rosenzweig et al., 1968), causing reduced learning abilities (e.g. Nithianantharajah and Hannan, 2006; Rosenzweig et al., 1968): potentially important because learning abilities increase attractiveness in several species (Boogert et al., 2011; Prokosch et al., 2009) and also help improve young adults' abilities to copulate as well as to care for their offspring (Broom and Fraser, 2007; Cauchard et al., 2013). Second, barren environments

induce stereotypic behaviours (e.g. Mason and Latham, 2004), in part by causing basal ganglia dysfunction (e.g. Muehlmann and Lewis, 2012). Bouts of stereotypic activity may be problematic if they interrupt normal intra-specific interactions (Rodriguiz et al., 2004; see Widholzer and Voss, 1978, for a zoo example). Furthermore, stereotypic behaviour typically reflects generalised changes in behavioural control that are manifest as abnormal tendencies to repeat actions and over-utilise fixed routines ('perseveration'). Such impairments in behavioural organisation characterise human disorders featuring stereotypic behaviour (Hill, 2004; Lam et al., 2008; Muehlmann and Lewis, 2012), and make affected subjects poor at the flexible 'give and take' of normal social interaction. These impairments are also widespread in stereotypic captive animals (e.g. Dallaire et al., 2011; Garner and Mason, 2002; Mason, 2006; Tanimura et al., 2008): perseveration has been found to be associated with stereotypic behaviour in all 15 species tested to date, with unknown implications for such animals' social, sexual and parental behaviours.

Adding environmental enrichment, i.e. welfare-enhancing stimuli to captive housing (e.g. Newberry, 1995), has therefore been proposed as a solution to wild animals' breeding difficulties (Carlstead and Shepherdson, 1994; Kiik et al., 2013; Peng et al., 2007; Poessel et al., 2011; Swaisgood et al., 2001). There are several recent indications that enrichment is a useful approach. Enrichment enhances male mating success in laboratory-reared flies (Díaz-Fleischer et al., 2009; Dukas and Mooers, 2003); prolongs reproductive lifespan in domestic hens (Leone and Estévez, 2008); and in mammals, enrichment seems to increase the number of infants succesfully weaned by females in laboratory mice (Whitaker et al., 2009) (though cf. Carvalho et al., 2009) and farmed mink (Buob et al., 2013). However, no study has

investigated the effects of enrichment in both sexes, through all stages of reproduction from mating inspections to weaning; nor has any study identified the mechanisms underlying any observed success. We therefore assessed whether raising captive Carnivora with enrichments improves their reproductive performance. We assessed our subjects from pre-copulatory phases (e.g. mate inspection) through to whelping, and also investigated the mediators of any effects. American mink (*Neovison vison*) were ideal model Carnivores because the enrichments that are beneficial for their welfare are well understood (e.g. Hansen et al., 2007; Mason et al., 2001); female behaviour and physiology suggest mate choosiness (Thom et al., 2004); their stereotypies are known to be accompanied by increased perseveration (Campbell et al., 2013; Dallaire et al., 2011); and small enclosures are commonly used for breeding endangered mustelids (Hellstedt and Kallio, 2005; Poessel et al., 2011; Santymire et al., 2007).

If barren rearing and housing conditions impair carnivore reproduction, then enriched-reared mink (henceforth simply 'enriched') should mate more and/or produce more offspring. If such effects are mediated by HPA activation, then enrichment should reduce faecal glucocorticoid metabolites (Belz et al., 2003), and/or adrenal gland weight (Abou-Ismail and Mahboub, 2011) and have beneficial effects on traits relating to the HPG axis, with these effects correlating with reproductive benefits. If barren environments reduce male attractiveness, then enriched males should be heavier, more symmetrical, and/or show evidence of improved immunocompetence (e.g. smaller lymphoid organs such as thymus and spleen [Corbin et al., 2008; Mitchell et al., 2006], both essential for cell-mediated immunity), with such changes in turn predicting increased mating success. Finally, if barren

environments affect psychological traits important in courtship and/or maternal care, then enrichment should boost relative brain weight and/or reduce stereotypic behaviours, with these changes in turn predicting enhanced reproductive abilities.

Methods and Materials

Animals, housing and differential rearing

Sixty-four multiparous pregnant mink of the Black colourtype born at the Michigan State University's research farm were moved into an indoor facility where they were housed in wire mesh home cages (61x76x46cm) with solid plastic sidewalls and an attached plastic nest box (31x25x25cm, bedded all year round). They were provided with *ad libitum* water via a nipple drinker and approximately *ad libitum* food once daily. Non-enriched (NE) mink (N=32) lived in unmodified versions of these, which resembled the smallest, least enriched enclosures that are used for breeding endangered mustelids (Hellstedt and Kallio, 2005; Poessel et al., 2011; Santymire et al., 2007). Every alternate cage was an enriched (E) cage. Each of these E cages was supplied with a wire mesh vertical stack (56x15x121 cm) that each of the 32 E mink could access via lockable exit ramps (see e.g. Dallaire et al., 2011; ***also Fig. 2, Chapter 2***). This vertical stack contained sloping ramps for the mink to climb, and led to a 13x244x13 cm overhead tunnel. The tunnel was connected to an identical stack at the other end, which mink could descend via sloping ramps to access a separate enriched compartment double the size of the home cage and provided with an extra empty wooden nest box (24x22x28 cm); novel objects (e.g. plastic toys) that were replaced with new items

every month (highly preferred by mink, Mason et al., 2001); a water channel (120 cm long) containing filtered circulating water for wading (mink are semi-aquatic [Dunstone, 1993] and highly motivated to access water baths [Mason et al., 2001]); and a variety of manipulable items (e.g. balls, chewable pet toys) plus tunnel/shelf-like structures (plastic pipes; a suspended, rigid plastic hammock) together known to reduce stereotypic behaviour and corticosteroid hormone output (Hansen et al., 2007). E mink thus had more space, more exercise opportunities, more sensory stimulation, and more access to reinforcement than NE mink. Parallel studies confirmed that these enriched compartments reduced stereotypic behaviour and corticosteroid outputs (Dallaire et al., 2011; Dallaire et al., 2012; Meagher, 2011), and that mink were highly motivated to use them (Dallaire et al., 2012).

When infants (kits) were born, access to the E compartments was prevented until the young animals had open eyes and reasonable mobility (at approximately five weeks). Half the E and half the NE cages were designated 'male' and the other half 'female', such that each female subject would be raised between two males, one E and one NE. In each 'male' litter, one random male infant was selected as our subject; a female was similarly selected in each 'female' litter. Each subject's family was removed when infants were 52+/- 3 days old; it was then given an unrelated same age, same treatment, opposite sex companion until seven months of age (early adulthood). This ensured that all of our subjects were well-socialised and identically raised in terms of access to opposite-sex conspecifics. At seven months, companions were removed (adults being naturally solitary, Dunstone, 1993). In total, our subject males (16 E, 16 NE) were differentially housed for 23 months, spanning two annual breeding seasons (after which they were killed).

Thirty-two females were equally divided into 'Year 1' and 'Year 2' choosers. Year 1 choosers (8E, 8NE) were exposed to these differentially-housed males as potential mates in their first breeding season, when they were 10 months old; and differentially housed until being killed at 15 months (after weaning). Year 2 choosers (8E, 8NE) were mated with non-experimental males in Year 1; exposed to the differentially-housed males as potential mates in Year 2 when 22 months old; and differentially housed until being killed at 28 months (after weaning their second litters).

Mating trials, and assessment of reproductive outcomes

Each female was exposed to one pair of males (one E, one NE); each male pair was exposed to two chooser females (one in Year 1, one in Year 2). The mating trials utilised an apparatus that allowed each female to visit her two potential mates freely. This apparatus comprised of a tunnel connecting her cage with her two male neighbours, with five openings: one allowing her to exit/enter her own cage, and two per male cage to reduce males' abilities to prevent her leaving (Fig. 1). These openings were designed to exploit this species' great sexual dimorphism (females have approximately half the body size of males, Dunstone, 1993; Thom et al., 2004), being custom cut so that only the females could pass through them. Between trials these openings were blocked with solid inserts; while during trials, these were retracted to reveal the custom-sized holes allowing free passage of each female (Fig. 1). Before trials commenced, each female was given five days of access to familiarize with her apparatus, and males were removed for brief periods (< 5 minutes, once or twice a day) to allow her to explore their cages. To ensure both males' cages would

appear similar to females during mate choice trials, vertical stacks with ramps were attached to NE cages (but locked and inaccessible), and any enrichments in E males' home cages were removed. During mate choice trials, each female was moved to a new cage so that her potential mates were equally unfamiliar rather than long-term neighbours, to avoid differential exposure acting as confounds (e.g. each NE female might be less familiar with her E neighbour than her NE one, because E males spent time away in their E compartments; see Martin and Shepherdson [2012] for the potential importance of familiarity). During trials, each E male was also locked out of his E compartment. Between trials, each female was returned to her own cage. The trials, each four hours long, were conducted daily for 21 consecutive days. During each trial, mink were scanned every 10 minutes to record visits by female to male; copulations (identified by the male holding the female in a position allowing intromission, the female's tail perpendicular to her body, Thom et al., 2004); and stereotypic behaviours. Escalated aggression, defined (subjectively, based on the observer's prior experience with fighting mink) as agonistic interactions involving prolonged, repeated high-pitched screams, was used as a cue to terminate a trial; however, only two matings ever needed splitting because of these behaviours.

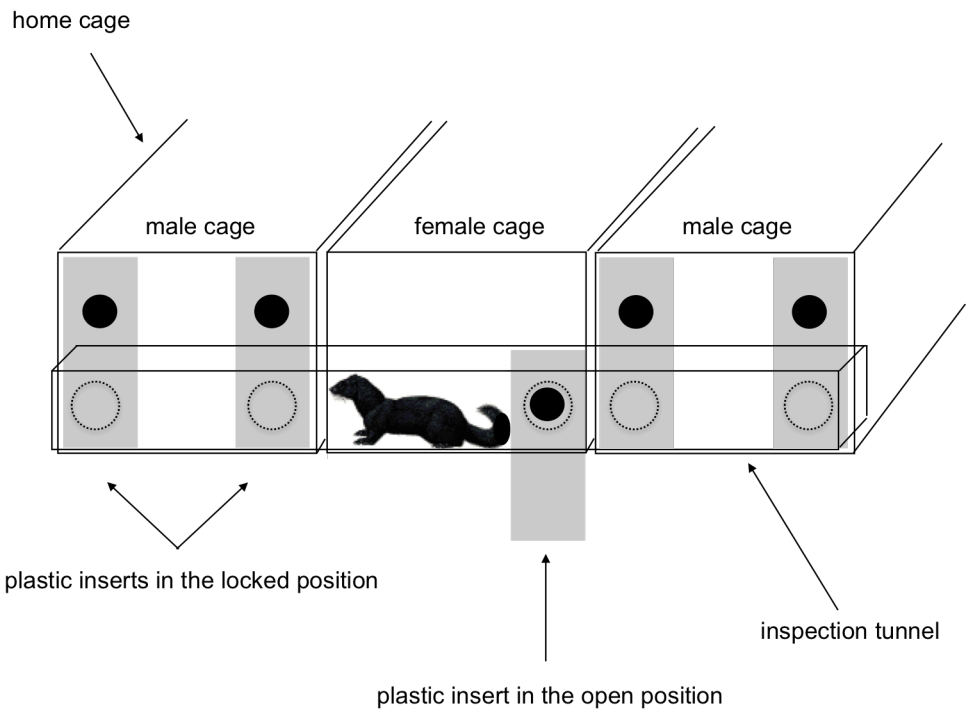


Figure 1. Diagram of the mate choice apparatus.

Parturition occurred approximately two months after mating. Kits were counted and weighed on post-natal day (PND) 1; plans to follow them for longer were abandoned, however, due to small sample sizes (see Results). They were pelted at nine months, and muscle samples collected to assess paternity (any dying earlier were similarly sampled; thus paternity data came from all kits). The PCR amplification conditions and quality control procedures for the paternity tests are described in Williams and Scribner (2009). In brief, 10 loci were used: Ma-2, Ma-19, Gg-7 (Davis and Strobeck, 1998), Mvis02, Mvis72, Mvis75, Mvis99, Mer09, Mer22, and Mer41 (Fleming et al., 1999). Allele counts, allele frequencies, expected and observed heterozygosities, and parentage analyses were calculated using CERVUS version 3.0 (Kalinowski et al., 2007). Each infant was assigned to one of his two

possible fathers (blind to their treatment) based on complete exclusion or 70% or higher confidence. To help validate our behavioural data on mating (which were not collected blind to treatment), the behavioural data were compared to paternity data (see Results), since paternity analyses were conduced blind to treatment.

Additional data collection from live subjects

Stereotypies were assessed over two 8-day periods (when mink first show these behaviours consistently, at 8 months old [Jeppesen et al., 2000; Mason, 1993], and then again at the end of our experiment, when the animals were 23 months old) (following methods in Dallaire et al., 2011; Dallaire et al., 2012; Meagher, 2011). These were typically locomotor, involving whole-body movements like pacing or 'nodding' with the upper body, repeated at least three times consecutively (Mason, 1993). Inactivity was scored so that stereotypies could be corrected for activity levels, to yield the measure that best correlates with perseveration (cf. e.g. Campbell et al., 2013; Garner, 1999).

After the second breeding season, faeces were collected and pooled over five consecutive days; stored in a -20°C freezer; then homogenized and processed (Palme et al., 2013). Extracts were assayed (blind to treatment) for cortisol metabolite concentrations using a mink-validated enzyme immunoassay (EIA) for 11ß-hydroxyaetiocholanolone (Malmkvist et al., 2011). We also measured androgen metabolites with a testosterone (T) and epiandrosterone (EPI) EIA: these assays measure 17β-hydroxy- and 17-oxo-androgens, respectively (Möhle et al., 2002; Palme and Möstl, 1994). Since neither EIA is validated

specifically for mink, and cross-reactivity with other steroids of adrenal origin is likely (indeed evident in our samples), we used glucocorticoid metabolite concentrations as covariates in all relevant analyses.

Post mortem data collection

Mink were killed as humanely as possible. Killing methods followed improved guidelines from the University of Guelph and Michigan State University. Since CO_2 is highly aversive to mink (Cooper et al., 1998), we anaesthetised the animals first. Each mink was placed individually in a transparent plastic 50 l container, into which 10 ml of isoflurane was sprayed. This rendered animals unconscious in less than 60 s with no obvious signs of stress or suffering. The container was then filled with CO_2 for three minutes to ensure death. Each was then weighed and measured, and certain structures removed. The following organs were weighed fresh to the nearest 0.0001g, after careful fat removal: brains (cut at the caudal end of the medulla, adrenals, testes, thymus and spleen. To assess fluctuating asymmetry (FA), mandibles were also removed. These were selected as the bones to measure due to their established anatomical landmarks (Badyaev et al., 2000; Klingenberg et al., 2003; ***Fig. 4, Chapter 2***); their known vulnerability to stress (Badyaev et al., 2005); and to avoid potential confounds, since development and FA in other skeletal bones could be influenced by stereotypic behaviours. After manual cleaning, morphometrical analyses of the mandibles' labial sides followed those of Galicia Paredes (Galicia Paredes, 2006). Landmarks were located using TpsDig software (v. 2.0, Rohlf, 2004; ***Fig. 4, Chapter 2***). They were processed by side rather than by individual (i.e. all left mandibles were processed first, then all right mandibles), to ensure that the second set of landmarks for each

individual was placed blind to the location of the first set. The presence of FA was verified, before quantifying the asymmetry between sides for each individual (Palmer and Strobeck, 2003): to do this, mandibular FA was calculated using Procrustes analyses to generate a landmark-based index ("FA18", Palmer and Strobeck, 2003) which quantified the shape differences between sides.

Finally, the last post mortem data came from the *os penis* (baculum). This was removed from each male, and scored for degree of development, since this bone's proximate knob (Fig. 2) grows with age in mink (Elder, 1951) and least weasels (*Mustela nivalis*) (Wright, 1950), and in the latter species its development is testosterone-dependent (Wright, 1950). To do this, five observers, each blind to mink treatment and previously trained using published images of young *vs.* mature mink bacula (Elder, 1951), independently ranked our 32 bones from the least developed (i.e. a score of 1) to the most developed (a maximum possible score of 32; ties were allowed). Spearman correlations confirmed concordance between the observers; an average rank was then calculated for each male[4].

[4] ***Opportunistically, and to explore other potential ways of measuring male mink libido, the plastic inserts from the mate choice apparatus (see Fig. 1) were also ranked for bite and scratch marks (see Appendix I)**.

a)

b)

Figure 2. Mink bacula. a) baculum with a well-developed proximal process (mean score = 30.2), and b) baculum with an under-developed proximal process (mean score = 1.8). Arrow indicates the proximal process. Scale = 1cm.

Statistical analyses

Most analyses involved General Linear Models (GLMs, Minitab v. 14: Minitab, 2008). Data were transformed where necessary; models contained all possible interactions to avoid pooling error terms (Bogartz, 2005); alpha was set at 0.05; and results are 1-tailed due to clear *a priori* predictions (Levine and Banas, 2003; Rice and Gaines, 1994). GLMs investigating treatment effects on reproduction contained Male and Female treatment (E *vs.*

NE) as independent variables, along with Year, and Male pair (a random effect) nested in Female Treatment. Logistic regressions were conducted for paternity, since this was a categorical variable.

We investigated first how treatment affected phenotypes in the relevant sex, and second, which of these specific housing-influenced aspect(s) of phenotype predicted any observed reproductive benefit. In practice enrichment only affected male reproduction. We therefore ran GLMs that used Male copulation number as the dependent variable, and each housing-influenced aspect of phenotype as an independent variable, blocking for Female treatment, Year, and Male pair nested in Female treatment ('Analysis 1'). For each variable that these tests identified as significant, we ran a second GLM ('Analysis 2'), using the proportion of each female's total number of copulations allocated to her E mating partner as the dependent variable; and the absolute difference in the trait of interest's magnitude between that female's E male and his NE rival as the independent variable. Analyses 1 and 2 involved continuous dependent variables that proved to be non-orthogonal (Grafen and Hails, 2002); we therefore used sequential sums of squares to calculate F ratios, placing the trait of interest as the last main effect after the blocking factors (Doncaster and Davey, 2007). These GLMs investigating underlying mechanisms generated 21 P values and we therefore used "false discovery rate" procedures (FDR; Benjamini and Hochberg, 1995) to reduce risks of Type I error. Previously significant results that did not pass these more stringent alphas are subsequently reported as trends[5].

[5] ***In the context of the thesis, we conducted further analyses on the published dataset regarding female preference and other copulatory variables (see Appendix II).***

Results

Copulation number: effects of individual and housing, and implications for paternity

Housing conditions did not significantly influence female mating behaviour (see Table 1), nor female chances to produce a litter (odds ratio of non-enriched:enriched=1.67, Z=0.57, p=0.283), nor litter size, nor offspring attributes -- although only 17 females whelped and only 10 had kits surviving past PND 4. Male treatment, however, affected the numbers of copulations obtained: NE males were less successful ($F_{1,14}$=3.20, p=0.047; see Table 1). Individual males' copulation numbers also covaried across the two test years ($F_{1,28}$=7.48, p=0.011), showing that it was, or reflected, a stable male trait.

Table 1. Effects of environmental enrichment on reproductive variables (see text for effects of enrichment on likelihood of producing progeny). Significant effects are in bold. Means are raw means.

		Males				**Females**			
		Mean	*SE*	*Statistic*	*p*	*Mean*	*SE*	*Statistic*	*p*
# copulations	E	**3.25**	**0.56**	**$F_{1,14}$=3.20**	**0.047**	1.97	0.43	$F_{1,14}$=2.27	0.154
	NE	**1.91**	**0.37**			3.19	0.52		
Litter size	E	2.25	0.92	$F_{1,13}$=0.47	0.505	2.83	0.56	$F_{1,13}$=0.32	0.580
	NE	2.92	0.52			2.33	0.48		
Kit weight at birth (g)	E	11.53	1.481	$F_{1,13}$=1.56	0.234	13.99	1.600	$F_{1,13}$=0.69	0.210
	NE	14.29	1.481			11.83	1.352		

Paternity analyses revealed that all the kits per litter were sired by just one male. Male treatment did not significantly predict likelihood of paternity (odds ratio of non-enriched:enriched=1, Z=-0.00, p=0.500); however, exploratory comparisons of paternity data with behaviour showed that in Year 2 (but not Year 1, p>0.10 in all analyses), mating more often predicted a higher probability of paternity ($F_{1,10}$=8.82, p=0.014).

