Refinement of the care and use of laboratory ferrets

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Refinement of the care and use of laboratory ferrets

Verfijning van de zorg voor en het gebruik van fretten als proefdier

(met een samenvatting in het Nederlands)

Proefschrift

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Promotor: Prof. dr. C.F.M. Hendriksen

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"Happy animals make good science"

Poole 1997

Contents

Chapter 1

General introduction

Ferrets are a frequently used research model, e.g. for influenza studies. However, science-based knowledge on husbandry and care of ferrets is lacking. Therefore, this thesis aims to provide insight into methods to refine the care and use of laboratory ferrets, with a focus on environmental enrichment and pain recognition. This general introduction contains seven sections and starts with an introduction of *ferrets* in general (**Chapter 1.1**). **Chapter 1.2** discusses *the ferret as an animal model*. **Chapter 1.3** gives an overview of the *legislation on laboratory ferret housing and husbandry*. **Chapter 1.4** discusses a framework that is solidly embedded in the legislation and is used to improve the welfare of laboratory animals: *the principles of the 3Rs* (Replacement, Reduction and Refinement). **Chapter 1.5 and 1.6** discuss two ways in which the care and use of laboratory ferrets can be refined, i.e. through *the provision of environmental enrichment* and through *adequate pain assessment*. **Chapter 1.7** gives a short *overview of the studies* that are described in this thesis.

Ferrets

Ferrets (*Mustela putorius furo*) are small, agile and energetic carnivores from the *Mustelidae* family. They belong to a fully domesticated species with no direct wild counterparts (Wolfensohn and Lloyd, 2008). Although the identity of the ferrets' precise ancestor is still uncertain, the most probable candidates are the European polecat (*Mustela putorius* putorius) and the Steppe polecat (*Mustela eversmanni*) (Fisher, 2006). Ferrets were domesticated as early as 2000-3000 years ago, to protect stored food materials from rodents and hunt for rabbits, mice and rats (i.e. "ferreting", Zeuner, 1963). Currently, their popularity as pet animals has increased considerably in Western countries. Additionally, ferrets are used as an animal model in the biomedical sciences, for example for studies on the virulence, pathogenesis and transmission of influenza viruses (Ball, 2006; Belser et al., 2011; Vinke and Schoemaker, 2012).

Biological characteristics

Ferrets are relatively small animals, with short, sturdy legs, five toes per foot, an elongated body and cranium and a short nose. Ferrets reach their adult weight when they are 16-25 weeks old. They are sexually dimorphic: adult males (hobs) weigh 0.7-2.7 kg and are 38-46 centimetres long (without tail) and adult females (jills) weigh 0.6-1.2 kg and are 33-36 centimetres long (Bulloch and Tynes, 2010; Fox et al., 2014). A ferrets' body weight shows seasonal variations: they can lose 30-40% of their body weight in spring, which they gain back in late November (Fox et al., 2014; Lloyd, 1999; Plant and Lloyd, 2010; Wolfensohn and Lloyd, 2008). Ferrets mould coincidently with the breeding season and have a sleek summer coat and a fluffy winter coat with thick undercoat (Fox et al., 2014). In captivity, the average lifespan of a ferret is six to eight years (Fox et al., 2014; Lloyd, 1999).

Figure 1.1 A black-eyed-white male ferret (left) and a sable/wildtype female ferret (right)

Reproduction

Ferrets reach sexual maturity at 8-12 months of age and they are seasonal breeders. Male ferrets are sexually active from December to July (Northern hemisphere), when short light periods cause the testicles to enlarge and descent into the scrotum. Male ferrets will remain in breeding condition if they are kept under 6-8 hour light periods (Fox et al., 2014). Adult female ferrets are seasonally polyestrous (March-August), stimulated by a marked increase in photoperiod to 14 hours of daylight. Female ferrets are induced ovulators and a jill will remain in oestrus indefinitely if she is not bred. The resulting continuously high oestrogen levels can lead to weight loss, alopecia and even bone marrow depression (i.e. aplastic anaemia, leukopenia and thrombocytopenia, Fox et al., 2014). Ovariohysterectomy or hormonal treatment (e.g. injection with hCG or GnRH) can prevent or cause regression of the signs of oestrus (Fox et al., 2014; Lloyd, 1999; Plant and Lloyd, 2010; Wolfensohn and Lloyd, 2008). Ferret copulation is vigorous, with the male grabbing the female at the nape and dragging her around the enclosure. After a gestation period of 42 days, an average of eight kits are born (range 1-18), which are weaned at six weeks old (Fox et al., 2014).

Behaviour

Domesticated ferrets sleep 50-70% of the time and they are polycyclic sleepers, with cycles of two to four hours (Boyce et al., 2001; Jha et al., 2006; Marks and Shaffery, 1996). Ferrets are mostly awake for short periods (less than 10 minutes) between sleeping bouts (Marks and Shaffery, 1996), during which they are very boisterous (Boyce et al., 2001; Jha et al., 2006). They sleep very soundly: their respiration and heart rate decrease and it takes them several moments to awake (Fisher, 2006).

Feral ferrets and their wild ancestors are solitary animals that show intrasexual territoriality, i.e. they do not accept conspecifics of the same sex in their territories. Two feral male ferrets will chase each other and fight aggressively when they meet, whereas a feral female ferret will generally not fight, but try to dominate an intruder that enters her territory (Clapperton, 1985). Domesticated (castrated) ferrets are said to be gregarious and will sleep together, share food and water and play vigorously (Fisher, 2006; Plant and Lloyd, 2010; Wolfensohn and Lloyd, 2008).

Ferrets play approximately 8% of the time, which involves jerky, bouncing movements, inhibited attacks and clumsy movements. Their play preferences change with age, with the amount of rough and tumble play declining from 7-8 weeks old to 15 weeks old and the amount of locomotor play increasing with age. Social play is mostly dyadic, independent of the number of animals in the enclosure. (Poole, 1978)

Husbandry and nutrition

In general, ferrets like to sleep in dark, enclosed areas, so a nest box or a comparable structure should be provided. Ferrets love to dig and burrow in bedding material, as well as explore their surroundings. For this purpose, rigid or flexible tubes can be used. Balls can be used to stimulate hunt and capture behaviour. Ferrets are also known to hoard toys or food and will select hiding places in their enclosure. (Bulloch and Tynes, 2010; Fisher, 2006; Lloyd, 1999; Wolfensohn and Lloyd, 2008)

Ferrets are obligate carnivores: their diet should contain 30-40% of mostly meat-derived protein, 18-20% fat, 22-44% carbohydrates, 2-5% fibre and adequate levels of vitamin E (Lloyd, 1999; Plant and Lloyd, 2010). They eat multiple (9-10) small meals per day, which in total amount to 200-300 Kcal/kg body weight or 135 g pellets/kg body weight (Bleavins and Aulerich, 1981; Kaufman, 1980). Generally, ferrets can be fed *ad libitum*, as they will eat to calorie requirement (Fisher, 2006). Ferrets drink 75-100 mL daily, spread over about 10 drinking bouts (Lewington, 2007; Lloyd, 1999; Wolfensohn and Lloyd, 2008).

Common diseases

Ferrets can suffer from the consequences of different viral, bacterial and parasitic infections and several non-infectious (e.g. neoplastic, cardiovascular, renal) diseases.

Ferrets are highly susceptible to the Canine distemper virus, which is a Morbillivirus that is almost always fatal in ferrets (Deem et al., 2000). This virus is highly contagious and is spread through aerosolisation of respiratory exudate. Ferrets can and should be vaccinated against this virus on an annual basis (Wimsatt et al., 2001). Ferrets are also highly susceptible to other viruses, including Aleutian disease virus (a fatal parvovirus that is transferred through body fluids; Palley et al., 1992), and *Coronavirus* (the causative agent for epizootic catarrhal enteritis and ferret systemic *Coronavirus* infection). Unfortunately, there is no vaccine or treatment for Aleutian disease, so prevention is key (Welchman D. et al., 1993). Quarantine, vaccination and cleaning procedures can be used to prevent infection (Williams et al., 2000). A fourth virus that ferrets are highly susceptible to is influenza virus, which is one of the reasons they are commonly used as an animal model for human influenza virus (see Chapter 1.2). Clinical signs of an influenza virus infection are less severe and less often fatal than canine distemper and Aleutian disease virus infection.

The most common bacterial infection in ferrets is *Helicobacter mustelae* infection, which causes gastric disorders and can be treated, although some animals may nevertheless die following severe infection (Erdman et al., 1997; Hillyer and

Quesenberry, 2012). The disease often manifests following exposure to stressors such as weaning, transport or experimental manipulations (Fox et al., 1997). Ferrets are also susceptible to other bacterial diseases, e.g. bacterial pneumonia, botulism (Harrison and Borland, 1973; Myllykoski et al., 2011; Quortrup and Gorham, 1949), campylobacteriosis (Fox, 1982), chlamydia (Fox et al., 1992; Francis and Magill, 1938), *Clostridium perfringens* Type A (Schulman et al., 1993), mycobacteriosis (Dunkin et al., 1929), mycoplasmosis (Kiupel et al., 2012; Koshimizu et al., 1982), and proliferative bowel disease (Fox et al., 1982; Fox et al., 1986).

The most common ectoparasite to be encountered in ferrets is *Otodectes cynotis*, (Nie and Pick, 1978; Sweatman, 1958), which causes ear mite infestation and can lead to otitis. Luckily, this ectoparasite can easily be controlled with topical use of ivermectin or selamectin (Patterson and Kirchain, 1999). Ferrets are also susceptible to several other parasitic diseases, such as protozoal enteric coccidiosis (Hoare, 1927; Pantchev et al., 2011), cryptosporidiosis (Abe and Iseki, 2003), heartworm (Supakorndej et al., 1992), nematodes, cestodes (Fox and Marini, 2014), flea infestation (Wenzel et al., 2008), sarcoptic mange (Sarcoptes scabiei), demodicosis (Beaufrere et al., 2009; Noli et al., 1996), ticks, and myiasis. (Bell, 1997; Fox and Marini, 2014).

The most common neoplasms in ferrets are endocrine tumours, of which the majority are pancreatic islet beta-cell tumours (i.e. insulinoma, Caplan et al., 1996; Chen, 2010; Pilny and Chen, 2004; Weiss et al., 1998) and adrenocortical neoplasms (Chen, 2010; Miwa et al., 2008; Simone-Freilicher, 2008). The third most common neoplasm in ferrets is lymphoma (i.e. cancer of the white blood cells, Ammersbach et al., 2008; Hess, 2005; Li and Fox, 1996; Williams, 1996; Wills et al., 2005). Neoplasms can also be found in the skin and subcutaneous tissue (Parker and Picut, 1993), with basal cell tumours accounting for up to 60% of skin tumours (Parker and Picut, 1993). Other common skin tumours in ferrets are mast cell tumours (Stauber et al., 1990; Williams and Nye, 1995), and tumours of the apocrine sweat glands. (Fox and Marini, 2014; Li et al., 1998)

The ferret as an animal model

Ferrets were first recognised as a valuable animal model in biomedical research in 1926, when Dunkin and Laidlaw studied canine distemper in ferrets (Dunkin and Laidlaw, 1926). In 1933, ferrets were the first animal species to be successfully infected with human influenza virus using human throat-washings, after unsuccessful attempts to artificially infect many different species (Smith et al., 1933). As the ferret lung shares many properties with human lung tissue and ferrets and humans share similar symptomatology, ferrets make a good model for the study of human respiratory viruses (Maher and DeStefano, 2004). Soon after the first successful artificial infection with human influenza visus, in 1935-1939, ferrets were also used to study other viral infectious diseases such as Rift Valley fever, acute meningo-pneumonitis and lymphocytic choriomeningitis (Dalldorf, 1939; Francis and Magill, 1935; Francis and Magill, 1938b). Nonetheless, it was only from the late 1960s, at the time of the Hong Kong Influenza (H_3N_2) pandemic (Kilbourne, 2006), that an increasing appreciation arose for the usefulness of the ferret as an animal model (Frederick and Babish, 1985; Hahn and Wester, 1969). Aside from being an excellent model for studies on viral respiratory infections, ferrets have also been used to study gastrointestinal disease (e.g. peptic ulcer disease, Patterson et al., 2003), for nutrition research (e.g. beta-carotene metabolism, Wang et al., 1992) and to study the emetic potential of drugs (Holmes et al., 2009). Moreover, they serve as an animal model for cardiovascular, neural development, auditory and visual system studies, skeletal research, and renal diseases (Ball, 2006).