Which aspects of male phenotype were affected by enrichment?

Housing-type affected male behaviour, physiology and anatomy (Table 2). NE males showed evidence of increased HPA activity and its predicted effects on reproductive

physiology: concentrations of excreted faecal metabolites were higher for glucocorticoids, and lower for testosterone and epiandrosterone. Bacular proximate knobs were also less developed (although not significantly so after FDR corrections, see Table 2). Furthermore, there was evidence of phenotypic changes that could affect attractiveness or social competence: NE males were lighter, had smaller spleens suggesting poorer cell-mediated immunity, and performed more stereotypy; they were also less symmetrical, although not significantly so after FDR corrections (see Table 2).

Table 2. Effects of environmental enrichment on male phenotype. Significant results are in bold. Means are raw means. High baculum development scores indicate a higher degree of development; high fluctuating asymmetry scores indicate a higher degree of asymmetry.

	E		**NE**			
	Mean	*SE*	*Mean*	*SE*	*Statistic*	*p*
Adrenal weight (g)*	0.1311	0.00698	0.1283	0.00676	$F_{1,29}$= 0.08	p=0.387
Fecal glucocorticoid metabolites (ng/g)	**185**	**42.5**	**299**	**41.6**	**$F_{1,29}$= 8.33**	**p=0.003**
Testis weight (g)*	6.769	0.2656	6.393	0.2656	$F_{1,30}$= 1.02	p=0.160
Testosterone (ng/g)[§]	**60.01**	**4.376**	**33.79**	**4.220**	**$F_{1,28}$= 16.53**	**p<0.001**
Epiandrosterone (μ/g)	**17.083**	**1.953**	**5.809**	**1.883**	**$F_{1,28}$= 15.34**	**p=0.001**
Baculum proximal process development score	**18.61**	**1.778**	**12.64**	**1.721**	**$F_{1,30}$= 5.37**	**p=0.014**
Thymus weight (g)*	1.156	0.1251	1.102	0.1211	$F_{1,29}$= 0.09	p=0.380
Spleen weight (g)*[¶]	**6.457**	**0.4160**	**4.339**	**0.4160**	**$F_{1,28}$= 12.95**	**p=0.001**
Body weight (kg)	**2.567**	**0.07625**	**2.218**	**0.07625**	**$F_{1,28}$= 10.51**	**p=0.003**
Fluctuating asymmetry	**0.03589**	**0.00285**	**0.04331**	**0.00295**	**$F_{1,29}$= 3.27**	**p=0.040**
Locomotory stereotypies	**2.510**	**0.756**	**33.19**	**7.15**	**$F_{1,30}$= 13.63**	**p=0.0005**
Brain weight (g)*	10.63	0.1938	10.33	0.1877	$F_{1,28}$= 0.266	p=0.133

*corrected for body length
[§]corrected for levels of fecal glucocorticoid metabolites
[¶]corrected for body weight

Which enrichment effects on male phenotype predicted copulation number?

Stereotypies significantly predicted copulation number (Fig. 3), with the most stereotypic males gaining the fewest copulations (Analysis 1: $F_{1,29}$=7.60, p=0.004; Analysis 2: $F_{1,24}$=4.25, p=0.029). Bodyweights also positively co-varied with copulation number in Year 2 (bodyweight data in Year 1 were not available) -- although not significantly after FDR corrections (see Table 3)[6]. No other aspect of enrichment-altered male physiology or anatomy covaried with copulation number.

[6] ***For additional analyses looking at the importance of body weight in mink copulation, see Appendix III.***

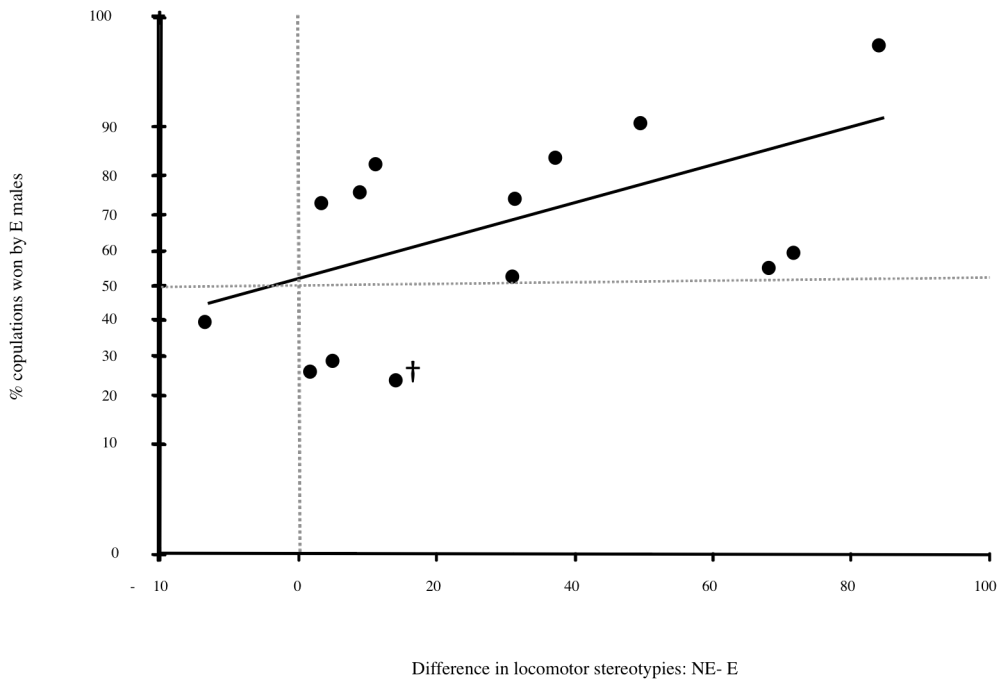


Figure 3. Relationship between locomotory stereotypies and number of copulations. Graph from Analysis 2. "†" indicates the one pair in which the NE male stereotyped (during two trials).

Table 3. Relationships between enrichment-affected male traits and male mating success. Significant results are in bold. - = negative relationship; + = positive relationship.

Traits	Statistical method	Copulation number
Stereotypic behaviour	Analysis 1	**$F_{1,29}$=7.60, p=0.004 -**
(Year 1 and Year 2 averaged)	Analysis 2	**$F_{1,24}$=4.25, p=0.029 -**
Body weight	Analysis 1	**$F_{1,14}$=3.46, p=0.042 +**
	Analysis 2	**$F_{1,10}$=6.87, p=0.013 +**
Baculum development	Analysis 1	$F_{1,14}$=1.76, p=0.103
Glucocorticoid metabolites	Analysis 1	$F_{1,13}$=1.21, p=0.146
Testosterone	Analysis 1	$F_{1,12}$=0.14, p=0.710
Epiandrosterone	Analysis 1	$F_{1,12}$=0.82, p=0.383
Spleen weight	Analysis 1	$F_{1,12}$=0.00, p=0.981

Discussion

Our results for male copulatory behaviour supported the long-standing, previously untested hypothesis that barren environments compromise breeding in captive carnivores. The poorer performance of non-enriched male mink compared to enriched conspecifics indicated weaker libido (Chenoweth et al., 1979) and/or reduced attractiveness to females (Birkhead and Møller, 1993): both problematic in the males of some captive-bred wild

species (Berejikian et al., 2001; Kiik et al., 2013; Mellen, 1991; Morato et al., 2001; Peng et al., 2007; Santymire et al., 2007; Zhang et al., 2004). Since multiple matings increase males' chances of fertilization in some species (Gibson and Jewell, 1982; Zhang et al., 2004), this result also suggests that barren environments could reduce abilities to father offspring. We found no direct evidence for this, but some indirect support: in Year 2, males that successfully sired litters also copulated more. Future work should now investigate this further, and also assess other potential benefits of enriching males, including improved sperm motility (Suarez et al., 1996), male libido (Grønli et al., 2005), attractiveness to females, and enhanced maternal investment in their offspring, by the dams mated to them (Loyau et al., 2007; Roed et al., 2007).

Housing conditions did not apparently affect female receptivity, in contrast. Housing conditions also did not seem to affect female fertility (although statistical power was low here because few females reproduced, likely reflecting deficits in the indoor lighting's brightness or spectral range, Murphy, 1974). This aspect of our work therefore requires replication, since the apparent absence of effects on females could be attributed to Type II error, especially given that environmental enrichment increases the number of infants successfully weaned by females in farmed mink (Buob et al., 2013). However, it is also possible that male reproductive success is genuinely more sensitive to the physical and sensory properties of their rearing and housing conditions. As evidence, over 95% of female mink successfully conceive litters on commercial fur farms (Sundqvist et al., 1989), suggesting that their reproduction is rather resilient to barren housing conditions; whereas the same is not true for males, since over 10% do not mate (G. Mason, unpublished data).

Furthermore, in non-enriched enclosures, breeding also appears to be more problematic for males than females in European mink, giant pandas and black-footed ferrets (Kiik et al., 2013; Wolf et al., 2000; Zhang et al., 2004); while likewise in flies, barren rearing environments decrease the copulations gained by males but not by females (Díaz-Fleischer et al., 2009; Dukas and Mooers, 2003).

We then investigated three non-mutually exclusive potential mediators that could explain why non-enriched males are handicapped in mate choice competitions. One widely-suggested problem for NE housed animals is that increased HPA activity will have knock-on detrimental effects on the HPG endocrine axis. Consistent with this, faecal metabolites revealed barren environments to elevate circulating concentrations of cortisol, and perhaps reduce androgen levels (the lack of fully-validated androgen assays for mink necessitating caution here). The bacula of non-enriched males also tended to have less well-developed proximal processes, a result that now needs replication. The second set of mediators investigated were enrichment-induced changes in male morphology or physiology that are likely to affect attractiveness. Mate choice research provides considerable evidence that developmental stress can create stunted, asymmetrical, immune-compromised phenotypes that females generally prefer not to mate with, yet these adverse implications of poor rearing environments are never considered by captive breeding programmes. Consistent with this hypothesis, non-enriched males tended to be more asymmetrical (significant until FDR corrections). They also had smaller spleens, suggesting that immunological benefits of enrichment should now be investigated in depth (e.g. via the histological examination of splenic germinal centre numbers and structure, Wong et al., 2003). Non-enriched males

were also lighter: an effect whose possible role in their poorer copulatory performance now needs replicating.

Stereotypy was at the heart of our third hypothesis: that barren environments make animals less socially competent by reducing intelligence and/or behavioural flexibility. Even though barren rearing conditions cause well-understood learning, memory and forebrain function deficits (Nithianantharajah and Hannan, 2006; Rosenzweig et al., 1968), the potential implications of such effects for courtship, copulation, maternal care and other intra-specific interactions have to date not been studied. We did not find the brain weight effects reported for rodents, but such data are prone to measurement error. Future studies should therefore assess subjects' learning abilities, and also section brains for histology, in order to measure cortical thickness and hippocampal volume (e.g. Rosenzweig et al., 1968). However, we did find strong evidence that captivity-induced basal ganglia changes have adverse socio-sexual implications: non-enriched males who, prior to the mating season, spent a relatively large proportion of their home cage active time budget repetitively pacing, nodding or head-twirling, went on to gain little mating success during mate choice trials. This negative impact of stereotypy on copulation number was not because highly stereotypic males performed stereotypies during the tests themselves (with bouts then directly interfering with sexual behaviour): stereotypy was only observed twice (in just one male) in all 504 mating trials. Instead, we suspect that subtle behavioural correlates of stereotypy are the key. As outlined in the Introduction, humans suffering from psychiatric or developmental disorders characterised by stereotypic behaviour (e.g. schizophrenia; autism) often have problematic or no sexual relationships (Barbato et al., 2004; Bhui et al., 1997; Stevenson et al., 2006) because

they are socially odd (Jones, 1999): behaviourally and conversationally inflexible, and poor at responding appropriately to others' actions, words or facial expressions. Furthermore, perseveration (inappropriate response repetition) is elevated, not just in these stereotypic humans, but in all stereotypic captive animal species tested to date -- including two other Carnivora (Vickery and Mason, 2004; Vickery and Mason, 2005) as well as mink (Campbell et al., 2013; Dallaire et al., 2011). We therefore suspect that non-enriched, highly stereotypic male mink are inappropiately repetitive or inflexible during courtship, perhaps in their courtship vocalizations ('chuckling', Gilbert, 1969) or play-like interactions with receptive females: a hypothesis that now needs to be tested directly.

Small, non-enriched cages are still common in breeding centres for European mink, black-footed ferrets and other endangered carnivores (Branvold et al., 2003; Hellstedt and Kallio, 2005; Kiik et al., 2013; Loeffler, 2011a; Peng et al., 2007). Currently, breeding problems in these and other captive wild animals are often addressed with reproductive medicine or technology (e.g. artificial insemination, Santymire et al., 2007; Wildt, 2009). Our results confirm what has been long-suspected (e.g. Wildt, 2009; Zhang et al., 2004): that enriching rearing conditions could provide an alternative solution (especially for males). This solution has the added ethical benefit of enhancing animal welfare. We suspect that our results may even have implications beyond captive wild carnivores, suggesting needs for additional research. Suboptimal housing has been suggested to cause breeding problems in other wild taxa (e.g. black rhinoceroses *Diceros bicornis* and white rhinoceroses *Ceratotherium simum*, Carlstead and Brown, 2005). Furthermore, stereotypic behaviours are extremely prevalent in other captive populations (including laboratory and farmed animals), being performed by

tens or even hundreds of millions of individuals (Mason and Latham, 2004). Additionally, small impoverished enclosures remain common for wild animals (Mason, 2010), and standard for billions of farm and research animals (Mason, 2010). These are likely to affect brain development even in species that tend not to stereotype (e.g. rats, Würbel, 2006). We therefore suspect that the perseveration of stereotypic individuals, as well as the learning impairments likely in captive-bred animals, may prove to have widespread socio-sexual consequences across diverse captive species, especially for complex courtship (e.g. duetting), maternal care, and other interactions with conspecifics that require responsiveness and flexibility.

A modified version of the following chapter is in preparation for submission to Scientifur as:

Díez-León, M, Mason, G. Effects of environmental enrichment on aspects of maternal behaviour, and infant growth and mortality, in American mink (Neovison vison).

Chapter IV. Effects of environmental enrichment on aspects of maternal behaviour, and infant growth and survival, in American mink (*Neovison vison*).

Abstract

The high rates of infant mortality (IM) observed in captive carnivores, in some cases surpassing those reported for wild populations, have been attributed to abnormal maternal behaviour brought about by captive conditions. Supporting this, environmental enrichment has been shown to improve litter size in American mink, as well as, in other species, to improve stress-affected aspects of maternal behavior that are important for infant development and survival (e.g. nest building, maternal licking and grooming). Enrichment also reduces stereotypic behaviours (SB), which in the American mink negatively correlate with infant growth rates. Low birthweights and slow growth rates are risk factors for IM, and can also result from other aspects of maternal investment potentially compromised by stress. We therefore tested two hypotheses: i) enrichment decreases IM by improving maternal behaviour and/or maternal investment; ii) maternal SB compromise maternal care, resulting in offspring reduced growth. We raised 22 American mink enriched (E) and non-enriched (NE) females (9E:13NE) in an artificially lit facility. We followed them for two breeding seasons (all primiparous in the first, 4 multiparous in the second; parity was controlled for statistically). Offspring-related variables were recorded at birth and at post-natal day (PND) 20, to cover the period when the altricial kits are fully dependent on their mothers. We recorded: litter size (counting stillbirths and live kits only), IM (at PND1 and PND20), litter sex-ratio (at PND1 only), and weight. Growth rates were calculated separately for males and females. Maternal behaviour was assessed by recording nest shape

(on PND1), and by assessing licking and grooming (LG; via video recordings made between PND1-PND7). Data on maternal SB were obtained from a previous experiment. Enrichment did not decrease IM. However, NE females had female-skewed litters at birth (including stillbirths), and enrichment tended to improve nest shape, although not levels of maternal LG (even though these showed very high individual consistency). As expected, maternal SB negatively correlated with offspring growth rates; however, the effect was not mediated by the maternal behaviours we recorded. Due to our small sample sizes, our study should be replicated, particularly to explore potential benefits of enrichment on nest building abilities. The possible implications of LG for offspring stress reactivity and fitness should also be explored.

Introduction

Infant mortality can be high in wild carnivores kept in zoos and conservation breeding centres (Clubb and Mason, 2007; Ginsberg, 1994; Marker and O'Brien, 1989). For example, neonatal deaths (<30 days of age) range between 54-57% for giant panda *Ailuropoda melanoleuca* (Xie and Gipps, 1997 as cited in Zhang et al., 2000), red panda *Ailurus fulgens* (Glatston, 2011), African wild dog *Lycaon pictus* (Ginsberg, 1994) and maned wolf *Chrysocyon brachyurus* (Maia and Gouveia, 2002). Infant mortality is also high for fur-farmed silver foxes *Vulpes vulpes*, artic foxes *Alopex lagopus* and American mink *Neovison vison* (Braastad and Bakken, 1993; Malmkvist et al., 2007). Four lines of evidence suggest that the captive environment, or some aspect of it, is responsible for the observed high rates of infant mortality in captivity. First, infant mortality in captive lions *Panthera leo* is higher than that observed in the wild (42% vs. 30%, Clubb and Mason, 2007; Schaller, 1972): surprising because predation, disease and starvation, the main sources of mortality in the wild (e.g. Steiger et al., 1989), are not issues in captivity. Second, across the Carnivora, natural home range size and stereotypic behaviours predict captive but not wild infant mortality (Clubb and Mason, 2003, 2007). Third, different captive populations differ in their infant mortality rates, suggesting that some aspect of housing and/or management in these captive settings influences infant mortality. For example, red panda infant mortality rates are much higher in North American zoos than in European or Australian zoos (40% vs. respectively 24% and 11%, Glatston, 2011), as is cheetah *Acinonyx jubatus* infant mortality in zoos compared to in situ captive breeding facilities (36.7% vs. 28.7%, Bertschinger et al., 2008; Laurenson, 1993; Marker and O'Brien, 1989). Fourth, one type of environmental enrichment -- an elevated resting shelf for nursing dams -- has been shown to reduce infant mortality in farmed

American mink (Buob et al., 2013), and environmental enrichment has long been suspected to be similarly beneficial for a range of other species (Carlstead and Shepherdson, 1994).

One likely mediator of these harmful effects of captivity is abnormal maternal behaviour. In several captive species, including black-footed ferrets *Mustela nigripes* (Bronson et al., 2007), cheetahs (Laurenson et al., 1995; Marker-Kraus, 1997), maned wolves (Maia and Gouveia, 2002), rusty-spotted cats *Prionailurus rubiginosus* (Dmoch, 1997), and red pandas (Loeffler, 2011a, b), neonatal deaths (from 60 to 87%) are caused by physical harm, cannibalism and/or neglect by the mother. Further, a small number of studies have demonstrated that environmental enrichment improved maternal behaviour and offspring development. Thus, providing naturalistic nesting materials improves kit retrieval responses in mink dams (Malmkvist and Palme, 2008) and reduces piglet crushing by sows *Sus scrofa* (Damm et al., 2010); social housing also improves nest-building behaviour by mouse mothers (Curley et al., 2009); and female rats *Ratus norvegicus* raised in physically enriched environments spend more time licking and grooming their infants (Baroncelli et al., 2010; Champagne and Meaney, 2007; Sale et al., 2004): a behaviour that helps newborns to urinate/defecate (e.g. Lovic et al., 2001; Metz and Metz, 1986), promotes sexual differentiation of males and females (e.g. Moore, 1995), and reduces infant stress reactivity by shaping their hypothalamic-pituitary-adrenal axes responses (reviewed in Cameron et al., 2005; Meaney, 2001). All of these findings are consistent with the well-studied deleterious effects of chronic stress (induced by forced exposure to aversive stimuli and/or assessed via corticosteroid levels) on maternal behaviour, which include reduced nest-building, and maternal licking and grooming, (in laboratory rodents, (Cameron et al., 2008; Kinsley and Svare, 1988; Meaney, 2001; Pérez-

Laso et al., 2008), and decreased active protective holding behaviour (gorillas *Gorilla gorilla*, Bahr et al., 1998).