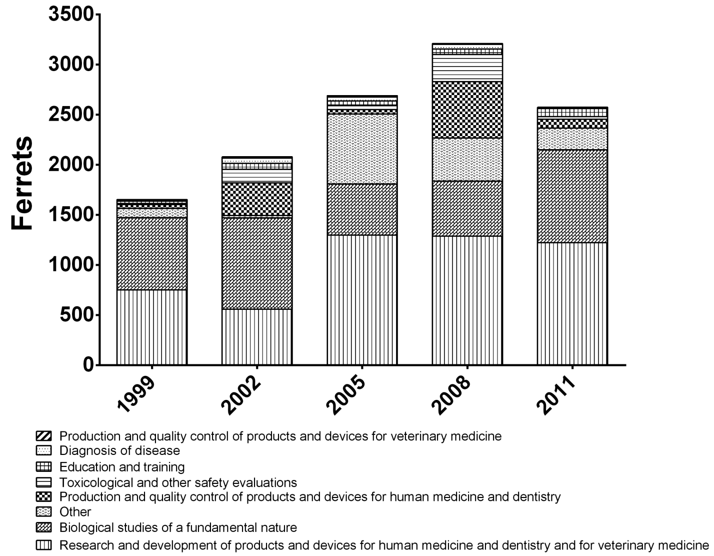

Figure 1.2 The number of ferrets used for experimental and other scientific purposes in the European Union from 1999-2011. (European Commission, 2003; 2005; 2007b; 2010b; 2013b)

Over the years, the number of ferrets used for scientific purposes increased until 2008, when 3208 ferrets were used for experimental and other scientific purposes in the European Union (European Commission, 2003; 2005; 2007b; 2010b; Figure 1.2). The number of ferrets that were used in 2008 are comparable to the number of cats, goats and reptiles that were used in that year and higher than the number of prosimians and new world monkeys that were used in 2008 (European Commission, 2010b). The rising numbers in ferrets that were used over the years are probably due to the occurrence of an avian influenza A (H5N1) pandemic in 2008 and an influenza A (H1N1) pandemic in 2009. This is also reflected in the purpose for which a large proportion (77-93%) of the ferrets were used, namely studies on human and animal diseases (of which 78% was used to study "other human diseases", 14% to study "human nervous and mental disorders", 5% to study "human cardiovascular diseases", and 3% for "studies specific to animal diseases". In 2011, the number of ferrets that were used in the European Commission decreased to 2573 (European Commission, 2013b; Figure 1.2), possibly due to the demise of the influenza pandemic or the ongoing search for alternatives to animal testing. It will only become clear whether this trend continues until a new EU report is due, at the end of 2019.

The use of ferrets is not equally divided over the member states of the European Union: the Netherlands (34%) and the United Kingdom (22%) together used more than half of the 2573 ferrets that were used in 2011 (European Commission, 2013a; Figure 1.3). Of the ferrets that were used in experiments for studies on human and animal diseases in 2011, 91% was used to study "other" human diseases (i.e. not related to cardiovascular disease, nervous and mental disorders or cancer, European Commission, 2013a). Therefore, the high number of ferrets that were used in the Netherlands and the United Kingdom might be related to the high number of influenza research facilities in these countries.

Ferrets are selected for use as an animal model based on their health status, age and weight. They are often screened for absence of Aleutian disease and antibodies to the virus that is the subject of the study, e.g. a specific influenza strain. Both males and females are used and even though the sable/wild type ferrets are still the most common, ferrets with all coat colours and types are used. As it is often difficult to individually identify ferrets, ear tags or microchips are needed. As the number of research ferret breeding facilities is limited, ferrets often have to be transported over long distances from the breeding facility to the research facility. (Ball, 2006)

Legislation on laboratory ferret housing and husbandry

When ferrets were first used as laboratory animals there was no legislation with guidelines on the housing conditions and management of these animals. A first description of housing conditions for laboratory ferrets was published in 1940 (Pyle, 1940). Here, outdoor cages similar to those used for rabbits were described for housing one or two ferrets. These cages were 45.7 cm wide, 55.9 cm long and 35.6 cm high mesh wire cages with a solid bottom and wood shavings as litter. For long term experiments, a larger cage with separate exercising run and housing compartment (with a nest box) was advised. (Pyle, 1940)

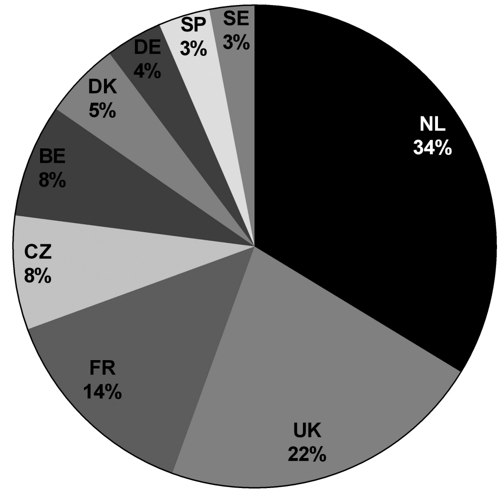

Figure 1.3 The number of ferrets used for experimental and other scientific purposes in the different member countries of the European Union in 2011. NL=the Netherlands, UK=the United Kingdom, FR=France, CZ= Czech Republic, BE=Belgium, DK=Denmark, *SP*=Spain, SE=Sweden. (European Commission, 2013a)

In 1986, the European Economic Community was the first to publish specific legislation regarding the use of animals for scientific research: Directive 86/609/EEC (European Economic Community, 1986). This legislation contained general guidelines that applied to all experimental animals, such as a ventilation rate of 15-20 air changes per hour, a relative humidity of 55±10% and continuous sound of moderate intensity (soft music). The Directive further stated that all animals should be able to satisfy their physiological and ethological needs (for example the need to climb, hide or shelter); should be checked daily as part of the health monitoring process; and pain and avoidable suffering, distress or lasting harm should be prevented and eliminated as quickly as possible. Additionally, the legislation included species-specific guidelines on e.g. cage dimensions, cage material and enrichment. However, specification on what the physiological and ethological needs of ferrets are and how suffering, distress and lasting harm can be recognised and prevented, was not included in this document. In fact, the only ferret-specific guideline in the Directive stated that ferrets should be kept at temperatures between 15°C and 21°C.

More than 20 years later, in 2007, the 1986 Directive has been supplemented with species-specific recommendations, wherein a section has also been devoted to ferrets (European Commission, 2007a). These recommendations state that ferrets should be provided with a complex, escape-proof enclosure that allows for the expression of a wide behavioural repertoire and meets the species- and breed-specific needs. Further specification describes that the ferrets should be kept between 15°C and 24°C under a natural 24h light-dark cycle or 8-16 hours of artificial light daily with at least low level night lighting. In addition, auditory stimulation should be provided and exposure to unfamiliar noise and vibration should be avoided. Ferrets should furthermore be regularly handled and housed in socially harmonious groups, where the enclosure allows them to exert control over social interactions. Separate areas for different activities should be provided (e.g. raised platforms and pen sub-divisions) and all ferrets should be provided with a warm and comfortable resting place (e.g. beds or platforms). Additional enrichment in the form of containers and tubes of cardboard or rigid plastic, paper bags, water baths and bowls, bedding and nesting material (hay, straw or paper) should be provided. The Directive also includes minimum floor area requirements per ferret (no enclosure should be less than 4500 cm^2, with a minimum height of 50 cm). This enclosure should have a rectangular rather than a square shape and should have a solid floor with a smooth non-slip finish.

When using ferrets as an animal model for infectious diseases, they can be housed under Animal Biosafety Level (ABSL)-II practices, equipment, and facilities until virus challenge. Infection with viruses with zoonotic potential and subsequent housing should be under ABSL-III conditions including respiratory protection, appropriate protective clothing and preventative vaccination protocols (Dyson, 2017; Fox and Marini, 2014; Maher and DeStefano, 2004).

In 2010, the 1986 Directive was replaced by a new one: Directive 2010/63/EU (European Commission, 2010a). The 2007 recommendation remained in place and compliments the 2010 Directive, in which additional ferret-specific guidelines on housing dimensions and acceptable killing methods (i.e. anaesthetic overdose) that were not listed in the old Directive are included. Additionally, the principles of the 3Rs (Replacement, Reduction, Refinement) are solidly embedded in the legislation, stating that all experiments using vertebrates or cephalopods should conform to the principles of the 3Rs.

The Principle of the 3Rs

The Principle of the 3Rs (Replacement, Reduction and Refinement) was first described by William Russell and Rex Burch in "The Principles of Humane Experimental Technique" (Russell and Burch, 1959). These principles aim to promote high quality science and guard laboratory animal welfare by avoiding the use of animals when alternative methods are available (Replacement), minimising the number of animals that are used (Reduction), and minimising the amount of suffering and distress in each individual animal (Refinement).

Replacement

Russell and Burch described the principle of replacement as "*any scientific method employing non-sentient material which may in the history of animal experimentation replace methods which use conscious living vertebrates*" (Russell and Burch, 1959). For a review on alternatives to animal testing, see Doke and Dhawale (2015). Russel and Burch further divided replacement in relative and absolute replacement. In the case of relative replacement, animals are still used, but not exposed to distress (e.g. non-recovery experiments or the use of isolated cells, tissues or organs). An example of relative replacement of ferrets is the development of a system to study the isolated trachea of ferrets to further understand and develop treatments for cystic fibrosis, instead of using live animals (Joo et al., 2001). In the case of absolute replacement, no animals are needed at any stage of the experiment (e.g. human tissues, cells and volunteers, mathematical and computer models or cell lines). An example of absolute replacement of ferrets is the use of *C. elegans* to screen agonists and antagonists for potential emetic liability, instead of using ferrets (Holmes et al., 2009). Even though total replacement of animals in research is the ultimate goal, this is not yet possible for all experiments. Therefore, as long as there are no acceptable alternatives, the principles of reduction and refinement should be applied to research using animals.

Reduction

Russel and Burch described the principle of reduction as "*any means of minimising, other than by replacement, the number of animals used to obtain information of a given amount and precision*" (Russell and Burch, 1959). This definition has been adapted to move away from the level of information that is needed and instead focus on the scientific needs and is redefined as "*any approach in scientific research, product testing or education that leads directly or indirectly to a decrease in the number of animals used while meeting the scientific requirements*" (de Boo and Hendriksen, 2005). The most apparent reduction strategies focus on reducing the number of animals needed for a specific experiment by e.g. testing clear hypotheses, using multifactorial and randomised block designs and controlling variation. However, supra- and extra-experimental reduction opportunities (e.g. education and training, re-use of animals, Good Laboratory Practice (GLP) and Good Manufacturing Practice (GMP) should also be exploited. See de Boo and Hendriksen (2005) for a review on reduction strategies.

An example of reduction of the use of ferrets is a tissue sharing agreement between a commercial biotechnology company (Aviron, Mountain View, California) and a neighbouring academic institution (Stanford University, Palo Alto, California). This agreement allows tracheas that are harvested from ferrets that are euthanised as part of federally mandatory product safety testing on a modified live influenza vaccine to be shared. Stanford University uses these ferret tracheas for *in vitro* assays to study cystic fibrosis (see the example of relative replacement). Over the course of three years, 270 ferret tracheas have been shared as part of this agreement, illustrating the importance of such agreements to reduce the amount of animals used in for experimental and other scientific purposes. (Broome et al., 2011)

Refinement

Russel and Burch described the principle of refinement as a "*decrease in the incidence or severity of inhumane procedures applied to those animals which have to be used*" (Russell and Burch, 1959). This definition has been redefined a number of times, culminating in the following: *"any approach which avoids or minimises the actual or potential pain, distress and other adverse effects experienced at any time during the life of the animals involved, and which enhances their well-being*" (Buchanan-Smith et al., 2005). This means that refinement should be applied to both direct and contingent harms (Russell and Burch, 1959). Direct harm includes pain caused as a result of research procedures, which for instance can be refined through proper recognition and alleviation of pain (Flecknell et al., 2007). Contingent harm includes distress caused as a result of housing animals in a research laboratory, which for instance can be refined through optimisation of the animals' housing condition by providing animals with the opportunity to express species-specific behaviours (Russell and Burch, 1959).
Refinement can oppose the rigorous standardisation that was advocated in an attempt to reduce the number of animals needed (i.e. reduce variation), often leaving the environment of the animals barren. It is now recognised that experimental designs that provide optimum conditions of animal care, and thereby minimising pain and distress, will result in more reliable data (Flecknell, 2002), i.e. "*happy animals make good science*" (Poole, 1997). Refinement is seen as the lesser obstacle by scientists (Fenwick et al., 2011) and it can have positive interactions with reduction and/or replacement. However, conflicts might also arise between refinement and reduction or replacement strategies. For example, the use of foetal bovine serum in cell cultures is an example of a replacement method that conflicts with refinement. The use of telemetry devices to gather more data from one animal is an example where a reduction methods conflict with refinement, while the use of buddy animals for social animals is an example of a refinement strategy that conflicts with reduction (De Boo et al., 2005).