Maternal stereotypic behaviour may also contribute to poor infant survivorship in captive animals. In American mink, kits from stereotypic mothers grow more slowly than those of more inactive dams (Mason et al., 1996; Meagher et al., 2012b), while anecdotally, in several other non-carnivore captive species, the performance of stereotypic behaviours by females has caused neglect of or harm to their infants (e.g. bank voles *Myodes glareolus*, Sørensen and Randrup, 1986; giant anteater *Myrmecophaga tridactyla*, Widholzer and Voss, 1978; chinchillas *Chinchilla lanigera*, M. Galeano unpublished data). It is also possible that the typically perseverative characteristics of stereotypic animals (see Chapters 2 and 3) could render their maternal behaviour abnormally repetitive and routine-prone, thence poor at flexibly adapting to infants' proximate needs.

Other aspects of the maternal response, such as prenatal investment or post-natal milk provision, could also be compromised in captive environments and should be investigated as potential risk factors for poor infant survivorship in carnivores. For example, milk deficiencies have been listed as partly responsible for neonatal mortality in captive red pandas (Loeffler, 2011b), effects that could be due to stress. Mink dams that undergo handling stress during lactation have slower growing offspring (Dobson et al., 2008), and being stressed while pregnant also decreases milk yield and milk quality in guinea pigs *Cavia aperea* (Klaus et al., 2013). Maternal stress has also been shown experimentally to have adverse pre-natal effects on offspring, for example reducing birthweights in mice *Mus*

musculus (Kinsley and Svare, 1988). It is unknown whether birthweights are reduced by stress in captive wild carnivores. However, low birthweights have been positively correlated with infant mortality in wild polar bears *Ursus maritimus* (Derocher and Stirling, 1996), and domestic cats *Felis catus* (Greco and Watters, 1990), and also suggested as a risk factor for infant mortality in cheetah (Beekman et al., 1999). Deficiencies in birthweight and growth, as well as in lactation, are also potential risk factors for infant mortality in a number of species (e.g. pigs, Pedersen et al., 2011; goats *Capra aegragrus*, Chowdhury et al., 2002; rats, Jennings et al., 1999).

This chapter tested whether environmental enrichment improved infant survival in a captive population of American mink. It further tested whether maternal behaviour and/or other aspects of maternal investment mediate such effects. We therefore predicted that long-term enrichment for dams will reduce their rates of infant mortality compared to those of non-enriched mothers, and that it will do so by increasing their maternal behaviours (nest building, licking and grooming of offspring) and/or other aspects of maternal investment (as evidenced by the birthweights and growth rates of their offspring). Additionally, we tested the hypothesis that the performance of stereotypic behaviour compromises maternal behaviour, predicting that stereotypic dams will build poorer nests, show lower levels of licking and grooming, and have slower growing kits.

Methods and Materials

The experiment took place at the Michigan State University Experimental Fur Farm during two consecutive breeding seasons (Year 1 and Year 2). Twenty two adult black American mink females whelped (Year 1, all primiparous; Year 2, all primiparous except for four that were multiparous females from Year 1; see Table 1). Husbandry and treatment conditions have been described in detail elsewhere (Campbell et al., 2013; Dallaire et al., 2011; Meagher and Mason, 2012; see also Chapter 2 and Chapter 3 / Díez-León et al., 2013). Our experimental females were born and raised in these environments (see Chapter 2 for more details). During both whelping seasons, enriched-raised (E) females where encouraged to whelp in the nest box of the home cage by not providing nesting material in the enriched nest box (this was successful). In addition, during whelpings and until the kits opened their eyes at five weeks of age, water was removed from the water channel to avoid any drowning and/or wet nests.

Table 1. Sample sizes across years and treatment levels. The left panel summarizes the number of dams that whelped each year (E = enriched; NE = non-enriched); "( )" represents multiparous dam subjects from the previous year. The right panel summarizes our sample size as used in analyses, since, for multiparous females that had data on both reproductive seasons, we only used data from their second year (see Statistical Analyses section).

	Number of whelpings		**Sample size as used in analyses**	
	E	**NE**	**E**	**NE**
Year 1	8	11	7	8
Year 2	1 + (1)	2 + (3)	2	5
		Total	***9***	***13***

Variables recorded

Offspring growth and infant mortality in the first three weeks

Born altricial (see Fig. 1), mink kits do not achieve independent thermoregulation until at least post-natal day (PND) 22 (Harjunpää and Rouvinen-Watt, 2004). In farms, they are also fully dependent on maternal milk until PND 21, when they start transitioning to solid food (Jørgensen, 1985). These first three weeks are also particularly important because, as well as being completely dependent on their mothers for nourishment and heat, kits are vulnerable to some diseases: kits that develop skin infections typically do so by PND10 (Hunter, 2008) and those that develop diarrhoea do so before PND20 (Jørgensen, 1985). Therefore, variables pertaining to offspring growth and mortality were recorded at PND1 (dams were not disturbed during the 24h following parturition to avoid stress-induced losses: Jørgensen, 1985; Malmkvist et al., 2007) and PND20, as follows:

- Litter size (LS) and infant mortality (IM): we recorded LS at PND1[7] to estimate pre-natal (in utero) investment, and at PND20 to estimate quality of post-natal care (see explanation in the Introduction). At PND1 we recorded both stillbirths and living kits. At PND20 the number of living kits per litter was counted. LS was also recorded to control statistically for number of kits in analyses examining licking and grooming rates (LG; see below), because maternal care per individual kit could be smaller in large litters than in small litters (Bechard et al., 2012). For these analyses,

[7] Since we checked litters for the first time 24h after whelping, it is possible that our LS and IM values are underestimates, as dams could have whelped, lost their litters and ate them in those first 24h. However, since we did find dead kits at PND1, we are fairly confident that our sample is representative of the reproductive output of our dams.

LS of living kits at PND1 was used. Infant mortality was calculated as the percentage of dead kits in a litter at PND1, and at PND20 as the percentage of the difference of living kits between PND20 and PND1.

- Litter sex ratio (LSxR): the sex ratio of each litter was recorded as the percentage of males to total offspring produced at PND1 (both including stillbirths and only living kits) and at PND20. This was recorded to control for possible sex bias in the amount of LG, since males can attract more (specifically more ano-genital licking: Baum et al., 1996; Moore and Chadwick-Dias, 1986; Moore and Morelli, 1979; Richmond and Sachs, 1984). For these analyses, LSxR of living kits at PND1 was used.
- Kit weight and growth rate: live kits were weighed at PND1 and PND20 to the nearest 0.01g (Pelouze® SP5 scale). Kits were not individually marked. Therefore, in order to obtain growth rates, weights were averaged for each litter, splitting by sex. Growth rates for each sex were calculated as the absolute difference in average kit weight between PND 1 and PND 20, divided by 19 days.

Figure 1. Mink development during the first three weeks of life, while completely dependent on their mothers. From left to right: post natal day (PND) 1, PND10, PND20.

Maternal behaviour and stereotypic behaviour

To assess maternal and stereotypic behaviour we recorded the following variables:

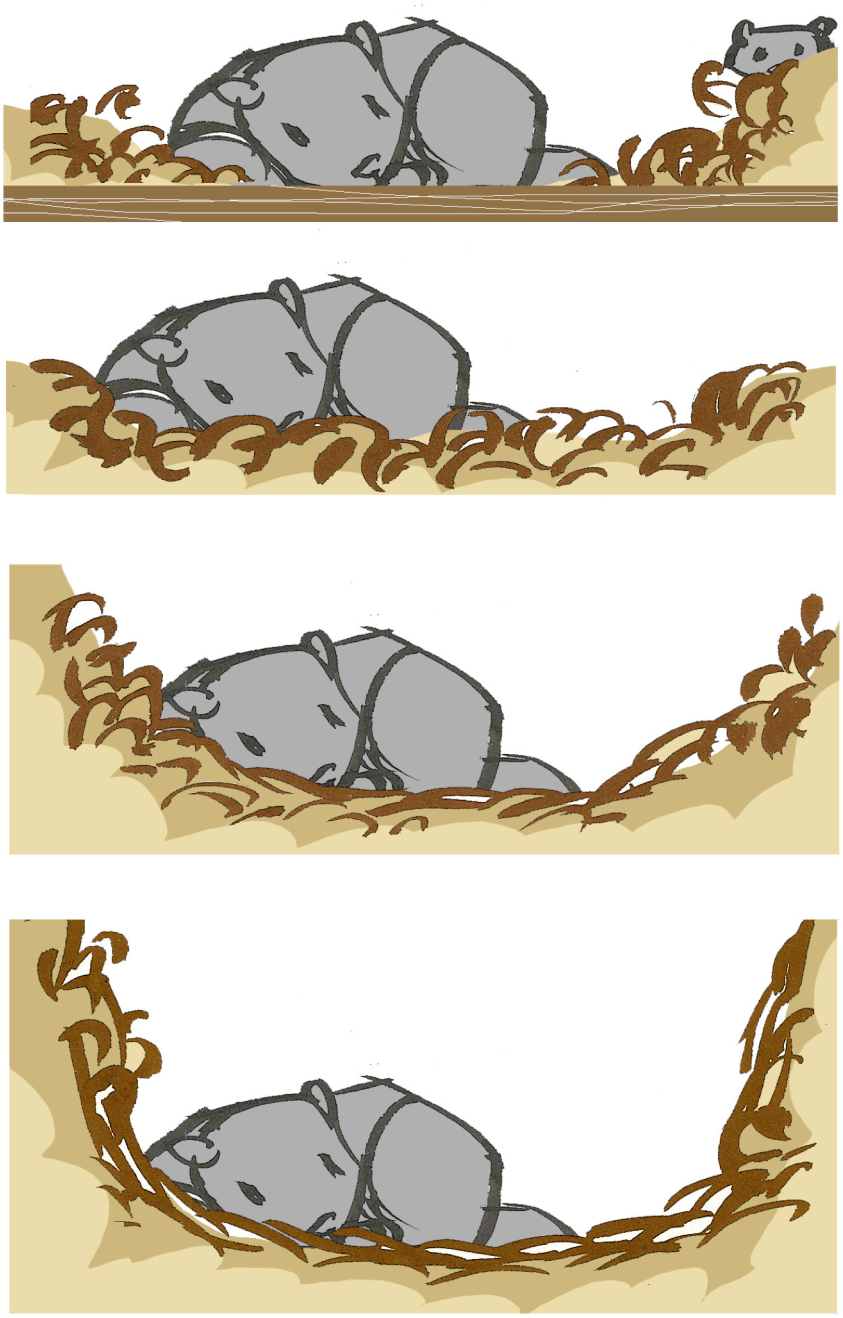
- Nest shape: recorded on PND1 on a 0-3 scale (based on Malmkvist and Palme, 2008; Meagher et al., 2012b), in which 0 represents a flat, unstructured nest and 3 a round nest with walls higher than the dam when she is curled inside (Fig. 2).
- Licking and grooming (LG): the amount of licking and grooming towards the kits was recorded from video recordings. Dams and kits (a.k.a. family units) were filmed in the nest box of the home cage. Of the 22 females that whelped, three dams in Year 1 (1E, 2NE) and three in Year 2 (1E, 2NE) lost their litters in the first few days following parturition. Of the remaining females, due to logistical constraints, we were only able to obtain week-long (PND1-PND7) videos for 15 of them (Year 1: 4E, 7NE; Year 2: 2E, 2NE). One NE female was recorded both years, but we used only data from her second year (see Statistical Analyses), which gave us an effective total of 14 recorded females (6E, 8NE). Cameras provided with infrared lights (Hawk Eye Nature Cam: www.birdhousespy.com) were placed above the nest boxes, resting on wire platforms that were 40cm high. Cameras fed into switchers (Deluxe AV Switch: www.birdhousespy.com) and DVD recorders (Magnavox DVD/VCR combo) in such a way that four family units were scan-recorded successively in one minute (15 seconds per family unit). Family units were recorded during mornings (8:00-12:00). A pilot study (Bechard et al. unpublished data) demonstrated this period to provide a good representation of their total daily (24h) behaviour. Two observers, one each year, both blind to our hypotheses and blind to treatment of the family units, watched the videos to calculate frequencies of licking and grooming as

percentage of their total time budget. Intra- and inter-observer reliability were both high ($p<0.01$ in all cases).

- Stereotypic behaviour: performance of stereotypic behaviour is greatly reduced with pregnancy (Bildsøe et al., 1990); therefore, we used recordings from winter of Y1[8] of these behaviours as an indicator of the subjects' overall levels of stereotypic behaviour. We recorded two sub-types: locomotory stereotypies and scrabbling (see Chapter 2). We pooled these and looked at their proportion over total observation time as in Mason (1996) and Meagher and colleagues (2012b) infant growth results. We also explored whether the proportion of locomotory stereotypies only and over active time inversely correlated with kit growth and maternal behaviour, since this measure more closely correlates with tendencies to perseverate (Campbell et al., 2013; Dallaire et al., 2011), and with poor copulation success, another socio-sexual behaviour, in males (Chapter 3 / Díez-León et al., 2013).

[8] Levels of stereotypic behaviour remained relatively constant between Year 1 and Year 2 (see Chapter 2).

Figure 2. Illustration of the nest shape scoring system. From top to bottom: flat, unstructured nest, some bedding material (0); flat, unstructured nest, significant bedding (1); saucer-shape indentation (2); round hole with sides higher than the dam when cuddled inside the nest (3). Scoring system from Malmkvist and Palme 2008 and Meagher 2012b. Illustration by Íñigo Izal Azcárate.



Statistical analyses

Statistical analyses were performed using Minitab (v. 14, Minitab, 2008) and JMP (v. 10.0, SAS Inc., 2012). Data were transformed when necessary. Alpha was set at 0.05. In almost all cases, 1-tailed results are presented as clearly directional hypotheses were being tested (Levine and Banas, 2003; Rice and Gaines, 1994); i.e. p values were halved when results were in the predicted direction. All analyses blocked for parity. For multiparous females that had data on both reproductive seasons, we only used data from their second year in order to increase our sample size so that analyses would run (see Table 1). This gave us an effective sample size of 22 for all variables measured at PND1 (i.e. weight, LS, IM, NS) and for IM at PND20, a sample size of 18 for assessing weight, growth rates at PND20, and a sample size of 14 for assessing LG. All General Linear Models (GLMs) that involved non-orthogonal variables used sequential sum of squares (Grafen and Hails, 2002), placing the factor of interest -- housing treatment of stereotypic behaviour -- last in the model (Doncaster and Davey, 2007). We generated a total of 31 GLMs; therefore, we used "false discovery rate" procedures (FDR; Benjamini and Hochberg, 1995) to reduce risks of Type I error.

Effects of enrichment on offspring development were tested using GLMs that had the relevant litter characteristic (i.e. birthweight, litter size, litter sex ratio, growth rate and weight at PND20) as the dependent variable, and treatment, parity and their interaction as blocking factors. Analyses considering kit weight were carried out separately for female and male kits. For analyses involving litter sex ratio, we also investigated if departures from a 50:50 ratio were statistically significant in any of our four sub-groups (enriched litters including

stillbirths; enriched litters only including live kits; non-enriched litters including stillbirths; non-enriched litters only including live kits) using t-tests. Effects of enrichment on infant mortality and nest shape (which needed to be log-transformed) were tested using GLMs with treatment, parity and their interaction as blocking factors. To determine if dams displayed consistent levels of LG, we ran split-half analyses (for which data are divided in two halves - e.g. odd and even days - and then correlated as a measure of internal consistency or reliability; Martin and Bateson, 2007) correcting for litter size and parity. To test for the effects of environmental enrichment on LG, and since the amount of LG did not vary with kit age or LSxR (data not shown), we ran a GLM with the LG average across days as the dependent variable and treatment, parity, litter size and the interaction between treatment and parity as factors.

In order to investigate the effects of stereotypic dam behaviour on offspring development, we ran GLMs in which stereotypic behaviours acted respectively as predictors of offspring development (infant mortality, weight at PND20, growth rate), and also whether locomotor stereotypies over active time predicted maternal behaviour (LG, controlling for litter size; nest shape).

Finally, in order to compare the magnitude of our effects with that observed in published studies (where available; i.e. E and LG: Champagne and Meaney, 2007; Sale et al., 2004), we calculated Cohen's d (Cohen, 1992) to obtain effect sizes.

Results

Enrichment effects on litter characteristics at birth

Before looking at infant mortality at PND20, we checked for any effects of enrichment on offspring characteristics at birth. There was no consistent effect of enrichment on birth weights or litter size (Table 2). NE dams had female skewed litters, but only if litter size was calculated counting stillbirths (non-significant after FDR corrections; Table 2). Litter sex ratios from enriched females, whether counting stillbirths or not, were not significantly different than a 50:50 ratio (two-tailed results, respectively t_8=0.476, p=0.647; and t_7=0.519, p=0.620). For non-enriched females, litter sex ratios calculated with stillbirths were female skewed (two-tailed t_{12}=2.841, p=0.015; non-significant after FDR corrections) while if only calculated with live kits, they were not (two-tailed t_9=1.885, p=0.092).

Table 2. Enrichment effects on offspring characteristics on post natal day 1. F: female offspring; M: male offspring. s = data include stillbirths; l = data only include living kits. Means are least square means. Litter size and litter sex-ratio results are 2-tailed.

		E		NE		Statistic	p
		Mean	*SE*	*Mean*	*SE*		
Body weight	*F*	11.79	1.68	11.88	1.19	$F_{1,16}$=0.32	0.582
	M	11.52	2.64	11.98	1.71	$F_{1,10}$=0.33	0.579
Litter size	*s*	4.08	0.97	3.70	0.70	$F_{1,18}$=0.01	0.932
	l	3.25	0.82	2.95	0.60	$F_{1,18}$=0.05	0.820
Litter sex ratio	*s*	**54.52**	**9.50**	**30.48**	**6.87**	**$F_{1,18}$=6.67**	**0.009***
	l	43.44	12.65	32.71	9.17	$F_{1,18}$=1.30	0.134

*non-significant after FDR corrections

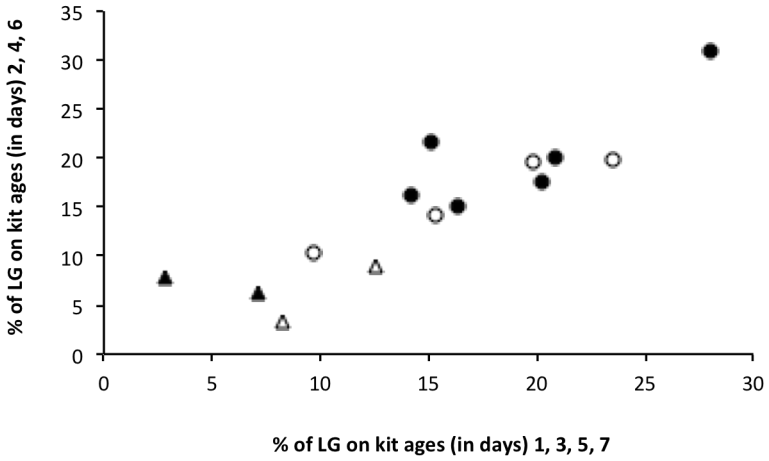
Enrichment effects on infant mortality and offspring growth

Infant mortality at PND20 did not differ between enriched and non-enriched families (including stillbirths: $F_{1,18}$=0.15, p=0.699; accounting for only living offspring: $F_{1,18}$=0.23, p=0.637). Enrichment did not significantly affect offspring weight at PND20 and daily growth rates did not differ significantly, on average, between the enriched and non-enriched groups (weight at PND20: females: $F_{1,13}$=1.41, p=0.128; males: $F_{1,10}$=0.66, p=0.218; growth rates: female: $F_{1,13}$=1.04, p=0.163; males: $F_{1,10}$=0.57, p=0.234).