An example of refinement of the use of ferrets is a systematic review of the cisplatin-induced ferret model of emesis, which has identified that the observation time required to identify anti-emetic effects can be reduced from 24 hours to only 4 hours, limiting the duration of the animal experiment and therefore limiting distress in the animals (Du Sert et al., 2011). Even though this is an excellent example of refinement of the use of ferrets for a very specific topic, there is still a lack of knowledge on how to refine experiments using ferrets. This thesis therefore aims to increase the amount of evidence-based information to reduce both indirect and direct harm through the provision of effective environmental enrichment (Chapter 1.5) and through adequate recognition of pain (Chapter 1.6).

Refinement through the provision of environmental enrichment

The provision of environmental enrichment is advocated as an effective way to refine animal experiments and improve the welfare of laboratory animals. Environmental enrichment is regarded as "*any modification or change of the environment that increases the complexity of an enclosure in order to provide the opportunity for animals to perform species-specific behaviours, with the goal of improving animal welfare*" (Hutchinson et al., 2005; Young, 2003). Any addition to an enclosure will increase the complexity of the environment, providing the animal with some level of control over its social and spatial environment and a greater choice of activity, which will benefit its welfare (Sambrook and Buchanan-Smith, 1997). It is more difficult to assess which enrichment items will provide animals with the opportunity to perform species-specific behaviour and meet the ultimate goal of improving animal welfare without compromising the research objectives.

Environmental enrichment that increases predictability and controllability

Often, laboratory environments are limited in their complexity due to standardisation (Ohl and Putman, 2014). Moreover, these environments are commonly highly predictable as events take place in a set order (i.e. contextual predictability) and on scheduled times (i.e. temporal predictability) to limit the influence of these factors on the outcome of the study. For long, there was a widely held view that these predictable captive environments were preferable as they offered security and would consequently reduce stress (Shepherdson et al., 1989). Indeed, occurrence of an unpredictable event leads to activation of the HPA-axis, which in turn increases circulating corticosteroids. This short term activation of the HPA-axis is adaptive, e.g. by suppression of reproductive and territorial behaviour and by facilitation of foraging and exploratory behaviour (Wingfield and Ramenofsky, 1999). It is true that predictability sometimes provides the animal with some kind of control as it allows the animal to prepare for what is coming, which can be beneficial to its welfare (for a review, see Bassett and Buchanan-Smith, 2007). This is most intuitive for negative stimuli, for example, rats that received unpredictable electric shocks showed more somatic stress reactions and more stress-induced pathology than rats that were given a reliable signal before receiving electric shocks (Seligman, 1968; Weiss, 1970). Additionally, cats subjected to unpredictable handling procedures played less and tried to hide more (Carlstead et al., 1993) and rats living under unpredictable housing conditions showed more negative cognitive bias than rats living in predictable housing (Harding et al., 2004). However, the positive effects of predictability not only apply to negative stimuli such as shocks and handling, but also to positive events such as the provision of enrichment. For example, piglets that were given a reliable cue before they were given enrichment played more, were less aggressive and suffered less physical injuries than piglets that received unpredictable enrichment (Dudink et al., 2006).

In addition to predictability, controllability in the sense that an animals' behavioural response reliably results in the desired outcome, is an important aspect in animal welfare (Bassett and Buchanan-Smith, 2007; Overmier et al., 1980). For example, rats that could control (i.e. avoid/escape) being shocked had less gastric ulceration than rats that could not control being shocked, regardless of whether they received a warning signal or not (Weiss, 1971). For the HPA-axis activation in response to a stressor to be adaptive, the animal must have some degree of control over its environment. If a behavioural response is impossible or when the response does not result in satisfaction of the motivation, this might lead to welfare problems (Hughes and Duncan, 1988). Again, this not only applies to control over negative experiences, but also to control over positive experiences. For example, infant rhesus monkeys that could control appetitive aspects of their environment through the use of a manipulable device showed less fear, more exploratory behaviour and better coping than monkeys without this means of exerting control over the environment (Mineka et al., 1986).

Environmental enrichment that allows for species-specific behaviours

To refine animal experiments, provision of environmental enrichment that allows for the expression of species-specific behaviours, i.e. enrichment that is functionally significant and biologically relevant to the animal, is recommended (Newberry, 1995) without any concrete evidence that the treatment represented an improvement for the animals. Others have used the term when the main beneficiaries may have been people rather than their captive animals. The criteria used to assess enrichment have also varied according to animal use (e.g. laboratory, farm or zoo animals. A distinction can be made between pseudo-enrichments (which are never biologically relevant and either neutral or detrimental to animal welfare), conditionally beneficial enrichments (which are biologically relevant, but may induce welfare problems if not properly managed) and beneficial enrichments (which are biologically relevant, beneficial to animal welfare, and rarely if ever associated with welfare problems; Würbel and Garner, 2007). Beneficial enrichments can be further divided into resources that meet physiological needs and resources that meet behavioural needs. Physiological needs encompass acquiring food and water, the opportunity to adapt to climatic changes (e.g. seeking shelter) and having a safe living space (Poole, 1992). Meeting these physiological needs is essential for the animal's survival, which is why these needs have been incorporated in older regulations concerning animals experimentation and are mandatory to take into account in the laboratory setting. Behavioural needs, on the other hand, encompass behavioural patterns that are the most important for the animal to perform, but are not necessary for immediate survival (Jensen and Toates, 1993; Poole, 1992). It is important for the animal to perform these behavioural patterns for the process of performing the behaviour, rather than for the outcome (Dawkins, 1990; Fraser and Nicol, 2011; Hughes and Duncan, 1988). These behavioural needs can be divided in the need for stability and security (i.e. exert some control), appropriate complexity (i.e. choice in activity), an element of unpredictability (e.g. exploration of novel objects/bedding or cage mates) and opportunities to achieve goals (e.g. training or foraging enrichment; Poole, 1992). These behavioural needs are more difficult to define for a specific animal species and are therefore less often met in laboratory settings, sometimes resulting in frustration and/or a negative psychological state (indicated by e.g. disturbed behaviour and/or HPA-axis activation; Jensen and Toates, 1993; Poole, 1992).

To determine which enrichments meet behavioural needs, the motivation that animals have to gain access to different enrichments should be quantified (Kirkden and Pajor, 2006). For this purpose, the frequency of occurrence of behaviours that are enabled by the enrichment, preference tests and motivation tests have been used as a measure.

Frequency of occurrence

The frequency with which a behaviour occurs does not necessarily correlate with the importance of the behaviour. Thus, behaviours that are performed infrequently might be more valuable to the animal than some frequently seen behaviours, thereby rendering frequency measurements unsuitable for the purpose of establishing importance of behaviour for the animal (Dawkins, 1983).

Preference tests

Preference tests were introduced as a means to determine an animal's motivation for different resources (e.g. Cruden, 2011). A preference tests consists of a free choice between two or more resources using e.g. a T- or Y-maze set-up. However, preference tests also come with several limitations. First, a preference test can only be used to test resources that represent different ways of satisfying the same behavioural need, i.e. resources that are substitutes (Nicol, 1997). Second, a preference test only provides information on the relative value of enrichments and can therefore not be used to determine the absolute value of these enrichments/behaviours (Kirkden and Pajor, 2006). For example, inaccurate results may be generated if the animal is asked to choose between resources that are all deemed to be of low value by the animal (Baumans, 2005a). In other words, a preference test assesses whether an animal "likes" or "prefers" (affective consequence) a specific enrichment item over another and not whether it actually "wants" or "needs" (is motivated to access) this item (Berridge and Robinson, 2003). Third, the time at which the animal is tested might not necessarily be the time of peak motivation for a certain resource (Fraser and Matthews, 1997). For example, hens will only show a high motivation for perching at night and will therefore show a preference for another commodity over a perch if tested during the day (Olsson and Keeling, 2002). Fourth, the choice the animal makes might not reflect long-term priorities (Baumans, 2005a), e.g. baboons showed a clear preference for heroin and brain stimulation over food when provided a free or low-cost choice (Elsmore et al., 1980; Hursh and Natelson, 1981). For a full review on the limitations of preference tests, see (Kirkden and Pajor, 2006).

Consumer demand studies

A measure that can give an indication of the importance of the behaviours that environmental enrichment stimulates is how much effort an animal is willing to put in ("pay") for access to this enrichment, i.e. how motivated an animal is to gain access to the resource. To establish the amount of effort an animal is willing to pay, a so-called consumer demand study can be used. This technique has been derived from human micro-economics and has been introduced in animal studies as a tool to accurately identify behavioural needs (Dawkins, 1983). A consumer demand study uses an operant conditioning task in which the "price" (e.g. number of responses) to gain access to a resource is systematically increased (Cooper and Mason, 2001; Dawkins, 1990). This approach can be used to compare resources that are not unidimensional substitutes, provides information on the absolute value of a resource (see requirement 2 below) and eliminates the issue of peak-motivation (see requirement 3 below; Kirkden and Pajor, 2006). Also, the choices made in a consumer demand study better reflect long-term priorities, e.g. the baboons from the abovementioned example reversed their preference (i.e. preferred food over heroin and brain stimulation) when the availability of the resources was restricted or the cost for the resources was increased (Elsmore et al., 1980; Hursh and Natelson, 1981).

There are three basic requirements to perform a consumer demand study:

1. A naturalistic task should be used, as the association between task and reward is more apparent when using a naturalistic task (e.g. walking through a narrow gap, pushing a weighted door or walking a long raceway) than when using an unnaturalistic task (e.g. lever pressing or lever pecking; Cooper and Mason, 2001). This greater ease of association between task an reward makes naturalistic tasks less prone to operant-reinforcer bias (i.e. differences in ease of learning the connection between an action and different rewards; Dawkins, 1990). It is assumed that animals do not need training to perform a naturalistic task and animals will prefer to perform this task over other tasks, e.g. pigs, blue tits and mink preferred and learned faster to perform a more naturalistic operant task (Hansen et al., 2002; Partridge, 1976; Young et al., 1994). Which task is most appropriate will depend on the species and its natural behaviour.
2. A negative and positive control should be measured to be able to interpret the price the animal pays in the consumer demand study correctly (Dawkins, 1983). As a negative control, the animals' motivation for an empty chamber is often measured, in order to quantify how motivated the animal is to reach additional space and/or perform the task itself. As a positive control, the animal's maximum push capacity (MPC), i.e. the maximum effort that the animal is able to exert to accomplish the task, is established. The animal's motivation for food or water are often used as MPC as these are essential needs, for which the animal will presumably perform to its maximum ability (e.g. Dawkins, 1983; Matthews and Ladewig, 1994).
3. The animal should be tested in a closed economy, i.e. it should not be able to gain access to the resources outside of the testing environment. If animal can perform the behaviour outside the test situation, the demand for this specific resource might be lowered in the consumer demand study (Ladewig et al., 2002; Mason et al., 1998). Therefore, to prevent the animal from performing the behaviour outside

of the test situation, animals often live in the consumer demand set-up. As this also has the implication that the animals are tested 24-hours per day over several days, it can be assumed that the animal will be tested at its peak motivation.

Several other factors, such as e.g. the age of the animal (Riber, 2010); previous experiences (with the enrichments specifically and life experiences in general; Cooper and Appleby, 1995; Faure, 1991; Holm and Ladewig, 2007); water, food and enrichment deprivation level (Jensen et al., 2004); the social environment the animal is tested in (Pedersen et al., 2002; Sherwin, 2003); the order in which the enrichments and costs are presented (Asher et al., 2009; Aw et al., 2011); and enrichment cues during the test (Duncan and Kite, 1987; Warburton and Mason, 2003) have been shown to influence the motivation the animals have for the enrichments and therefore require careful consideration.

There are three indices that are commonly used to interpret the behaviour of the animals in a consumer demand study: the price elasticity of demand index, the consumer surplus index and the maximum price paid index.

Price elasticity of demand index
The price elasticity of demand index measures the rate of decline in consumption per unit increase in cost. This index has several disadvantages: the resource does not always have a single elasticity value; demands for resources can be similar over a large price range; resources for which quick satiation takes place will be overvalued; and resources with high initial consumption will be underestimated (Kirkden et al., 2003; Kirkden and Pajor, 2006).

Consumer surplus index
The consumer surplus index measures the area under the demand curve of price versus amount consumed/visit number (i.e. travel cost consumer surplus) or price versus number of subject prepared to pay each price (i.e. aggregate consumer surplus, e.g. Mason et al., 2001). This index removes some of the limitations of the elasticity of demand index, but unfortunately, as the elasticity curve is used to calculate consumer surplus, it also shares most disadvantages (Kirkden et al., 2003).