Enrichment effects on maternal behaviour

Levels of LG showed remarkable within-individual consistency as well as individual differences (LG per litter: $F_{1,6}$=18.79, p=0.005; LG per kit: $F_{1,7}$=50.78, p<0.001; see Fig. 3); however, environmental enrichment did not significantly affect LG ($F_{1,9}$=0.18, p=0.677). It also did not significantly affect nest shape (although enriched dams tended to build better nests; $F_{1,18}$=2.89, p=0.053, see Fig. 4).

a)



b)

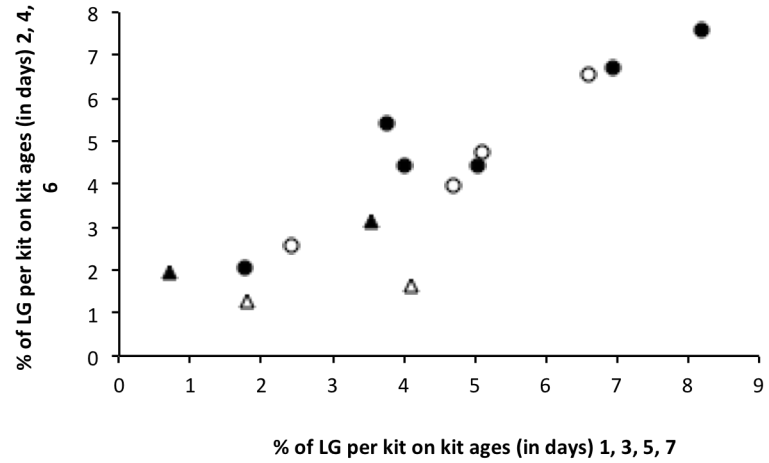


Figure 3. Dams' consistency in their levels of LG expressed as percentage of time budget spent on the behaviour: a) on the litter as a whole (r^2 adjusted: 79.26%); and b) per kit (r^2 adjusted: 84.89%). Circles: first parity dams; triangles: second parity dams. Black symbols: non-enriched individuals; open symbols = enriched individuals). Tests were 2-tailed.

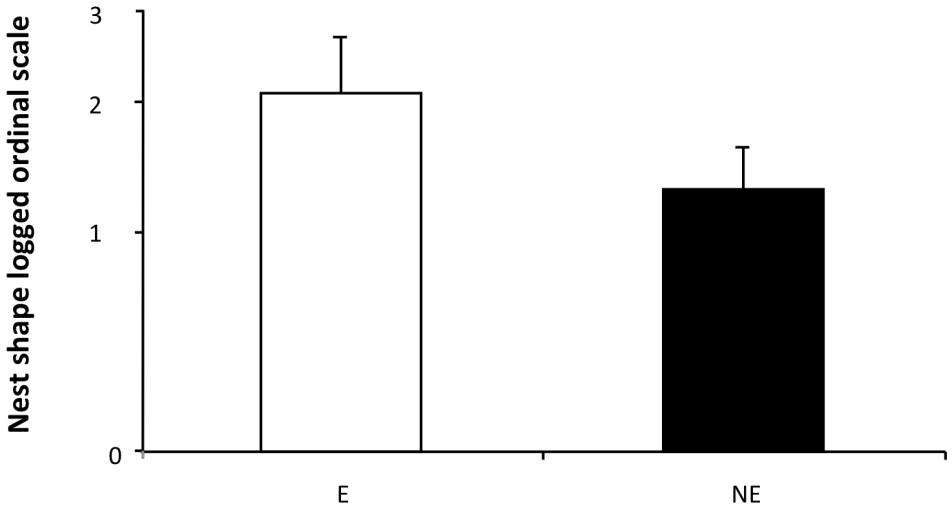


Figure 4. Nest shape differences between enriched and non-enriched dams. Bars represent standard errors. Higher scores represent nests that were more bowl-shaped. Open bars: enriched (E) dams; solid bars: non-enriched (NE) dams.

Relationships between stereotypic behaviour and offspring growth and maternal behaviour

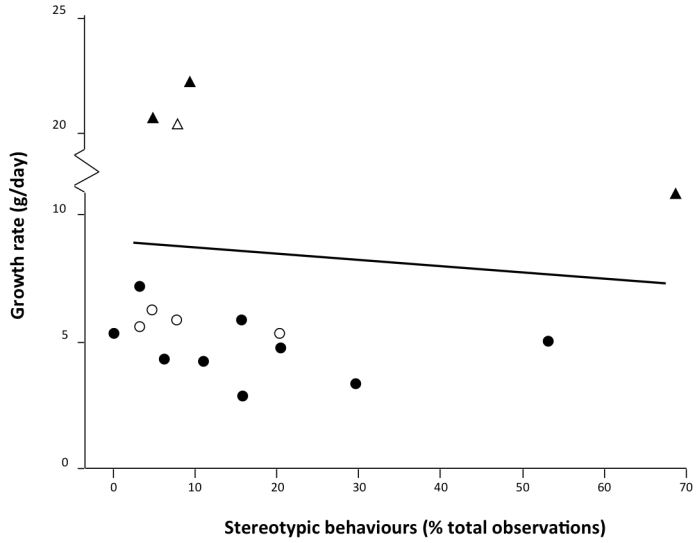
As expected, stereotypic behaviours correlated negatively with offspring growth rate (see Table 3 and Fig. 5; non-significant after FDR corrections). However, although enriched females were known to be less stereotypic (see Chapter 2), their levels of stereotypic behaviours did not predict any measured aspect of their maternal behaviour.

Table 3. Effects of stereotypic behaviours on maternal behaviour and kit development. F: female; M: male. Analyses for LG and kit development related variables controlled for littersize (see Statistical Analyses section in the Methods). Significant results in bold. A minus sign following a p values indicates the relationship was negative. Most kit growth related analyses required splitting the data set by parity; in such cases, results are given in italics (top row = primiparous dams; bottom row = multiparous dams).

		STEREOTYPIC BEHAVIOURS *(over total observations)*		**LOCOMOTORY STEREOTYPIES** *(over active observations)*	
		Statistic	*p*	*Statistic*	*p*
Kit growth	F	**$F_{1,9}$=5.33**	**0.023*-**	*$F_{1,8}$=0.02*	*0.878*
				$F_{1,2}$=11.39	***0.039*-***
	M	*$F_{1,6}$=4.28*	***0.042*-***	*$F_{1,6}$=2.67*	*0.076*
		$F_{1,1}$=19.47	*0.071*	*$F_{1,1}$=3.28*	*0.160*
Maternal behaviour	LG			$F_{1,7}$=0.01	0.458
	Nest shape			$F_{1,15}$=0.91	0.355

*non-significant after FDR corrections

a)



b)

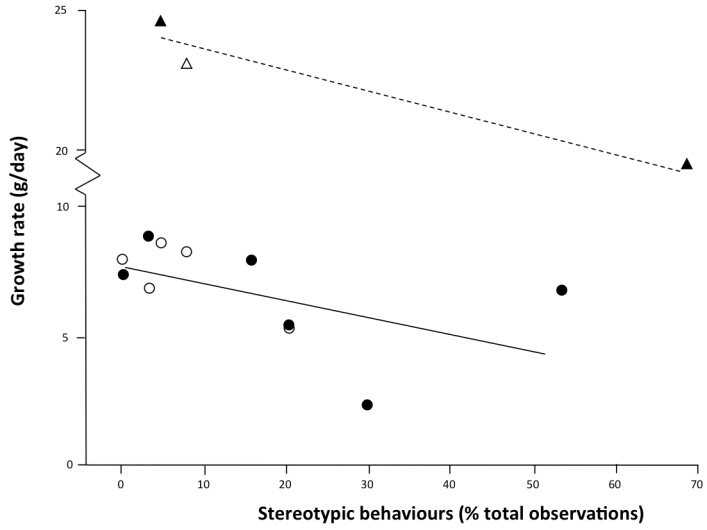


Figure 5. Relationships between female (a) and male (b) offspring growth rates and the stereotypic behaviour of their mothers. Each point represents a family. Circles represent first parity mothers/dams. Triangles represent second parity dams. Solid symbols represent non-enriched families. Open symbols represent enriched families. In a), the solid line represents the trend line for female offspring born to both first and second parity dams (parity not significant in the model, see Table 3; r^2 adjusted: 96.13%). In b) the solid line represent the trend line for males born to first parity dams (significant, see Table 3; r^2 adjusted: 77.83%) and the dashed line the trend line for males born to second parity dams. NB. graphs do not faithfully reflect the GLMs run (i.e. they do not consider all the blocking factors in the model). Average stereotypic behaviours (±SE) of dams: E=10.55±4.87; NE=21.21±5.90.

Effect size comparisons

Due to our small sample size and largely non-significant results, we calculated effect sizes from previous studies that found enrichment to significantly affect maternal licking and grooming and compared them to our own effect sizes. Compared to studies of enrichment and LG in rodents, our effect size for mink was much smaller, the variation in our sample was much larger, and the effect was also in the opposite direction (Fig. 6).

study	effect size	confidence interval at 95%	
	Cohen's d	lower	upper
Sale et al. 2004	2.52	1.08	3.96
Champagne and Meaney 2007	1.11	-0.52	2.75
our study	0.05	-2.70	2.71

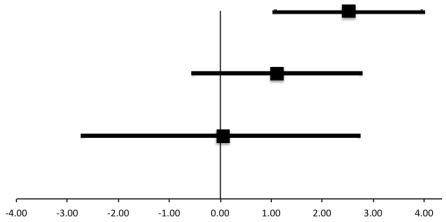


Figure 6. Forest plots of Cohen's d for the effect sizes of enrichment on maternal licking and grooming.

Discussion

This study aimed to assess whether highly-preferred enrichments, known to be very effective at reducing stress and perseveration (see Chapters 2 and 4; also Campbell et al., 2013), would enhance maternal behaviour and/or kit weights and growth rates, thus reducing infant mortality. Although infant mortality was not significantly reduced by enrichment in this

experiment, the study did identify some potential benefits of enrichment, especially on nest-building, litter sex ratios and, albeit indirectly, perhaps also kit growth.

Overall, this study was unable to replicate findings of the previous larger scale study of mink enrichment (Buob et al., 2013), in which females on commercial farms given a shelf during lactation weaned more kits due to reduced mortality. This difference is perhaps due to the fact that Buob and colleagues measured infant mortality at weaning (6 weeks), three weeks later than in our study; it is therefore possible that enrichment might have had benefits on offspring independent of maternal behaviour during the lactation period. However, we suspect it is more likely due to our small sample size, since a comparison of effect sizes with studies that found a significant effect revealed large variability within our sample. We had given the mink far more complex, stimulating enrichments that we hoped would increase effect sizes, yet this was not the case. However, sample sizes were far lower than planned because such a low proportion of females whelped (see Chapter 3 for discussion), litter sizes were also smaller than typical for farms (Jørgensen, 1985), further suggesting unusually low conception rates, perhaps because they had higher fat content (see Chapter 2) or our lighting regime was far from ideal (see Chapter 3) and/or high pre-term losses.

We found no apparent effects of enrichment on neonate weight at post natal day 1, but we did find an unexpected sex ratio effect at the same age (a result significant until FDR corrections). Our sex ratio finding was that right at birth, non-enriched mothers had female-biased litters. Thus they seemed to bear fewer males (perhaps suggesting in utero reabsorption of male fetuses, e.g. Labov et al., 1986, as may be induced by stress in other

species (e.g. Braastad, 1998). However, this result was only significant when we took stillbirths into account, and on commercial farms, as in our non-enriched animals, live kit sex ratios are 50:50. The biological significance of this finding is thus unclear, and replication is needed before strong conclusions can be drawn.

Over the following three weeks, non-stereotypic dams' kits grew significantly more quickly than those of stereotypic dams. Stereotypic behaviours were also successfully decreased by our enrichment paradigm (see Chapter 2). However, despite this, the kits of enriched mothers did not grow more quickly – an effect again likely reflecting our small sample sizes. Buob and colleagues (2013) similarly failed to find that enrichments given to commercially farmed mink boosted kit weaning weights but, in their study, the shelves used had negligible effects on stereotypic behaviour. We therefore think that the hypothesis that large complex, enriched cages would boost kit growth rates is a plausible idea that should now be tested with a more powerful design. For example, as well as increasing sample sizes, future studies could focus on dams with extreme phenotypes in terms of stereotypic behaviour.

That our kits with non-stereotypic dams grew more quickly (a result significant until FDR corrections) is in agreement with the findings of two previous studies (Mason et al., 1996; Meagher et al., 2012b). The first of these used cross-fostering between high and low stereotypic dams, confirming that dam traits (rather than kit characteristics) are driving the effect on growth rates. The second also looked at kit retrieval, finding that low-stereotypy, inactive dams are faster at this, at least for male kits. Here, we found no such relationships with our two measures of maternal behaviour: nest-building and infant licking. This could

be again a byproduct of our small sample size, or instead stem from our choice of maternal behaviour measures. However, we did find indirect evidence that the perseveration associated with stereotypic behaviour is not the key determinant of reduced kit growth. Locomotor stereotypy expressed as a proportion of activity was a much weaker predictor of kit growth than was total stereotypic behaviour (including scrabbling) expressed as a proportion of total time, even though only the former is linked to perseveration in mink (Campbell et al., 2013). Future work should more directly investigate the mechanisms underlying slow growth in the kits of high stereotypic dams by measuring dams' perseveration directly, and also by assessing other potential mediators such as correlated aspects of maternal care (especially responses to kits needing to be retrieved [Meagher et al., 2012b]; but also lactation), and body fat, which is higher in non-stereotypic dams and may affect lactation (Meagher et al., 2012b).

Future studies should also look at dam enrichment and improved nest building ability, because our enriched dams tended to build better nests (a result trending to significance until FDR corrections). This is consistent with previous findings that low stress and/or more naturalistic housing enhances nest-building in mice and pigs (see Introduction), and is potentially important because good nests are crucial for altricial species, including mink (Malmkvist and Palme, 2008). In addition, or alternatively to increasing sample size, more statistical power could perhaps be achieved by providing dams with nesting materials (e.g. straw) that would allow them to display a wider range of nests (Malmkvist and Palme, 2008). The role of improved nest-building in the reduced infant mortality of enriched dams on commercial farms (Buob et al., 2013) should also be investigated.

Licking and grooming has been little studied in mink. We found that mink licking was very like rodent licking in some ways: highly consistent for each dam and also very variable between individuals (cf. e.g. Champagne et al., 2003). This behaviour is worth studying because of its important effects on offspring phenotypes, at least in rodents (see Chapter 6 for more details). In rats this behaviour can also be affected by environmental enrichment (see Introduction). We did not find a similar enrichment effect, however. This could be a true lack of effect. For example, not all rodent studies find a difference (e.g. Welberg et al., 2006), and even those that do, do not find it in all phenotypes (i.e. enrichment affected dams selected for low-licking rates but not those selected for high-liking rates, Champagne and Meaney, 2007). However, as discussed, a Type (see Chapter 3 / Díez-León et al., 2013 and Chapter 6) error is also likely, since again a comparison of effect sizes showed a large variation within our small sample. Our data also help refine methodologies for any future work: our method of only recording them in the morning yielded data that predicted dams' licking and grooming around the clock; the individual differences across dams were also so stable that future studies could collect data over fewer days than we did (e.g. even days only) and still obtain a representative picture of dam behaviour.

In conclusion, our experiment needs to be replicated addressing the methodological issues stated above, especially the low power. However, the previous enrichment effects on mink infant mortality (Buob et al., 2013), and our hints that nest-building and infant growth could both be improved by enrichment, do suggest that this is worthwhile. Furthermore, research in primates and rodents shows that maternal behaviour not only influences offspring

survival, but also has profound effects on offspring adult health, stress reactivity and reproduction (reviewed in Beery and Francis, 2011; Cameron et al., 2005; Meaney, 2001). Therefore, determining if environmental enrichment affects carnivore maternal behaviour in a way that positively impacts offspring development, survival and lifetime fitness would be the first step in improving animal welfare and production efficiency in both conservation captive breeding centres and fur farms.

A modified version of the following chapter is in preparation for submission to Behavioural Brain Research as:

Díez-León, M, Villegas, S, Duprey, R, Lewis, M, Mason, G. Effects of environmental enrichment on basal ganglia function and perseveration in male mink (Neovison vison).

Chapter V. Effects of environmental enrichment on basal ganglia function and perseveration in male mink (*Neovison vison*).

Abstract

Enrichment-induced neural changes in the forebrain are thought to explain its behavioural effects such as decreases in stereotypic behaviours (SB), which are hypothesised to arise from dysfunction in inhibitory cortico-basal ganglia circuitry. This hypothesis is supported by: i) neurophysiological evidence in deer mice, where cage-induced SB are linked to reduced neuronal activity in inhibitory basal ganglia (BG) regions; and ii) behavioural evidence in several species, where cage-induced SB are linked to perseveration (inabilities to suppress previously rewarded responses upon cessation), as also occurs in BG dysfunction. BG changes associated with perseveration have been invoked to explain the reduced copulatory success of stereotypic individuals in one model carnivore, the American mink. Despite the importance of understanding the neural basis of SB and how these may compromise other aspects of the animal's life, no study has investigated the links between BG dysfunction, the development of routine-like tendencies (perseveration and SB), and how these relate to animals' competence at natural behaviours. We tested the hypothesis that enrichment-induced BG changes correlate with routine-like tendencies and copulatory behaviour. We raised 32 male American mink in non-enriched (NE) *vs.* enriched (E) cages. After c.1.5 years, their perseveration in a spatial task was assessed via reversal learning (sensitive to affective and stuck-in-set perseveration) and extinction (affected by several different types of perseveration) was recorded. After their second breeding season (at c.2 years of age), they were killed and their brains extracted. Neuronal activity was analysed via cytochrome

oxidase (CO) staining in two areas of the main input region of the BG (caudate and putamen), as well as two inhibitory BG regions: globus pallidus externus, GPe; and subthalamic nucleus, STN. CO activity in the globus pallidus internus (GPi, an excitatory region) was also assessed to calculate the ratio of activity between the GPe:GPi as a measure of relative activation of the inhibitory/excitatory pathways. Data on SB and copulatory behaviour were obtained from a previous experiment. NE mink had a lower GPe:GPi, suggesting a relative underactivation of inhibitory pathways. However, no single BG region was affected by enrichment, and nor did GPe:GPi correlate with any cage behaviour. While reversal learning was unaffected by enrichment, NE mink tended to take longer to extinguish previously learned responses and extinction negatively correlated with number of copulations gained in mate choice tests. Overall, we found only moderate support for a link between BG dysfunction and SB in mink, yet our finding that both SB and perseveration in extinction are linked to copulatory behaviour suggests that routine-like tendencies compromise the animals' reproductive performance via a common mechanism.

Introduction

Understanding the neural basis of stereotypic behaviours and how they relate to animals' overall behavioural competencies when learning tasks and interacting with conspecifics is important to refine the use of stereotypic behaviour as a welfare tool (see Chapters 1 and 2). The forebrain changes that environmental enrichment induces at the cellular and molecular level (e.g. neurogenesis, synaptogenesis, increases in neurotrophic factors, spine density, and receptor density and affinity; Nithianantharajah and Hannan, 2006; Sale et al., 2009; van Praag et al., 2000) are thought to explain the behavioural changes observed at the whole organism level. For example, enrichment-induced neurogenesis in the hippocampus (a region critical in learning and memory processes [Burgess et al., 2002] as well as emotional responses [Campbell and MacQueen, 2004]) seems to mediate the beneficial effects of enrichment on hippocampus-dependent learning tasks, memory retention and anxiety-like behaviours [Bruel-Jungerman et al., 2005; Kempermann et al., 1997; Santarelli et al., 2003; but cf. Meshi et al., 2006]). As discussed in previous chapters, environmental enrichment is also known to reduce the performance of stereotypic behaviours, which have been hypothesised to result from alterations in the neural control of behaviour that make subjects more repetitive and routine-prone in everything they do (e.g. Garner et al., 2003a; Lewis et al., 2006; Mason, 2006). However, in contrast to the well-studied links between environmental enrichment, hippocampal structural changes and learning and memory effects, there has been little attention on the relationship between enrichment, neurological changes in brain regions important for behavioural control and stereotypic behaviours.

Cage-induced stereotypic behaviours are thought to occur due to alterations specifically in cortico-basal ganglia pathways that inhibit behaviour (Garner and Mason, 2002; Lewis et al., 2006). The basal ganglia are subcortical brain nuclei that regulate behaviour via "cortico-striato-pallido-thalamocortical loops" (henceforth, cortico-basal loops; reviewed in e.g. Alexander et al., 1986; Obeso et al., 2008; see Fig. 1 and Table 1) that also intervene in associative learning, planning, working memory and emotion (e.g. Alexander et al., 1986; Middleton and Strick, 2000). The basal ganglia's main input region, the striatum, is typically subdivided in two main areas - dorsal (caudate + putamen) and ventral (nucleus accumbens + olfactory tubercle) - each receiving projections from different cortical and subcortical areas (see Fig. 1 and Table 1). Correct behavioural control by this circuitry depends on the balanced activity of two anatomically and functionally different neural pathways within each loop: one excitatory (the direct or striatonigral pathway) and one inhibitory (the indirect or striatopallidal pathway) (e.g. DeLong and Wichmann, 2007; Graybiel, 1998; Redgrave et al., 1999; see Fig. 1). Briefly, the excitatory pathway travels from the striatum through the internal segment of the globus pallidus (GPi) and the substantia nigra (pars reticulata, SNr) to the thalamus, which relays excitatory signals back to the cortex, thus promoting behaviour; whereas the inhibitory pathway acts on this thalamo-cortical connection through inputs from the external segment of the globus pallidus (GPe) and the subthalamic nucleus (STN) (see Fig. 1). Alterations in either or both pathways can lead to movement disorders. For example, Parkinson's disease is characterised by 'hypokinesia' and difficulties producing behaviour resulting from an underactivation of the excitatory pathway in the motor loop, while Huntington's disease is characterised by 'hyperkinesia': difficulties in inhibiting behaviour resulting from the underactivation of the equivalent inhibitory pathway (reviewed in e.g. DeLong and Wichmann, 2007; Obeso et al., 2008).

Evidence for the neural basis of stereotypic behaviours primarily comes from pharmacological studies and studies of human disorders where stereotypic behaviours are symptomatic (e.g. autism, obsessive-compulsive disorder), and have linked steoreotypic behaviours to structural and functional changes in the basal ganglia (e.g. Hollander et al., 2005; Welch et al., 2007), especially the indirect pathway. Thus, for example, alterations in these pathways are involved in the stereotypic behaviors of patients with Obsessive Compulsive Disorder and Tourette's syndrome (Adler et al., 2000; Berardelli et al., 2003), as well as those elicited by psychostimulant drugs (e.g. Canales and Graybiel, 2000; Lyon and Robbins, 1975). Furthermore, pharmacologically inhibiting the GPe (using GABA antagonists) induces stereotypic behaviours in non-human primates (Grabli et al., 2004), while stimulating the activity of the STN decreases them (Baup et al., 2008). Disrupted cortico-basal circuitry can also affect cognitive function and flexibility beyond the performance of stereotypic behaviour. For example, individuals with lesions in either the caudate, lenticular nucleus (putamen+GP) or STN show 'perseverative' responding in behavioural tasks (humans, Nys et al., 2006; cats *Felis catus*, Olmstead et al., 1976; rodents, Chudasama et al., 2003), as do, amphetamine treated laboratory rodents (e.g. Evenden and Robbins, 1983; Lyon and Robbins, 1975). Perseveration refers to the repetition of previously rewarded responses in the absence of an appropriate reward under test conditions (e.g. Luria, 1965; Turner, 1997) and alterations in different cortico-basal loops can result in different types of perseveration, classified according to what the individual perseverates (e.g. motor response: recurrent perseveration; attentional set: stuck-in-set perseveration; previous stimulus-reward association: affective perseveration; see Table 1). Perseveration can, however, also be caused by other forebrain changes not related to cortico-basal circuitry and

therefore perseveration does not necessarily diagnose basal ganglia dysfunction (e.g. Duffy et al., 2008).

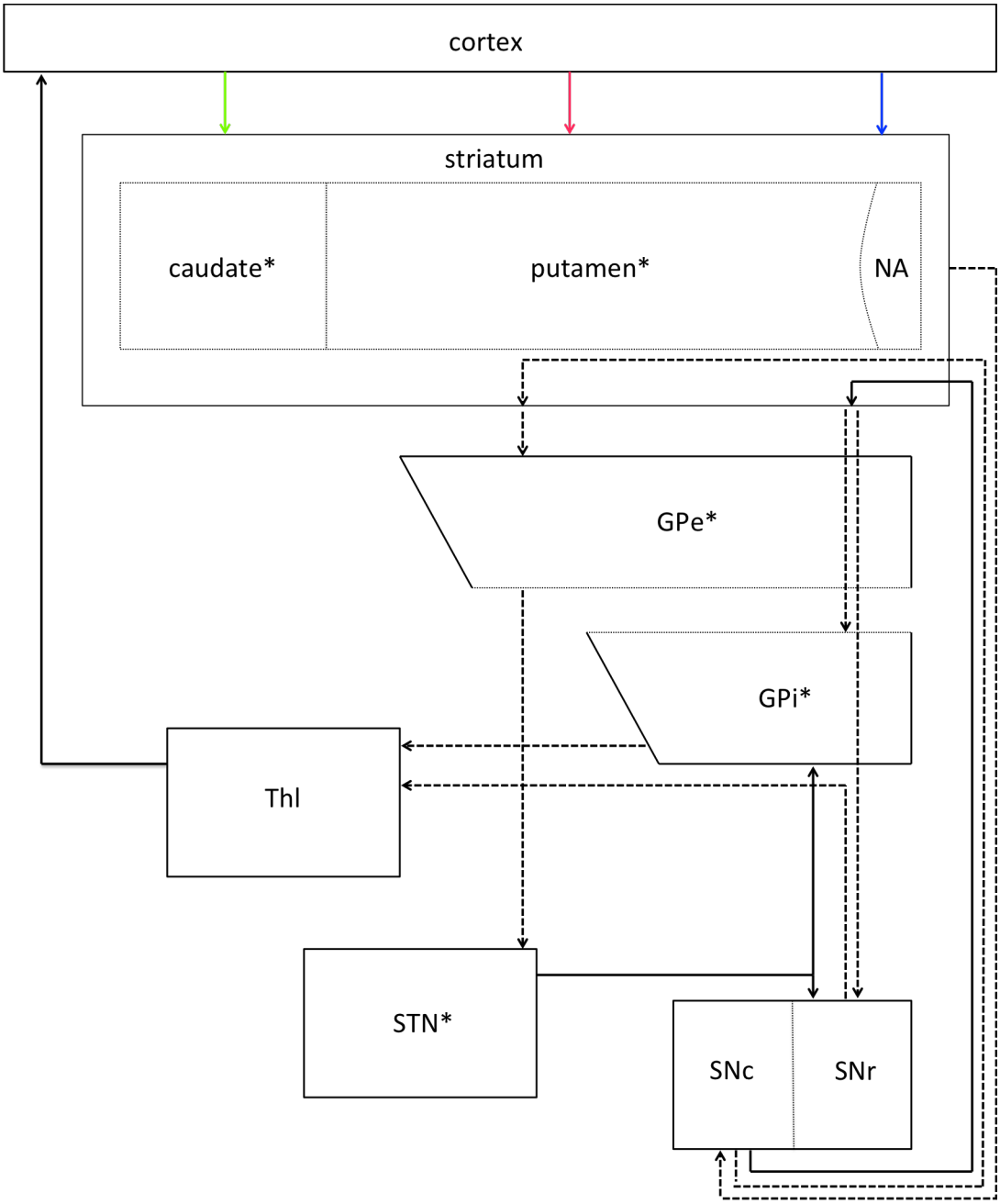


Figure 1. Simplified diagram of the cortico-basal circuitry (modified from a diagram by Joe Garner in Lewis et al. 2006; Obeso et al., 2008; DeLong et al. 2007). NA: nucleus accumbens; GPe: globus pallidus externus; GPi: globus pallidus internus; Thl: thalamus; STN: subthalamic nucleus; SNc: substantia nigra pars compacta; SNr: substantia nigra pars reticulata. Solid lines represent excitatory projections; dashed lines represent inhibitory projections. Different coloured cortical projections exemplify different functional loops (see Table 1): prefrontal (green), motor (red), limbic (blue). GPe and GPi are physically divided here for clarity. Asterisks denote areas sampled in our experimental subjects.

Table 1. Cortico-basal loops: relay regions, associated perseveration types and examples of the behavioural tests used to measure these. Loop names follow those of Krause et al. 2012 (names in parentheses indicate the original names given by Alexander et al. 1986). Each loop starts and ends in the same cortical area, which might be different for each loop (e.g. the arcuate premotor area takes part in both the motor and the prefrontal loops, but the somatosensory cortex only takes part in the motor loop; see Krause et al. 2012 for more details).

cortico-basal loop	loop relays	associated perseveration type	expected outcome in the associated behavioural test
dorsolateral (prefrontal)	cortex + caudate + GPi/SNr + thalamus + cortex	stuck-in-set[1,2] (poor abilities to shift task/attention)	in the intradimensional-extradimensional set-shifting task, subject impaired in the extradimensional aspect but not the intradimensional one[1,2] / impaired in extinction learning[3]
frontal eye field (oculomotor)	cortex + caudate + GPi/SNr + thalamus + cortex	oculomotor[4] (repeated eye movements)	slower blinking rate[4]
supplement motor (motor)	cortex + putamen + GPi/SNr + thalamus + cortex	continuous and recurrent[5,6] (repeated of motor actions)	repeated actions in the gambling task[6] / impaired extinction learning[3]
lateral orbitofrontal (limbic)	cortex + caudate + GPi/SNr + thalamus + cortex	affective[1,2] (poor abilities to disengage from super salient external stimuli associated to reward) and stuck-in-set[7]	inability to not focus on the stimuli during the detour task[8] / impaired at reversal learning[1,2,7,9] / impaired extinction learning[10]
anterior cingulate (limbic)	cortex + ventral striatum + GPi/SNr + thalamus + cortex		

[1]Dias et al. 1996
[2]Clarke et al. 2005
[3]Thullier et al. 1996
[4]Schiller et al. 1980
[5]Sandson and Albert 1984
[6]Ridley et al. 1988
[7]Chase et al. 2012
[8]Wallis et al. 2001
[9]Yawata et al. 2012
[10]Izquierdo and Murray 2005

Several studies show that being raised in impoverished conditions can affect basal ganglia development. For example, isolation-reared primates have decreased dopaminergic function in the dorsal striatum, GP and SN compared to group-housed individuals (Martin et al., 1991). Furthermore, deprivation- and cage-induced stereotypic behaviours have been associated with alterations in the neurotransmitters that regulate these basal ganglia pathways (e.g. dopamine, serotonine) in at least four species (rhesus monkeys *Macaca mulatta*, Lewis et al., 1990; bank voles *Clethrionomys glareolus*, Ödberg et al., 1987; horses, McBride and Hemmings, 2005; deer mice *Peromyscus maniculatus*, Wolmarans et al., 2013). This suggests that environmental enrichment could reduce stereotypic behaviours by normalizing aspects of cortico-basal circuitry. The best evidence on the effects of environmental enrichment on basal ganglia neurophysiological dysfunction and stereotypic behaviour comes from work in the deer mouse by Dr. Mark Lewis' lab at the University of Florida. Enriched deer mice show evidence of increased neuronal metabolic activity (assessed via cytochrome oxidase, CO) in the STN and SN (Tanimura et al., 2010). Additionally, enriched, non-stereotypic deer mice have more neuronal activity in the motor cortex, dorsal and ventral striatum, globus pallidus, thalamus and hippocampus (Turner et al., 2002), as well as greater dendritic spine density in the motor cortex and dorsal striatum (Turner et al., 2003), and higher levels of the brain derived neurotrophic factor in the dorsal striatum and globus pallidus (Turner and Lewis, 2003). Consistent with underactivation of the inhibitory indirect pathway, stereotypic behaviours in deer mice negatively correlate with neuronal metabolic activity in the STN and SN (Tanimura et al., 2011; Tanimura et al., 2010), and positively correlate with the ratio between two opiate neurotransmitters in the dorsal striatum: dynorphin (specific to neurons of the excitatory pathway) and enkephalin (specific to neurons of the inhibitory pathway) (Presti and Lewis, 2005). However, these studies (Tanimura et al., 2011; Tanimura

et al., 2010) also found significant relationships between stereotypic behaviours and neural activity (again assessed via cytochrome oxidase activity) in the ventral striatum, suggesting that alterations in the limbic loop might also play a role. Furthermore, none of these studies have conclusively shown that changes in cortico-basal circuitry are the sole cause of stereotypic behaviours, nor that these changes account for all aspects of the behaviour (reviewed in Mason, 2006). Therefore the extent to which this type of neural dysfunction plays a role in the development of cage-induced stereotypic behaviours is yet to be fully determined.

It has been suggested that non-enriched housing in a non-laboratory species, the American mink, may have similar effects on cortico-basal circuits to those identified in deer mice (Campbell et al., 2013). This is so because in mink, as well as in other species (e.g. bank voles *Clethrionomys glareolus*, Garner and Mason, 2002; deer mice, Tanimura et al., 2008; horses *Equus caballus*, Hemmings et al., 2007; dogs *Canis familiaris*, Protopopova et al., 2014; laboratory mice, Garner et al., 2011; Malayan sun bears *Helarctos malayanus* and Asiatic black bears *Ursus thibetanus*, Vickery and Mason, 2005), cage-induced stereotypic behaviours have been linked to perseveration (Campbell et al., 2013; Dallaire et al., 2011). Dallaire and colleagues (2011) found that mink stereotypic behaviours positively correlated with recurrent perseveration but not affective perseveration (thus suggesting involvement of the motor loop but not limbic loop, see Table 1). However, although providing enrichment reduced stereotypic behaviours in these adult, barren-raised mink, it did not decrease their perseverative tendencies (Dallaire et al., 2011). A subsequent study (Campbell et al., 2013), found that raising young animals with enrichment did decrease recurrent perseveration as

well as stereotypic behaviour. However, this latter study failed to find a correlation between perseveration and stereotypic behaviours. Although not the first study to find no relationship between perseveration and stereotypic behaviours (cf. Gross et al., 2011; Latham and Mason, 2010), since the age of the mink varied between studies (c. 2 year old in Dallaire et al., 2011; c. 1 year old in Campbell et al., 2013), Campbell and colleagues (2013) suggested that perhaps the correlational relationship between perseveration and stereotypic behaviours develops over time. The copulatory success of mink (assessed as number of copulations) raised for c. 2 years using the same enrichment paradigm as these two studies is both positively affected by enrichment and negatively predicted by their levels of stereotypic behaviours (Chapter 3 / Díez-León et al., 2013), a relationship that likewise seems to only develop as animals mature (D. Campbell, as well as our own unpublished data, see additional results in Chapter 3). This negative relationship between stereotypic behaviours and copulatory success has been hypothesised to reflect perseveration-like changes (Chapter 3 / Díez-León et al., 2013), yet this hypothesis had not been tested.

To date, no study in mink (or indeed any other species) has looked at how enrichment-induced cortico-basal circuitry alterations affect both perseveration and stereotypic behaviours. Moreover, it is unknown whether any such changes and/or perseverative tendencies might mediate the known link between stereotypies and reduced copulation number in mature male mink. We therefore tested the hypotheses that i) environmental enrichment provided from birth would improve behavioural control by enhancing neural activity in cortico-basal ganglia circuitry, particularly the inhibitory pathway, and ii) that such changes would relate to the overall control of behaviour and therefore affect other aspects of

the animal's performance (e.g. sexual behaviour). We specifically predicted that enrichment would increase neuronal metabolic activity (assessed using CO metabolism, see Methods) in the dorsal striatum (caudate and putamen) and two regions involved in the inhibitory pathway: the subthalamic nucleus and globus pallidus externus (cf. Tanimura et al., 2011; Turner et al., 2002). Additionally, we predicted a higher ratio of neuronal metabolic activities of the two segments of the globus pallidus (externus:internus or GPe:GPi) in enriched animals, indicating a relatively more active inhibitory pathway (cf. e.g. Heimer et al., 2006; Presti and Lewis, 2005). We also predicted that any changes effected by enrichment would negatively correlate with the performance of stereotypic behaviour and positively with copulation success. Further, we predicted that enrichment would reduce perseverative tendencies; that enrichment-induced changes in neuronal metabolic activity (including the ratio of activity between inhibitory and excitatory pathways) and behavioural control would negatively co-vary; and that highly perseverative mink will be both highly stereotypic and gain fewer copulations in mate choice tests.

Methods and Materials

Subjects and housing

Subject animals were 32 adult black American mink males (see Table 2 for the sample sizes for different variables) housed at the Michigan State University research fur farm (see previous chapters). Half of our subjects had been reared from birth indoors in non-enriched

(NE) or enriched (E) conditions. Details on the rearing treatments can be found in Dallaire and colleagues (2011), Díez-León and colleagues (2013), and Chapters 2 and 3.

Behavioural variables

Perseveration testing

To assess perseveration, we first trained mink with a modified Wisconsin General Testing Apparatus (e.g. Izquierdo and Murray, 2007) in a simple discrimination task, where half the mink were trained to enter the left side of the apparatus to obtain a reward, and half the mink were trained to enter the right side. The apparatus consisted of a wire mesh and plastic cage that attached to the nestbox hole of the home cage (see Fig. 2) for mink to interact with voluntarily. From here, mink could access a vestibule (45x45x37.5cm) where a plastic divider was raised (Fig. 2a), presenting the subject with two metal doors on either its right or left side. The mink could then push either door with its head to access two short tunnels (15x30x15cm; Fig. 2b) to receive a food treat (~0.75 g of hot dog) from a hole at the end of the tunnels (Fig. 2c). The observer delivered the food treat using a custom-made wire skewer (Fig. 2c). Food treats was kept as far as possible from the starting box, and also equidistant from both arms to avoid the mink using odour as a cue for choosing one arm over the other. To avoid behavioural cues from the tester, the apparatus was also designed to visually occlude the tester from the mink during trials (e.g. Hediger, 1981; Martin and Bateson, 2007). Incorrect choices were punished with a 30s timeout. All testing was conducted in the fall of Year 1 (see Chapter 2, Fig. 2), when subjects were 18 months old. Testing was conducted between 8:00 and 17:00 by one tester (SV) blind to the subjects'

stereotypic levels but not housing treatment. After mink chose to enter the apparatus, they were then locked in for the duration of each trial. Individuals were tested across six consecutive days with 20 trials each per day (120 trials/mink in total). Testing order was randomised across individuals and treatment. Which arm was rewarded was also randomized across individuals. Each trial commenced when the plastic slide was raised and concluded when the mink chose a door and its nose touched the food hole. If a mink did not enter either side of the apparatus within 3 min it was deemed a mistrial (not punished) and the slide lowered. The criterion for acquisition was set at 80% of correct responses in 3 consecutive sessions (the probability of reaching this criterion by chance using binomial tests is $p<0.0001$).