Maximum price paid index
The maximum price paid index measures the price at which the consumption falls to zero. This index does not share the disadvantages listed for the other two indices and has the advantage of being relatively insensitivity to external cues (Warburton and Mason, 2003); being useful for the testing of “all-or-none” goods (Olsson and Keeling, 2002); and the ability to use qualitative rather than quantitative increases in price, thereby omitting the need to make assumptions about subjective value of a task (Cooper, 2004). Therefore, it is advised to use the maximum price paid index to interpret the animals’ responses in a consumer demand study (Jensen and Pedersen, 2008).

When these requirements and factors are taken into account, consumer demand studies can be used to identify those enrichments that are most important to the animal and will also most likely have a positive effect on the animal’s welfare. For example, swimming water was identified as an important enrichment for mink, as they were as motivated to push a weighted door for access to swimming water as to reach food (Mason et al., 2001). A further study showed that swimming water indeed had a positive effect on the behaviour of juvenile mink, as play behaviour increased upon provision of swimming water compared to animals that were provided with a cylinder and a platform (Vinke et al., 2005). Additionally, depriving mink of access to a swimming pool (after having had prior experience with it) resulted in increased urinary cortisol (-creatinine ratio) levels (Korhonen et al., 2003; Mason et al., 2001) and increased repetitive intensive scratching at the cage wire mesh, i.e. “scrabbling” (a frequently seen repetitive behaviour in mink; Warburton and Mason, 2006), showing the negative impact of depriving animals from swimming water.

It is expected that a consumer demand study can be used successfully to determine the behavioural priorities of ferrets, a phylogenetically close relative of mink. Unfortunately, research on behaviour and behavioural priorities of ferrets is scarce (for a review, see Vinke and Schoemaker, 2012). Therefore, we have developed multiple consumer demand studies for ferrets and assessed whether all motivation tests are the same, i.e. whether the results of a consumer demand study are different depending on the number of enrichments that the ferrets can pay for simultaneously (Chapters 2-3). Subsequently we have assessed which environmental enrichments might allow for the expression of species-specific behaviours in a 7-chamber consumer demand study set-up (Chapter 4).

The effects of the provision environmental enrichment
Often, the term enrichment is applied to different types of environmental change and increases in complexity (e.g. social, physical, sensory), rather than to the actual outcome of the provision of added stimuli. However, changes to the environment should only be considered enrichment when their use ultimately results in an improvement in the welfare of the animals (Newberry, 1995). Therefore, it is important that appropriate enrichment options are identified and critically evaluated in terms of immediate and long-term effects on the welfare of animals (Baumans, 2005b;

Benefiel et al., 2005). An animal is considered to be in a positive welfare state when "it has the freedom to adequately react to hunger, thirst or incorrect food; thermal and physical discomfort; injuries or diseases; fear and chronic stress, and thus the freedom to display normal behavioural patterns that allow the animal to adapt to the demands of the prevailing environmental circumstances and enable it to reach a state that it perceives as positive" (Ohl and Van der Staay, 2012). Often, physiological and/or behavioural measures are used to assess whether environmental enrichment improves the welfare of an animal, some of which are discussed below.

Cortisol

When an animal is presented with a challenge, the HPA-axis will be activated, releasing glucocorticoids and catecholamines, allowing the animal to adequately react to challenges (Matteri et al., 2000; Wingfield and Ramenofsky, 1999). However, cortisol is not only released under negative stressors, but also positive stressors such as courtship, copulation and hunting will cause an increase in cortisol levels (Broom and Johnson, 1993). Additionally, chronic stress can lead to HPA hypoactivity rather than hyperactivity under certain conditions (Miller et al., 2007). However, when cortisol measures are viewed within their context (i.e. are combined with behavioural measures), they can be a valuable indicator of stress in animals (Broom and Johnson, 1993; Lane, 2006). Cortisol levels can be measured in plasma, saliva (Kirschbaum and Hellhammer, 1989), urine, faeces (Hay and Mormede, 1998; Wasser et al., 2000), and hair (Meyer and Novak, 2012). Plasma and saliva measurements specifically have the limitation that these are point samples that vary as a function of circadian rhythms (de Jong et al., 2000; Irvine and Alexander, 1994). This can be overcome by taking repeated saliva or blood samples, but this is highly labour intensive and can cause great discomfort to the animal (which in turn also influences cortisol release). For example, restraint and handling required for blood sampling may cause sharp increases in peripheral glucocorticoid concentrations within minutes (Hopster et al., 1999; Moe and Bakken, 1997; Willemse et al., 1993). Additionally, factors such as food intake and exercise can influence cortisol levels in plasma and saliva (Gibson et al., 1999; Lane, 2006). Urine and faeces collection, on the other hand, are non-invasive and can thus be used to avoid some of the downsides of plasma and saliva collection. However, these samples still only provide information on a limited time period (24 hours or less) and collection of individual samples from group-housed animals can be challenging (Wasser et al., 2000). Moreover, faeces is not homogenous and bacteria and enzymes may influence the hormone levels. A new, promising method to gain long-term information on cortisol levels in animals is to measure cortisol in the hair of an animal (Meyer and Novak, 2012). However, the exact mechanism by which cortisol is incorporated in the hair is unknown (Meyer and Novak, 2012); exposure to shampoo and water may compromise the cortisol in hair (Hamel et al., 2011; Li et al., 2012); and cortisol concentrations are influenced by hair pigmentation (Bennett and Hayssen, 2010). Moreover, measuring hair cortisol proved to be impractical in ferrets, due to seasonal moulting and inter-individual differences in the rate of hair growth (Reijgwart et al., unpublished research).