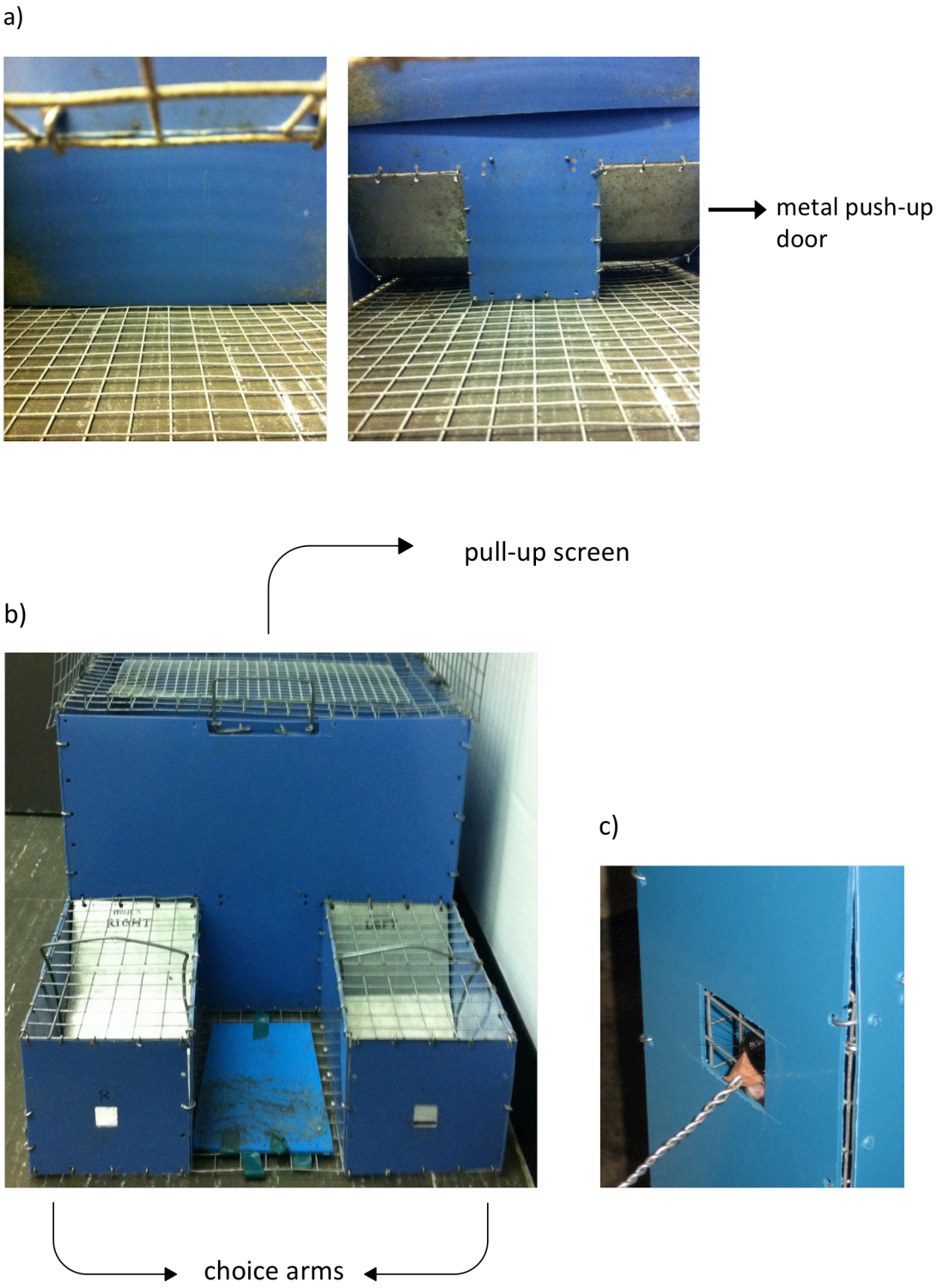


Figure 2. Apparatus used to test perseveration. a) Mink's view of the apparatus from the home cage, with the screen down (left) and up (right); b) tester's view of the apparatus; c) mink getting rewarded with a piece of hot dog at the end of one of the choice arms.

Perseveration was assessed in two ways: the number of responses taken to reverse the learned task (as a measure of affective perseveration[9], Table 1); and to extinguish performing the learned task (a measure sensitive to several types or perseveration, Table 1). Other authors have successfully employed similar methods to assess perseveration and find correlates of stereotypic behaviour (e.g. reversal learning: Tanimura et al., 2008; extinction learning: Garner and Mason, 2002; Vickery and Mason, 2005). Once mink met the criterion for acquisition, we started reversal trials, switching the reward to the opposite arm. Once mink re-met the criterion, we proceeded to extinguish responding. For this, we stopped rewarding either arm until mink dropped to 70% of previously correct responses (a criterion initially chosen because it was not significantly different from choosing the previously rewarded door 50% of the time) in three consecutive sets of ten trials. However, there were actually three possible outcomes during extinction (choosing the previously correct door, choosing the alternative door, and not responding at all), and so we did not use the number of sessions taken to reach this point to calculate extinction rates. Instead, we regressed the percentage of previously correct responses against the number of sessions to obtain the slope of the regression for each mink (a steep slope representing the rapid abandonment of formerly correct choices). During reversal learning, our sample size dropped, and was further reduced during extinction trials, because some animals never met the corresponding criterion, e.g. refusing to enter the apparatus after some trials (see Table 2).

[9] At the time this experiment was conducted it had not been tested whether locomotor stereotypies in mink were linked to affective perseveration as had been argued for mice (Cabib et al. 2006). However, Dallaire et al. 2011 subsequently showed that affective perseveration does not seem to be linked to minks' stereotypic behaviours.

Table 2. Sample sizes for the different variables recorded. For variables related to learning rates, sample sizes are given after the learning trials for acquisition, reversal and extinction were run (sample sizes at the start of each type of learning trial are given between brackets). STN: subthalamic nucleus; GPe: globus pallidus externus; GPi: globus pallidus internus. Square brackets indicate brains for which data were not reliable and so not used in analyses (see main text).

Type of variable		Enriched raised	Non-enriched raised	Total
Acquisition learning		11 (15)	10 (15)	**21 (30)**
Perseveration in:	Reversal	7 (11)	8 (10)	**15 (21)**
	Extinction	6 (7)	8 (8)	**14 (15)**
Copulatory behaviour		16	16	**32**
Stereotypic behaviours		16	16	**32**
Cytochrome oxidase activity of:	Caudate	15	15	**30**
	Putamen	15	15	**30**
	STN	12 + [2]	13 + [2]	**25 + [4]**
	GPe	13	13	**26**
	GPi	12	12	**24**

Mating behaviour

Data on the number and proportion of copulations gained by E and NE males (see Statistical Analyses section) were obtained from a previous mate choice experiment in which females had the choice to enter the cage of an E or NE male blind to their rearing condition (Chapter 3 / Díez-León et al., 2013). This particular measure was selected because it both increased with enrichment and negatively correlated with stereotypic behaviour (see Introduction). Only the copulatory behaviour data from Year 2 was used here, as closer in

time to our perseveration data (Year 1 copulatory behaviour data were collected 8 months before perseveration trials, whereas these preceded Year 2's mating season by only 2-3 months; see Chapter 2, Figure 2).

Stereotypic behaviours

Stereotypic behaviours were recorded when subjects were c.23 months old (i.e. at the end of the whole study; for details, see Chapter 2). Of the two types observed in this mink population -- locomotor stereotypies and scrabbling (see Chapter 2) -- only locomotor stereotypies were considered, since only they have been previously shown to correlate with recurrent perseveration (Campbell et al., 2013; Dallaire et al., 2011; Vickery and Mason, 2003) and copulatory success (Chapter 3 / Díez-León et al., 2013). Locomotor stereotypies were recorded as percentage of active time budget, since in mink this measure correlates best with both perseveration (Campbell et al., 2013) and success in mating competitions (Chapter 3 / Díez-León et al., 2013); it has also been used in previous studies on other species successfully linking stereotypic behaviours to perseverative tendencies (Garner, 1999).

Neurophysiological variables

Metabolic activity of the basal ganglia

Brains were collected from all 32 subjects. The killing and extraction method have been described elsewhere (see Chapter 2). Following death, brains were cut at the caudal end of the medulla, extracted, flash frozen and preserved in dry ice for transport from the farm and

then stored in a -80°C freezer until further processing. Neuronal metabolic activity in the basal ganglia was assessed via cytochrome oxidase (CO) histochemistry, an index of oxidative energy metabolism shown to correlate with long-term dendritic activity (Poremba et al., 1998; Wong-Riley, 1989; Wong-Riley et al., 1998). All CO histochemistry analyses were carried out by research assistants of Dr. Mark Lewis at the University of Florida. Activity of CO was assessed in the following basal ganglia nuclei, as selected by our collaborators: caudate, putamen, GPe, GPi, and STN. Details of CO histochemistry analyses can be found in Turner and colleagues (2002), and Tanimura and colleagues (2011). Briefly, CO assays were performed ten times to stain all relevant tissue. For each assay, the same number of E:NE brains were removed simultaneously from the - 80° C freezer. Each brain was coronally cryosectioned (-20 degrees C) at 20μm, until the whole of the basal ganglia were captured. Start and end of the basal ganglia were determined using a polecat brain atlas (obtained from the Michigan State University Brain Biodiversity Bank). Sections were then mounted on slides (4 sections per slide), which were kept in the -80° C freezer overnight to be stained the following day. Brains were stained with diaminobenzidine following the protocol in Gonzalez-Lima and Jones (1998). As stated in the Introduction, CO activity was assessed in the following regions: caudate, putamen, subthalamic nucleus (STN) and globus pallidus externus (GPe). The CO activity in the globus pallidus internus (GPi) was also assessed to be able to calculate the GPe:GPi ratio (see Introduction). CO activity was quantified by optical densitometry (OD; where higher densities correlate with higher CO activity (Gonzalez-Lima and Jones, 1994) using ImagePro (Media Cybernetics) on selected areas of digitized images by an experimenter blind to treatment conditions and stereotypic levels. An average of eight such samples per region of the basal ganglia was obtained. Measures were taken from both left and right hemispheres and then averaged.

The reliability of each staining batch was also checked by correlating OD readings for a standard brain (i.e. an homogenate brain processed so that there are no distinguishable structures that would stain differently) smeared at different homogenate thicknesses (from 10 to 100μm), since staining density should increase linearly with slice thickness. Using this method, four brains (2E:2NE) were identified for which the staining of the STN was sub-optimal. Therefore, those brains were excluded from any analyses involving the STN (see Table 2 for final sample sizes for each region. Note too that due to problems in processing [e.g. tears in the tissue], the are not data for all brains nor for all basal ganglia nuclei for each brain).

Statistical analyses

Statistical analyses were performed using JMP (v. 10.0, SAS Institute, 2012). When data did not meet the assumptions of parametric testing, they were transformed (e.g. number of sessions in extinction and number of copulations were log-transformed). Alpha was set at 0.05. Since clearly directional hypotheses were being tested, 1-tailed results are presented (cf. Levine and Banas, 2003; Rice and Gaines, 1994). Given the relatively low number of tests (13), and their subdivision into three small families of hypotheses (5, 2 and 6 tests in each family), unlike elsewhere in the thesis (Chapters 2-4), we did not deem it necessary to use "false discovery rate" procedures (FDR; Benjamini and Hochberg, 1995) to reduce risks of Type I error.

All analyses involving CO activity controlled for differences in degrees of staining between different staining batches by adding the values from each batch's standard homogenates at 20μm thickness (chosen as it is the thickness at which the brains for CO analysis were cut) as a covariate in the models. Data on enrichment effects on basal ganglia metabolic activity were analysed with models in which CO activity was the dependent variable and treatment (E/NE) was the independent variable. Similarly, data on enrichment effects on locomotor stereotypies, learning rates and perseveration were analysed with three models in which each behavioural variable acted as the dependent variable respectively, and treatment as the independent variable. Since neither enrichment nor level of stereotypic behaviour affected acquisition rates ($p>0.10$, data not shown), perseveration analyses do not control for learning rates. Relationships between enrichment-affected basal ganglia physiology and perseveration measures were analysed by means of General Linear Models (GLMs) in which the perseveration measure was the dependent variable and treatment, basal ganglia metabolic activity, and their interaction acted as blocking factors.

Finally, to investigate the relationships between enrichment-affected basal ganglia physiology or perseveration, and locomotor stereotypies, we ran GLMs in which locomotor stereotypies acted as dependent variable, and treatment, metabolic activity (in this case corrected by assay standard by using residuals to have enough degrees of freedom) or perseveration measure, and their interaction as blocking factors. To investigate relationships between enrichment-affected basal ganglia physiology and mating behaviour, we ran two types of GLM (see 'Analysis 1' and 'Analysis 2' in Chapter 3 / Díez-León et al., 2013): one with male copulation number as the dependent variable, and basal ganglia physiology as the independent variable,

blocking for female treatment, and male pair nested in female treatment, and their interaction; and a second GLM using the proportion (arcsine square root transformed) of each female's total number of copulations allocated to her E mating partner as the dependent variable, and the absolute difference in the trait of interest's magnitude between that female's E male and his NE rival as the independent variable, also blocking for female treatment, male pair, and their interaction. For perseveration, unfortunately, we did not have enough data for both males in many pairs of rivals. Therefore, in order to investigate the relationship between perseveration and reproductive performance, we ran a simple model in which just copulation number acted as the dependent variable, and just female treatment as a blocking factor and perseveration as the independent variable.

Results

Enrichment effects on neural correlates of locomotor stereotypies

Our predictions that enrichment would increase the metabolic activity of the basal ganglia regions implicated in the inhibitory pathway of the motor loop, as well as decrease perseverative tendencies, were partly supported. Enriched mink had greater GP ratios (GPe:GPi) of CO activity than non-enriched mink ($F_{1,20}$=5.840, p=0.012). However, no other basal ganglia region was significantly affected by environmental enrichment (see Table 3). The groups also showed no differences in reversal learning ($F_{1,13}$=0.412, p=0.265). However, non-enriched mink tended to take longer than enriched mink to extinguish previously learned responses ($F_{1,12}$=2.859, p=0.058).

Table 3. Effects of enrichment on basal ganglia metabolic activity (optical density scores). E: enriched; NE: non-enriched; STN: subthalamic nucleus; GPe: globus pallidus externus; GPi: globus pallidus internus. Significant results in bold. Means are least squared means; SE is the standard error. Analyses corrected for staining batch effects (see text).

	E		NE		Statistic	p
	Mean	SE	Mean	SE		
Caudate	0.119	0.007	0.111	0.007	$F_{1,27}$=0.670	0.210
Putamen	0.103	0.007	0.097	0.007	$F_{1,27}$=0.429	0.258
STN	0.198	0.008	0.194	0.008	$F_{1,26}$=0.102	0.376
GPe	0.108	0.008	0.107	0.008	$F_{1,23}$=0.01	0.455
GPe:GPi	**0.596**	**0.013**	**0.552**	**0.011**	**$F_{1,20}$=5.840**	**0.012**

* even though we did not have predictions involving metabolic activity in the direct pathway, given that the ratio of activity between the GPe and GPi was statistically different in E and NE animals, here we offer the statistical results for treatment effects on the GPi alone in case they are of interest: E: 0.185±0.016; NE: 0.197±0.015; $F_{1,19}$=0.310; p=0.584.

Enrichment-affected neural effects and their relationships with locomotor stereotypies and copulation success

Our prediction that enrichment-induced basal ganglia changes and perseverative tendencies would correlate with stereotypic behaviours and individual copulatory success was also partially supported. GPe:GPi did not predict the performance of locomotor stereotypies ($F_{1,19}$=0.891, p=0.178) nor the number of copulations gained by these males (total # of copulations: $F_{1,20}$=0.119, p=0.266; % of copulations gained: $F_{1,4}$=0.278, p=0.312). Extinction rates also did not correlate with GPe:GPi ($F_{1,8}$=0.070; p=0.797) or predict the performance of locomotor stereotypies ($F_{1,10}$=0.194, p=0.334). However, extinction rates negatively predict the number of copulations gained ($F_{1,11}$=4.965, p=0.024; Fig. 3).

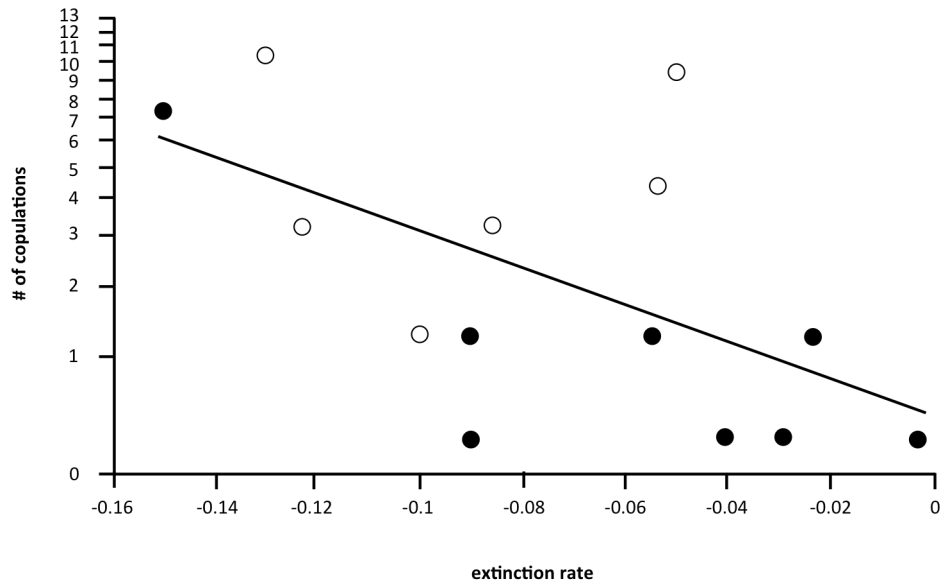


Figure 3. Relationship between extinction rates in a learning test (for calculation see Methods) and number of copulations in a mate choice test. Solid symbols represent non-enriched subjects. Open symbols represent enriched subjects. Y axis is in a log scale. Stats: $F_{1,11}$=4.965, p=0.024.

Discussion

Overall, we found only moderate support for an association between basal ganglia dysfunction and the cage-induced stereotypic behaviours of American mink. However, our finding linking perseveration with copulatory success, coupled with our previous finding that non-stereotypic animals also gain more copulations, does suggest there is a common mechanism behind these effects.

Consistent with Campbell and colleagues (2013), and as predicted, mink raised with enrichment tended to be less perseverative. This was seen only in extinction: enriched mink stopped responding in the absence of reward c. 30% faster than non-enriched mink, while reversing the learning paradigm no faster. The subtlety of the effect of enrichment on extinction is perhaps due to the fact that by this final stage of the learning task the sample size was greatly reduced by animals refusing to enter the apparatus. Nevertheless, even with this small number of subjects, perseveration in extinction predicted reduced copulation rates, supporting the hypothesis raised in Chapter 3 (Díez-León et al., 2013) that non-enriched, highly stereotypic mink were less successful with females likely because they were more perseverative. Enriched animals also had greater GPe:GPi ratios, confirming the predicted higher inhibitory activity in their basal ganglia. However, this effect was also subtle: individually, neither GPe nor GPi CO values differed between the groups and therefore, our prediction that these regions would have higher values of CO activity in E mink was not met. There was also no evidence of enhanced activity in the other indirect pathway region sampled: the STN. Indeed, environmental enrichment did not significantly increase metabolic activity in any of the basal ganglia nuclei examined. Therefore, we could not replicate in mink what has previously been found in the dorsal striatum and subthalamic nucleus of differentially housed deer mice (Tanimura et al., 2010; Turner et al., 2002). Furthermore, the one effect detected in the mink, an increased GPe:GPi ratio, seemed unrelated to the behavioural effects of enrichment, failing to correlate with stereotypies, performance in extinction, or copulation number.

Taken together with Dallaire and colleagues' (2011) results, our finding suggests that only alterations in the motor loop but not the limbic loop are involved in the perseverative tendencies observed in captive mink (Table 1). Similar to what has been recorded for mink using a different measure of affective perseveration (Dallaire et al., 2011), in our sample, non-enriched mink reversed the learning paradigm as fast as enriched mink did. This is in contrast to what has been found in deer mice, where enriched mice reversed faster than their non-enriched counterparts (Tanimura et al., 2008). Activation of the ventral striatum (along with other areas associated to the limbic loop) seems to be a key aspect of reversal learning (e.g. Cools et al., 2002; Frank and Claus, 2006). However, since extinction learning is not specific of any one type of cortico-basal loop dysfunction (e.g. Izquierdo and Murray, 2005; Thullier et al., 1996) and therefore acts more like a general marker of inappropriate behavioural inhibition (Garner, 2006; Izquierdo and Jentsch, 2012; Mason, 2006), it is still possible that more than just the motor cortico-basal loop is compromised in these animals. Future studies should investigate whether other types of perseveration occur in mink (e.g. stuck-in-set perseveration by looking at subjects' abilities to make attentional shifts, Dias et al., 1996), as well as use additional measures of potential underlying neurophysiological mechanisms (e.g. neurotrophic factors; Turner and Lewis, 2003).

Individual levels of stereotypic behaviours have been linked to levels of perseveration in at least 15 species, including mink (see Introduction). However, in our study, minks' individual levels of stereotypic behavior did not correlate with their perseverative tendencies measured in extinction. This is in agreement with Campbell and colleagues (2013), but conflicts with the findings of Dallaire and colleagues (2011). To explain this discrepancy, Campbell and

colleagues suggested that the link between stereotypic behaviours and perseveration might need time to develop and therefore it could not be found in their 10 months old males, while it was consolidated in Dallaire and colleagues' +2 year old females. Since we used older males (between 18-20 months old at the time of testing), and both stereotypic behaviours and extinction rates negatively predict copulatory success in these animals (see below for further discussion), we believe that the most parsimonious explanation for our lack of result is a Type II error (see Chapter 6 for further discussion). We suspect this because of the small number of males (see Table 2) that met the extinction criterion, and because perseveration under extinction was measured in our males five months earlier than both copulatory behaviour and stereotypic behaviours, which in contrast were recorded relatively close in time to each other (see Methods section; also Chapter 2, Fig. 2). Future studies of mink should therefore use larger sample sizes, especially if the methodology, like ours, requires subjects to undergo different training procedures in sequence; or perhaps increase motivation using mild food deprivation. Alternatively, they could use a perseveration testing regime that does not involve frustration at the absence of expected rewards and therefore likely to result in lower drop-out rates (e.g. the proportion of subjects not completing procedural and reversal learning in our experiment was almost double than that of other studies that measured recurrent perseveration in mink using a gambling task where reward rate remains fairly constant; cf. Campbell et al., 2013; Dallaire et al., 2011). Future studies should also assess perseveration, stereotypies and behaviour in mating tests much closer together in time.

To date, no study had investigated the consequences of perseverative tendencies on the performance of animals' natural behaviours. We found that mink who took longer to extinguish previously learned responses gained fewer copulations in mate choice trials. Along with our previous finding linking stereotypic behaviours to reduced copulatory success (Chapter 3 / Díez-León et al., 2013), this suggests that, similar to what is observed in autistic patients (e.g. Barbato et al., 2004), generally repetitive behavioural tendencies are associated with impaired social interactions. Now this needs further testing by investigating what other aspects of these animals' courtship (e.g. 'chuckling', Gilbert, 1969) or other normal, non-stereotypic behaviours (e.g. maternal care, cf. Chapter 4) are also altered.

We also for the first time investigated the relationship between enrichment-induced changes in the basal ganglia and enrichment-induced changes in repetitive behavioural tendencies. GPe:GPi ratios did not correlate with either stereotypic behaviours, perseveration in extinction or copulatory success. This was even though all these variables were affected by environmental enrichment (enrichment reduced GP activity ratios, stereotypic behaviours and perseveration in extinction, and increased copulatory success), and even though GPe:GPi activity should predict how readily inappropriate behaviours are inhibited (see Introduction). There are a number of possible interpretations of this lack of effect, and indeed lack of enrichment effects on the CO data. First, measurement errors could have arisen while working with a species for which a brain atlas and specific CO assay protocol do not exist. Another explanation for our lack of effect, perhaps more likely given the complexity of the cortico-basal circuitry, is that enrichment might be affecting mink basal ganglia in ways that we did not measure. For one, it is possible that measuring neuronal

activity per unit area does not capture changes that could be occurring in regional volume. To illustrate, compared with normal humans, autistic patients have larger striatal volumes that are associated with the performance of stereotypic behaviours (Hollander et al., 2005). In addition, other relevant aspects of cortico-basal ganglia circuitry might be implicated in cage-induced stereotypic behaviours in mink. For example, the neuronal activity (assessed again by CO activity) of the substantia nigra is increased in non-stereotypic enriched deer mice (Tanimura et al., 2010; Turner et al., 2002), with the CO activity in the SN negatively correlating with the performance of stereotypic behaviours (Tanimura et al., 2011). Looking outside of the motor loop also seems a logical next step since enrichment has been shown to induce neurogenesis in the prefrontal cortex of guinea pigs (Fan et al., 2014) and alter the cholinergic system in the prefrontal cortex of rats (Del Arco et al., 2007; Segovia et al., 2008); also in the latter species, lesions in this cortical area have been linked with increases in stereotypic behaviours (Lipska et al., 1998). Alternatively, forebrain regions not implicated in the cortico-basal ganglia circuitry might be implicated in caged mink repetitive tendencies. For example, as mentioned in the Introduction, environmental enrichment increases neurogenesis in the hippocampus (e.g. van Praag et al., 2000) and, in rodents, dysfunction in the hippocampus can also give rise to perseveration (Duffy et al., 2008; Whishaw and Tomie, 1997), and hippocampal lesions also seem to induce stereotypic behaviours (Wood et al., 1997). Given that enriched mink show behavioural signs of increased hippocampal function (Campbell et al., 2013), and, at least in birds, the hippocampus also seems to be important for reproductive success (e.g. Boogert et al., 2011), as well as learning and memory (e.g. van Praag et al., 2000), future studies could look at the relationship between enrichment, hippocampal function, stereotypic behaviours and copulatory success in mink.

Overall, our results cautiously indicate that there might be some involvement of the motor loop inhibitory pathway in the stereotypic behaviours of American mink, suggesting that future studies should investigate anew the basal ganglia dysfunction hypothesis (e.g. by looking at more diverse measures of neural function as well as other forebrain areas). Our results also confirm our previous hypothesis (Chapter 3 / Díez-León et al., 2013) that the lower performance of stereotypic animals in mate choice tests is linked to their perseverative tendencies, yet it is not clear how or if basal ganglia dysfunction mediates this effect. Further work is now needed to determine the role that brain dysfunction plays in the stereotypic behaviours of mink given the importance of understanding the basis of these behaviours to refine their use as welfare diagnostic tools.

"Our knowledge of circumstances has increased,

but our uncertainty, instead of having diminished,

has only increased"

- Carl von Clausewitz (1780-1831)

Chapter VI. Problems in the captive breeding of wild mammals: insights from studying the roles of environmental enrichment and stereotypic behaviours in the reproduction of a model carnivore, the American mink.

The breeding of captive wild mammals faces problems that have been attributed to the effects that non-stimulating environments can have on stress physiology. These raise animal welfare concerns about the individuals involved in conservation breeding programs as well as those kept in zoological parks for display purposes. In addition, stereotypic behaviours are commonly observed: a sign of group-level poor welfare, yet the welfare significance of individual differences in their performance is not very clear. Both reproductive problems and stereotypic behaviours are common in carnivore species, and so welfare-ameliorating modifications such as environmental enrichment have been suggested or used to tackle these issues, albeit typically without using *a priori* validated enrichment items. In this thesis, I therefore aimed to investigate the links between environmental enrichment, stereotypic behaviours and breeding success in a model captive carnivore, the American mink *Neovison vison*. My research hypotheses were:

I. environmental enrichment increases individuals' reproductive success by reducing stress and / or normalising brain function

II. stereotypic individuals have compromised reproduction due to brain alterations induced by the impoverished captive environments that result in impaired behavioural control

I tested these hypotheses by raising mink in either enriched or non-enriched environments from birth until c. 2 years old. I tested predictions regarding how enrichment affected stress-related physiology, certain aspects of brain function, and the performance of stereotypic behaviours. I then tested predictions about how these enrichment-affected aspects of the minks' phenotypes co-varied with each other and with aspects of reproduction (copulatory behaviour; maternal care; offspring survival and development). The results of this study and their significance have already been discussed in detail in each of the previous chapters. Here, I will focus on the three most important contributions of my thesis and suggest where to take those findings next. Next, I then discuss some unexpected or disappointing findings and say how they could be investigated further.

1. Environmental enrichment improves lymphoid organ development, skeletal symmetry and brain function in mink

In my thesis, I showed for the first time that certain effects that enrichment has on laboratory rodents and rabbits can be found in a model carnivore. Thus in addition to reduced glucocorticosteroid levels and stereotypic behaviours, enriched mink had heavier lymphoid organs (suggesting enhanced immune response), less skeletal asymmetry (suggesting reduced developmental stress), and reduced perseverative tendencies as well as a seemingly more normal functioning of some of the neural circuitry controlling how behaviour is sequenced (although results pertaining to brain function should be taken with care due to their preliminary nature). These findings are important for three main reasons. First, they confirm that environmental enrichment has positive effects for carnivore welfare

(including farmed mink) beyond ameliorating stereotypic behaviours and reducing glucocorticosteroid levels. Second, they open new avenues for research on enrichment effects on the immune system and overall health of captive carnivores (see below), an issue important for captive breeding managers and fur farmers alike. Finally, they highlight the potential that farmed mink have as models for future carnivore work, something particularly important for the conservation breeding programs of two highly endangered mustelids, the European mink *Mustela lutreola* and the black-footed ferret *Mustela nigripes*, where invasive procedures or research that involves killing individuals is not feasible.

Future research on mink

Research should continue so as to allow us to better understand these enrichment-affected variables. For example, the relationships between lymphoid organ weight and actual immune function and disease resistance now need to be investigated. Tests could be run to investigate the effects of immune challenges to both enriched and non-enriched mink, with the prediction that enriched mink will have a higher number of anti-body producing splenic cells (Persson, 1977) and/or increased contact sensitivity responses, which are thymus-dependent (Pritchard and Micklem, 1972). To see whether this affects function at the whole animal level, experiments should be run to see if non-enriched animals have higher infection rates and sustain heavier parasitic loads.

Continuing research on skeletal asymmetry should also occur: an important topic for two reasons. First, from a welfare point of view, fluctuating asymmetry represents a potential

new welfare indicator in carnivores, whose well-being is typically determined by recording just stereotypic behaviour or fecal corticoisteroids, both of which have their problems (see Chapters 1 and 2). In order to use fluctuating asymmetry more reliably in welfare assessment, future studies should look at more than one structure with bilateral symmetry (e.g. skull, limb bones). This is because within the same individual, different structures might show different degrees of fluctuating asymmetry (e.g. Knierim et al., 2007), and therefore, using just one measure could add noise to results. This type of study could additionally identify if there is any particular structure (e.g. mandibles, or perhaps some other bone) that is a good marker of overall skeletal fluctuating asymmetry in mink, thus helping optimise future work both in mink and mustelids. Furthermore, identifying the causal mechanisms underlying the development of asymmetrical bilateral structures has important consequences for their use as welfare indicators: if low fluctuating asymmetry reflects a stress-resistant genotype, then it can be taken as a good indicator of current welfare, while if it merely reflects a low stress early rearing environment, then it might not be useful when assessing current rather than past well-being. Second, from a conservation breeding point of view, it is important to identify characteristics that could make males attractive, in order to plan matings so as to preserve genetic diversity. Even though I did not find support for a link between skeletal symmetry and copulatory success, skeletal asymmetries could still negatively impact male reproduction if they impair aspects of it. For example, an asymmetrical bite could result in a less efficient scruff bite; facial asymmetries *per se* could also play a role in mate choice as observed in other species (Chapter 3 / Díez-León et al., 2013); and body asymmetries could result in poor performance during the initial play-like fight typical of mustelid copulation. Therefore, I would suggest testing this hypothesis in more detail with a

larger sample size, additional measures of fluctuating asymmetry, and looking at specific aspects of courtship that could be impaired as a result of an asymmetrical phenotype.

Finally, although I found some indication that enrichment did have positive neurological effects, most of my predicted results were non significant. Rather than dismissing the role of brain function in the stereotypic behaviours of non-enriched animals, and given the vast literature of enrichment effects on brain function, I believe that my rather subtle findings reflect more the preliminary nature of this part of my research (e.g. methodological shortcomings) than a true lack of effect. Therefore, I would suggest future studies should explore in more detail the effects of enrichment on brain function, both in terms of neurophysiology and using additional tests that measure behavioural flexibility (see Chapter 5). These studies should explore the basal ganglia further (by, for example looking at properties of the striatal matrix and striosomes, cf. Canales and Graybiel, 2000; Tanimura et al., 2011), as well as other brain areas, with one obvious candidate being the hippocampus (see Campbell et al., 2013; and Chapter 5).

2. Environmental enrichment improves male reproductive performance by reducing behavioural repetition

One of the problems reported for captive wild carnivores is that, despite being physically fit, many animals fail to breed, and if they do, their offspring survival is compromised (reviewed in Chapters 1, 3 / Díez-León et al., 2013, and 4). My study showed for the first time that

barren environments negatively impact the copulatory success of male mink. Moreover, it looked at mechanisms by which enrichment produced this effect, something no other study had done. I had hypothesised that enrichment effects on reproduction could be brought about by three non-mutually exclusive mechanisms: i) reduced effects of stress on reproductive physiology; ii) increased phenotypic attractiveness due to reduced developmental stress; and/or iii) improved brain function. Of these, although enrichment positively affected variables implicated in all three (Chapter 3 / Díez-León et al., 2013), only two proxy measures for brain function turned out to be predictive: locomotor stereotypies and perseverative tendencies both negatively predicted the copulations gained by mink males. This finding is the first to suggest that the behavioural changes underlying captivity-induced stereotypic behaviours impair the overall social competence of the animal, in a similar manner to that observed in humans with autism or primates reared in isolation. This has serious implications, as it raises questions about the social, sexual and maternal competence of the millions of captive animals displaying stereotypic behaviours.

Future research on mink

The role of brain function and behavioural flexibility in reproductive behaviour should be explored further. There are at least two hypotheses to test here. First, behaviourally inflexible animals might be bad at the 'give and take' of socio-sexual behaviour, and/or bad at adjusting their behaviour to that of their partners. In rodents, for example, routine-prone males that are trained to expect a male intruder (whom they attack) cannot inhibit their attack response when familiar females are presented to them (Benus et al., 1990). Similar experiments in mink could test how social interactions –between potential mates, potential

male rivals, playmates, and mothers and their offspring -- are compromised by behavioural inflexibility. For example, during mating season, would a highly perseverative male mink that encounters an oestrous female, then respond equally (i.e. would it try to copulate/fight or perhaps ignore, whichever its first response is) to an anoestrous female/unfamiliar male presented in an inspection tunnel?. Also, the courtship behaviour displayed by mink might have mate quality assessment components: are non-enriched animals abnormally repetitive in some of those components? One candidate could be the vocalizations mink perform during courtship (also known as 'chuckling', which are also performed when playing, Gilbert, 1969), which in bird species have been linked to both courtship effectiveness and brain function (reviewed in Nowicki and Searcy, 2005; Petkov and Jarvis, 2012). For example, future studies could test if non-enriched, stereotypic mink' courtship vocalizations sequences are more repetitive and less flexible, and whether the different elements of their courtship behaviour are initiated at higher frequencies (e.g. Garner and Mason, 2002; Garner et al., 2003a) and/or have fewer elements than those of non-stereotypic mink (e.g. Clubb, 2001; Mason, 1993). Studies with more detailed observations and using continuous sampling rather than just scan sampling could answer these questions.

In my study, I also found signs that, independently of repetitive tendencies, testosterone-related traits play a role in the copulatory success of male mink: mink whose bacular proximate processes were more developed tended to gain more copulations (see Chapter 3 / Díez-León et al., 2013). Similarly, a large on-farm enrichment study in mink found that higher levels of androgens (both testosterone and epiandrosterone) predicted longer copulations (Meagher et al., submitted). In addition, vocalizations have been linked to

testosterone levels in other carnivore species (e.g. giant panda *Ailuropoda melanoleuca*, Charlton et al., 2011). Given that mate choice is a multi-modal process, yet few studies address more than one signalling modality together (reviewed in Coleman, 2009), future studies could explore the interaction between testosterone-dependent traits (e.g. bacular morphology; also ano-genital distance, e.g. Graham and Gandelman, 1986), testosterone-related odours and vocalizations in helping to determine the reproductive success of male mink.

One major unknown in my work was also whether traits that predicted success did so by correlating with libido (male sexual motivation), by making males more attractive to females, or both. Those traits that can potentially make males more attractive to females should be identified in mink as well as other carnivore species, especially as research in other species has shown that high quality males seem to have more/high quality offspring as well as elicit more maternal care from their female mates (e.g. Mashoodh et al., 2012), something important for conservation captive breeding as well as the farming industry. Therefore, future studies could test if high stereotypic males are actually less attractive to females (via e.g. place preference tests)**,** and if so, whether their mates then have smaller litters, slow growing and/or lighter offspring and female-skewed litters with heightened stress reactivity.

3. Different forms of stereotypic behaviour in mink are differentially affected by enrichment, have different reproductive correlates, and therefore might have different underlying mechanisms and/or welfare implications

Stereotypic behaviours in mammals are a heterogeneous group of different forms: pacing, swaying, head twirling, tongue rolling, fur plucking, etc. (see Chapter 1, also Mason, 2006). Some forms are more prevalent in some species than others: carnivores typically perform locomotor stereotypies (e.g. pacing) while ungulates commonly perform oral forms (e.g. tongue rolling) (Mason et al., 2007), and these differences are hypothesised to reflect different underlying motivations (e.g. foraging *vs.* ranging) and species-typical modes of behaviour (e.g. grazing *vs.* hunting) (Bergeron et al., 2006; Clubb and Vickery, 2006). Within species, drugs and enrichments can also affect different forms differently. For example, apomorphine (a dopamine agonist) significantly induced pacing-like and oral stereotypies in deer mice but not repetitive jumping (Presti et al., 2002). Turning to enrichment, deer mice housed in large cages provided with different enrichments (e.g. wheels, hiding places), while still performing less stereotypic behaviours than conspecifics housed in smaller, non-enriched cages, significantly develop more pacing relative to their non-enriched conspecifics, who perform more jumping and backward somersaulting instead (Powell et al., 1999). In primates, foraging enrichments reduce pacing and other whole-body stereotypic behaviours, but do not reduce self-directed, injurious behaviours (Bayne et al., 1991; Novak et al., 1998). Some of these differences in the form of stereotypy might reflect physical constraints (i.e. non-enriched small cages might be too small for animals to pace in them, e.g. Powell et al., 1999), or the fact that frustrations are also heterogeneous in nature, so a particular enrichment paradigm might be tackling the underlying motivations of some forms, but not others.

However, it has also been suggested that some forms of stereotypic behaviours might be harder to tackle by enrichment if they reflect forebrain pathologies induced by early experience rather than frustration induced by the current environment (e.g., Mason, 2006). Consistent with this, some forms, but not others, have been linked to altered forebrain function. In the case of cage-induced stereotypies, non-enriched somersaulting deer mice were more perseverative in reversal learning compared to stereotypic jumpers (Tanimura et al., 2008); in rhesus macaques, head twirling, but not pacing, positively correlated with perseveration in extinction (Pomerantz et al., 2012b); and in mink, locomotor stereotypies (a combination of forms, including pacing and head twirling) positively correlate with the number of errors in tests measuring recurrent perseveration, while scrabbling does not (Campbell et al., 2013; Dallaire et al., 2011). In Campbell and colleagues' study (2013) locomotor styereotypies also correlated with fast repeats in the guessing task employed (as expected if they reflect loss of behavioural inhibition), while scrabbling correlated with all round slowness. Furthermore, a recent study also found signs that scrabbling mink in non-enriched cages show symptoms of boredom, while mink showing locomotor stereotypies do not (Meagher and Mason, 2012), suggesting that perhaps scrabbling is more informative about the current welfare status of the animals. My study built on these findings by showing two further ways in which these two forms of stereotypic behaviours differ in mink: i) enrichment use tended to negatively correlate with the performance of scrabbling but not locomotor stereotypies, and scrabbling was also more sensitive to current housing conditions than locomotor stereotypies; and, ii) locomotor stereotypies had negative reproductive consequences for males, while the same did not seem to be true for scrabbling.

Understanding the different underlying mechanisms and consequences of different forms of stereotypic behaviours is important because it allows us to determine which forms might allow a better assessment of current welfare within a given environment, as well as to devise specific means to alleviate different forms. Also statistically speaking, if different stereotypic behaviours are biologically heterogeneous, pooling them together for analysis could lead to Type II errors.

Future research on mink

My data suggest that scrabbling in mink is more sensitive than locomotor stereotypies to current conditions (while locomotor stereotypies seem more sensitive than scrabbling to past conditions) and, although some of my findings would need replication (as they were only statistical trends), future experiments manipulating such conditions could test this hypothesis. For example, studies looking at the performance of stereotypic behaviours under conditions of enrichment loss/gain could be set up. If scrabbling tracks changes in the current environment, its performance should increase over that of locomotor stereotypies when enrichments are removed (it should appear before locomotor stereotypies in time, coming to be displayed by both a larger percentage of animals as well as performed at higher frequency within individuals), and decrease more rapidly and/or more completely than locomotor stereotypies when enrichments are provided. Locomotor stereotypies should instead remain fairly constant in both scenarios. Although not something that I would necessarily want to do, another way of manipulating early environments would be to manipulate weaning age, as we already know that early weaning increases tail-biting in mink (Mason, 1994): does it also increase locomotor stereotypies more than scrabbling?. Also, the motivational basis

underlying the different forms could be tested. For example, similar set-ups to those employed in rodents (Nevison et al., 1999) could be used to test if scrabbling represents escape attempts from an aversive stimulus within or outside the cage by giving mink access to "escape routes" (e.g. a covered overhead tunnel) and manipulating the stimuli within/outside the cage; or if they represent motivations to access some external stimulus, which could also be tested by manipulating cues inside and outside the enclosures and giving mink options to interact with the stimulus (e.g. if scrabbling when the food cart arrives represents hunger motivation rather than a escape motivation, an automatic feeder inside the cage that mink could activate should abolish or at the very least decrease individual levels of scrabbling).

Additionally, the locomotor stereotypies of mink should be further divided at least into pacing and head twirling in future studies, as there is evidence that they might have different motivational bases (pacing is performed pre-feeding while 'stationary stereotypies', including head twirling, are most commonly observed post-feeding, Mason, 1993). Future studies could also investigate whether, similar to what has been observed in primates (cf. Pomerantz et al., 2012b), it is the latter and not the former that correlates with perseverative tendencies. Finally, changing the topic to an abnormal behaviour that was not seen at all in my subjects, but which does occur at a low prevalence on fur farms, if the fur-plucking observed in mink (e.g. Mason, 1994; Vinke et al., 2002) is similar to the barbering observed in mice, fur-plucking mink should correlate with stuck-in-set but not recurrent perseveration (cf. Garner et al., 2011).

Unexpected results, and what to do about them in future research

There were several unexpected results in my study. As already discussed, the many non-significant results from the preliminary cytochrome oxidase brain study was surprising and needs to be followed up with more work to investigate why. In addition, enrichment did not increase mink brain weight. This was surprising because it is a common effect reported in the enrichment literature (e.g. Rosenzweig et al., 1968), which includes one carnivore example (cats *Felis catus*, Beaulieu and Colonnier, 1987). Studies comparing wild, free ranging carnivores to zoo or farm-housed conspecifics have also found that the former have increased brain weights (e.g. lions *Panthera leo* and tigers *Panthera tigris*, Yamaguchi et al., 2009; American mink, Kruska and Schreiber, 1999). Future studies should therefore aim to use better, more naturalistic enrichments (e.g. water channels could be filled with freshwater crustacea; semi-natural outdoor encloses built, similar to those in Rauch et al., 2014), especially if long-term, which may then induce more dramatic brain changes (as well as probably more pronounced reproductive effects). Alternatively, non-enriched cages could be made more aversive.

Also, most of the enrichment-affected stress-related variables did not co-vary, something I was expecting if enrichment was causing all these effects through a common mechanism, namely, reduced HPA activation. I suspect a lack of statistical power might be to blame, since some of them did (e.g. adrenal weight and faecal glucocorticoid metabolite levels). Alternatively, my lack of significant results might mean that the relationship between these variables is not linear.

Perhaps the most disappointing finding was that environmental enrichment did not seem to affect any aspect of female reproductive behaviour. This was surprising given the vast literature on stress effects on female choice, female reproduction and investment (see Chapters 3 / Díez-León et al., 2013, and Chapter 4). It is possible that the lack of treatment effect on female choice responds to shortcomings in the methodology I used. For example, during mate choice trials, enriched females had to be locked out of their enriched compartments for a few hours every day during 3 weeks, while in contrast, non-enriched females experienced some form of enrichment during that time. The negative effects of enrichment deprivation (see Chapter 2) and the positive effects of enrichment provision on animal welfare and stress physiology might have masked treatment effects on female choice. Alternatively, the effects of captivity on non-enriched females might not have been so severe as to impair mate discrimination. As for the lack of effect on female reproduction and maternal behaviour, I believe it to be the result of lack of statistical power: only half of the females conceived (likely due to suboptimal lighting regime in our experimental facilities), and not all of these weaned their litters (see Chapters 3 / Díez-León et al., 2013, and Chapter 4). Therefore, these results should be taken as preliminary and need to be replicated.

Maternal care in mink and other species is a research avenue that should be followed due to the potential effects that it has on offspring's future stress reactivity and temperament, and their sexual development and adult health (e.g., reviewed in Meaney, 2001; Monclus et al., 2011; Ward and Renz, 1972). Future studies could determine the effects of enrichment on nest building abilities by providing females with different substrates (e.g. hay, and/or

artificial substitutes). In order to do this, and at least in mink, my preliminary study suggests that careful consideration should be given to sample sizes and lighting regimes. Furthermore, as recommended before, more complex and motivationally stimulating enrichments could be sought out and used, to try and induce bigger effect sizes.

Additional studies should further test the significance of maternal licking and grooming in mink, which proved to show high within-individual consistency. If similar to the licking and grooming behaviour observed in rodents, it could affect hippocampal function (and therefore spatial learning and memory) as well as program offspring stress resistance as adults (e.g. Bredy et al., 2003; van Hasselt et al., 2012). If this were so, manipulating the levels of maternal behaviour that offspring get (by either genetic selection for high licking dams; and/or cross-fostering offspring to high licking dams; and/or ameliorating captive environments if studies using larger sample sizes show that enrichment has the same effects in licking and grooming than it does in rodents) could be a way of altering offspring stress-responsiveness and, in the case of mustelid species bred for conservation, improve reintroduction success (see below).

Looking ahead: animal welfare and conservation

Captive breeding for the conservation of any species (i.e. not only carnivores, and not only mammals) aims to reintroduce *ex situ* propagated specimens that are capable of reproducing in order to establish self-sustaining populations in the wild (e.g. Philippart, 1995).

Thousands of captive bred mammals are released into the wild as part of conservation projects (e.g. 20 years ago, over 2300 captive bred individual mammals were released in c. 50 projects, Beck et al., 1994); yet, even when animals undergo pre-release training (e.g. to hunt/avoid predators), the reintroduction success of captive-bred animals is still notoriously low (Beck et al., 1994; Fischer and Lindenmayer, 2000; Griffith et al., 1989; Jule et al., 2008; Mathews et al., 2005; Reading and Clark, 1996; Sjöåsen, 1996; Wolf et al., 1998). In addition, a recent survey on mammalian rehabilitation centers worldwide seems to show that stereotypic individuals are still commonly released back into the wild (Guy et al., 2013). The biological reasons given for reintroduction failures include: i) inadequate dispersal behaviour, with animals dispersing either too much (increasing the chances of traffic collision or of being hunted, Bremner-Harrison et al., 2004) or too little (and therefore reducing animals' chances of finding adequate resources and/or mates, Bright and Morris, 1994); and ii) inadequate aggressive behaviour, with animals being too aggressive or not enough (to for example defend a territory, Braithwaite and Salvanes, 2005; Mathews et al., 2005). Furthermore, in some species, reintroduced captive-bred females show poor maternal behaviour, as evidenced by nest abandonment rates (Sage et al., 2003). It is only recently that long-lasting negative effects of the captive environment are being considered as a possible explanation for such failures (Braithwaite and Salvanes, 2005; Jule et al., 2008; Teixeira et al., 2007).

My findings suggest that compared with wild conspecifics, released captive-bred carnivores could be subject to impairments in behavioural control that have negative consequences for their reproductive capabilities, as well as likely negative impacts for survival. For example,

perseverative individuals would be more likely to keep using the same resource patches even when resources are depleted or decrease their value; and/or they would be more likely to navigate through the same routes, even when these are altered in a negative way (e.g. new roads being built). Thus, my results highlight the importance of understanding how the captive environment alters brain function and how that in turn impacts captive mammals' reproduction in captivity and the wild, as well as reintroduction success. If captive-bred animals are at a reproductive disadvantage in the wild, as well as having worse survivorship, the self-sustainability of some endangered species maybe hard to achieve if they need to rely on constant supply of captive-bred individuals to maintain population numbers. Further research should investigate if, for example, enrichment of captive-bred endangered mustelids improves reproductive success by making males less stereotypic and/or aggressive (as it does in farmed mink, Meagher et al., submitted), and if it improves females' maternal behaviours. Additionally, studies should test whether enrichment improves the reintroduction success of wild animals by improving hippocampal function (cf. Campbell et al., 2013), by assessing for example the performance of individuals in spatial learning tasks that could be implemented as enrichment or as part of their pre-release training.

Conservation scientists and managers often focus on entire populations or even species, which usually conflicts with the interests of animal welfare scientists whose focus is the individual (e.g. Teixeira et al., 2007). In recent years, there have been attempts to bridge the fields of animal welfare and conservation (see e.g. Compassionate Conservation initiative at http://compassionateconservation.net). This responds to a growing recognition that there needs to be a coordination between *ex situ* and *in situ* efforts to protect species and also to a

greater understanding of the effects of stress in different aspects of the conservation of species, from *ex situ* breeding, to the translocation of wild specimens and the reintroduction of captive-bred ones, to the effects of habitat loss in wild individuals (e.g. Martinez-Mota et al., 2007). After all, it is in the best interest of everyone involved -- conservation managers, animal welfare scientists and the general public -- that the animals that are under our care enjoy the best possible welfare and strive when released into the wild.

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Appendix I. Methodological approaches to measuring libido in male mink: inter-observer reliability when scoring marked plastic inserts from the mate choice apparatus.

Opportunistically, and after our mate choice experiment was finished (see Chapter 3 / Díez-León et al. 2013), we observed that the solid plastic (high density polyethylene) inserts that allowed/prevented access to females/males in the mate choice apparatus (see Fig. 1 in Chapter 3 / Díez-León et al. 2013) were bitten and scratched around the holes, and to a range of extents: some had pronounced markings while others were almost unmarked (see Fig. A below). We thought this evidence of differential scratching and biting directed towards the access point for the females could indicate the strength of motivation to mate by the males, and thus serve as an indicator of their libido. Due to the *a posteriori* nature of this observation, we cannot exclude other possible explanations for such markings (e.g. escape motivation) or circumvent experimental limitations (e.g. females as well as males might have performed the markings). However, we could at the very least determine if the inter observer reliability (IeOR) of insert-scoring is good enough so that future studies can develop and refine this method to assess male libido.

Rating method and statistical analysis

We presented 15 slides to two independent observers who did not consult, and asked them to rank the slides from least (15) to most (1) marked in two ways: by looking at marks

present only in the inner side and by looking at marks on both sides. Ties were allowed. Spearman correlations were run in JMP (v. 10.0, SAS Institute) to determine concordance between observers.

Results

The ranks of our two scorers correlated strongly for both, scoring one side ($F_{1,13}$=81.37, $p<0.0001$; r^2=85%) or both sides ($F_{1,13}$=78.54, $p<0.0001$; r^2=84.7%) of the insert (see Fig. B).

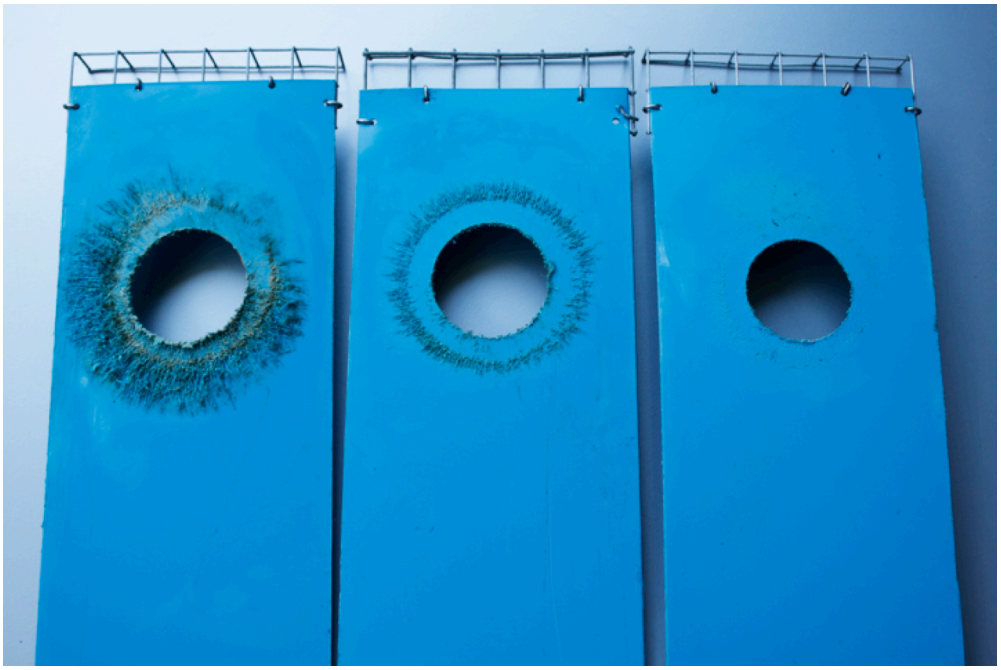
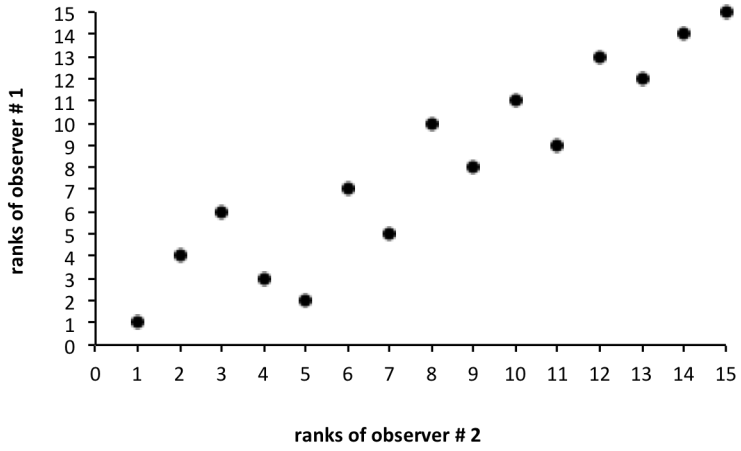


Figure A. Markings on the inner side of the plastic inserts from the mate choice apparatus.

a)



b)

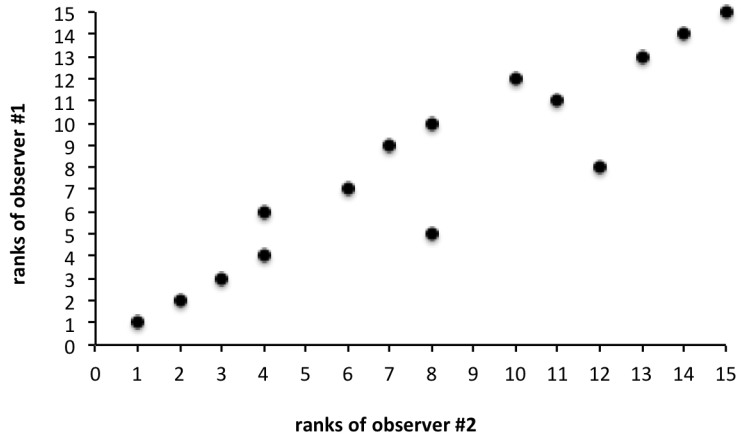


Figure B. Relationship between the ranks scored by both observers when a) only markings on the inner side of the inserts were considered; and b) when markings on both sides were considered.

Conclusion

There was a strong correlation between both scorers in both situations, and therefore IeOR is high for this simple method of assessing the amount of scratch marks on the inserts of the mate choice apparatus. Nonetheless, future studies should perhaps consider using different, more precise scoring methods, such as % of a given area that is marked, or measure the radius of the affected area, or even using imaging software to determine density of the markings; as well as video record the mating events to identify individual markings.

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Appendix II. Further analyses on the copulatory and stereotypic behaviours of male mink.

In the context of the thesis, we conducted further analyses on the published dataset. These additional analyses sought to investigate i) additional copulatory variables that might be of interest for future studies; ii) if the effects of stereotypic behaviours on number of copulations were stronger as males matured (see Chapter 5); and iii) the reproductive correlates of the other form of stereotypic behaviour assessed in our mink - scrabbling (see Chapter 2). Therefore, following the same statistical analyses outlined in this chapter (Díez-León et al., 2013), we investigated the effects of enrichment on female preference (assessed as number of visits allocated to each male, standard practice in experiments on mate choice and assumed to indicate investment into mate assessment, Poulin, 1994) and the following copulatory variables: duration of copulations (as time spent copulating has been positively correlated with amount of ejaculate in mink, Jørgensen, 1985); number of fertile copulations (i.e. copulations over 30 minutes, Kukla, 1977 as cited in Sundqvist et al., 1989); last male mated (in mink and other species mating last in the season can have reproductive advantages in terms of paternity of the offspring, e.g. Johansson and Venge, 1951; Jørgensen, 1985). Of these, enrichment only had an effect on duration of copulations, and number of fertile copulations (Table 1); and locomotor stereotypies only predicted duration of copulations ($F_{1,24}$=7.91, p=0.005).

Table 1. Effect of environmental enrichment on female preference and additional copulatory variables. E: enriched; NE: non-enriched. Significant results in bold. Means are least squared means; SE is the standard error.

		Mean	SE	Statistic	p
Number of visits	E	7.97	1.37	$F_{1,14}$=0.22	0.33
	NE	7.00	1.68		
Duration of copulations (minutes)	E	102.2	21.7	$F_{1,14}$=4.10	0.031
	NE	48.9	9.78		
Number of fertile copulations	E	1.81	0.37	$F_{1,14}$=4.12	0.031
	NE	0.84	0.20		
		Odds ratio		Statistic	p
Last male mated		1.37		Z=0.31	0.377

The effects of stereotypic behaviour on number of copulations seem to require mature males, as they were not present if data from Year 1 only were analysed (both Analysis 1 and Analysis 2 were non-significant) but if data from Year 2 only were used in analyses, the effect was significant (Analysis 1: $F_{1,14}$=6.60, p=0.011; Analysis 2: $F_{1,10}$=7.41, p=0.010).

As for scrabbling, environmentally enriched males did not show reduce levels of this behaviour ($F_{1,30}$=2.45, p=0.064), nor did it relate to any copulatory variable significantly affected by environmental enrichment (p>0.10 for all measures).

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Appendix III. Relative importance of male body weight *versus* stereotypic behaviours, and of body weight alone on the reproductive success of male mink

We found that male body weight had an effect on copulation success (see Chapter 3 / Díez-León et al. 2013). Although this result was demoted after FDR corrections, we thought important to investigate it further given the strong negative correlation found between body weight and stereotypic behaviours (see Chapter 3 / Díez-León et al. 2013); and the potential of forced matings in sexually dimorphic species (i.e. sexual coercion: Smuts, 1987), since we did not find clear evidence of pre-copulatory mate choice in our experiment (see "Additional analyses" section at the front of Chapter 3).

We first looked at the relative predictive value of body weight vs. stereotypic behaviours on copulatory variables by including both traits in a GLM, alternating which was the last main effect. With both terms in a GLM using sequential sums of squares, whichever term – locomotory stereotypies or body weight – was placed earlier in each model was significant. When locomotory stereotypy was second after body weight, it tended to still predict copulation number ($F_{1,12}$=2.74, p = 0.062) and duration ($F_{1,12}$=2.88, p=0.058), while in contrast if body weight was the second term it was not significant. Path analyses were also conducted in Amos v.5 (Arbuckle, 2003) to investigate which intervening variable best explained the reproductive data; here, model fit was assessed using the chi-square test, RMSEA (Root Mean Square Error of Approximation), NFI (Normed Fit Index) and AIC (Akaike Information Criterion) statistics. Path analyses revealed that the only model that fitted the data included both locomotory stereotypies and body weight. However, the

regression coefficient for locomotory stereotypies was slightly higher, and therefore explained the variance slightly better (for number of copulations: 27% *vs.* 24%; for duration of copulations: 24% *vs.* 23%).

Finally, we looked at the importance of body weight alone by testing the hypothesis that the copulatory success of heavy males reflected their weight advantage over their female partner. This hypothesis predicts that i) if males gain copulations just by outweighing the females, then lighter females would receive more and/or longer copulations and ii) the % of copulations won by the heaviest male in a pair would negatively co-vary with both female weight and also with the difference in weight between the male and the female. We thus run two different analyses: first, a GLM that had female body weight as a predictor of the behavioural reproductive variables; and second, a GLM of the sort of Analysis 2 (see Chapter 3 / Díez-León et al. 2013) in which the dependent variable was the % of copulations won by the heaviest male of a pair of rival males, and where the independent variables were respectively female weight and the weight differential between the male and the female. All of these analyses were non-significant.

These results confirm that stereotypic behaviours are a better predictor of male mink copulatory success; that body weight alone does not explain reproductive success in mink; and also indirectly support previous findings that suggest that dimorphism in mink responds to niche overlap rather than sexual selection (Thom et al., 2004).

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