Neutrophil/lymphocyte ratio

It has been suggested that the response of leukocytes to stress is a better indicator of long-term stress than more direct glucocorticoid measurements (Davis et al., 2008). Increased levels of serum cortisol will cause lymphocytes to migrate from the blood circulation to other tissues and cause neutrophils to migrate from bone marrow to the blood circulation and reduce migration from the blood to other parts of the body (Dhabhar et al., 1996; Leonard, 2005). Thus, a high neutrophil/lymphocyte ratio (N/L ratio) in the blood is and indicator of high cortisol concentrations and therefore high HPA axis activity (Davis et al., 2008). The N/L ratio has a positive correlation with the magnitude and number of stressful events and subsequent levels of glucocorticoid release (Davis et al., 2008; McFarlane and Curtis, 1989). Chronic elevation of glucocorticoid release due to chronic stress may thus lead to long-term rises in N/L ratios. Whereas hormonal responses to stress occur within seconds or minutes, the initial leukocyte response starts hours to days (depending on the taxon) after a specific stressor (Burguez et al., 1983; Davis et al., 2008). Such response times allow the acquisition of baseline samples with no influence of acute sampling distress. If environmental enrichments helps animals to adapt and cope with a challenge, cortisol will provide negative feedback to the hypothalamus, resulting in a subsequent decrease in cortisol concentrations and N/L ratio (Kant et al., 1987; Leonard, 2005). For example, N/L ratios and cortisol levels in farmed mink without nest boxes for several months were higher than in mink provided with nest boxes (Hansen and Damgaard, 1991). Similarly, it is expected that N/L ratios can be used as a reliable physiological indicator of long-term stress in ferrets.

Stereotypic behaviours

Stereotypic behaviours are defined as "repetitive behaviours induced by frustration, repeated attempts to cope and/or C.N.S. (brain) dysfunction" and are probably the most well-known example of behaviour which occurrence can be reduced by the provision of environmental enrichment (Mason and Latham, 2004). For example, the expression of stereotypies such as pacing and scrabbling in mink has been reduced by the provision of enrichment (Dallaire et al., 2012; Hansen et al., 2007). It is assumed that animals perform stereotypies when a need is frustrated (Dawkins 1990, Mason 1991). However, it should be taken into account that the animals in barren environments that do not perform stereotypies (but e.g. show particularly low levels of activity) might be worse off than the animals that do (Mason & Latham 2004). These non-stereotypic animals are suggested to have a different (reactive or inactive) coping style and will show changes in activity rather than perform stereotypies, e.g. inactive phenotypes have been associated with elevated baseline cortisol levels (Bildsøe et al., 1991).

Inactivity

Animals with a reactive coping style might not reduce time spent on stereotypic behaviour, but instead reduce the time they spent inactive in reaction to the provision of enrichment, e.g. provision of enrichment has been shown to result in a decrease in inactivity in mink (Hansen et al., 2007). However, a reduction in inactivity is not necessarily a positive welfare indicator, as it does not only reflect negative states, like stress or fear, but also includes states related to relaxation, such as sleep or rest (Meagher et al., 2013). For example, different subtypes of inactivity have been suggested to have different motivational backgrounds in mink, i.e. the time spent lying inactive while awake is suggested to reflect a boredom-like state, while being inactive in a nest box was proposed to reflect anxiety-induced hiding (Meagher and Mason, 2012; Meagher et al., 2013).

Fear and anxiety

Fear and anxiety is a trait that is well-studied in mink, by measuring the reactions of the animals to a stick (i.e. stick test, Hansen and Møller, 2001; Hansen, 1996) or gloved finger (i.e. glove test, Meagher et al., 2011) being extended into their home cage through the wire mesh, or a gloved hand being extended into the cage trying to touch the animal (i.e. Trapezov hand test (Malmkvist and Hansen, 2002; Trapezov, 2000). Fear has been shown to be detrimental to animal welfare (Hemsworth, 2003) and can be reduced by environmental enrichment. For example, mink that were provided with enrichment were less aggressive and showed decreased fear in a stick and glove test (Meagher et al., 2014).

Agonistic behaviour

Next to the physical risks involved with high aggression levels, agonistic behaviour is an important cause of social stress (Blanchard et al., 2001). Therefore, enrichment is often advocated to reduce the occurrence of this behaviour. However, enrichment seems to have a dual effect on agonistic behaviour, as some studies report a reduction in agonistic interactions after enrichment (e.g. Abou-Ismail, 2011; van Loo et al., 2002), whereas others report an increase in agonistic behaviour (e.g. Marashi et al., 2003; McGregor and Ayling, 1990). It is suggested that environmental enrichment results in

more competition when animals start to defend territories, facilitated by the structural elements in the enclosure, or try to monopolise the resources, potentially leading to an increase in aggression (Akre et al., 2011; Howerton et al., 2008). In contrast, enrichment has been suggested to decrease the number of agonistic encounters by compartmentalising the enclosure and thereby reducing the chances of accidental physical contacts and increasing the possibilities for seeking refuge (Abou-Ismail, 2011).

Play behaviour

Concurrent with a decrease in agonistic encounters, providing animals with greater control over their spatial, physical and social environment through the provision of enrichment can allow the animals to relax and express (social) play behaviour, which is regarded to be an indicator of positive welfare (Boissy et al., 2007; Held and Špinka, 2011). Play behaviour is regarded as a positive welfare indicator as it is easily intervened by other behaviour with a higher fitness-associated priority (Held and Špinka, 2011); mostly occurs when an animal is in a relaxed state and familiar conditions (Burghardt, 1999; Fagen, 1981); and is not expressed when an animal suffers from severe stress (Hinton and Dunn, 1967; McCune, 1992). To illustrate the effect of enrichment on play behaviour, provision of environmental enrichment has been reported to enhance play in both juvenile farmed mink (Vinke et al., 2005)but without swimming water", "type" : "article-journal", "volume" : "14" }, "uris" : ["http://www.mendeley.com/documents/?uuid=7bf646e2-4378-4058-8d29-66110aa2ed4a"] }], "mendeley" : { "formattedCitation" : "(Vinke et al. 2005 and pet ferrets (Talbot et al., 2014)the aims of this study were to investigate the relationship between housing and management and the incidence of play and undesirable behaviours in order to ultimately improve welfare in the domestic ferret. An online survey was constructed which focused on different housing and management strategies utilised by ferret owners and required owners to score the frequency of a range of behaviours observed. Ferret owners were also able to report what behavioural aspects they believed particular ferret behaviours were associated with. There were 466 ferret owners who participated in this survey study. Generalised linear mixed models (GLMMs).

Unfortunately, research on the effects of environmental enrichment on the welfare of ferrets is scarce. Therefore, we have assessed the effect of preferred and non-preferred environmental enrichment on several physiological and behavioural parameters in laboratory ferrets (Chapter 5).

Refinement through the minimisation of pain

Studies on diseases have the potential of causing severe discomfort, as the animals have to undergo a disease process. Pain is an important component of discomfort and is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (International Association for the Study of Pain (IASP) Task Force on Taxonomy, 1994). Timely recognition and treatment of pain in animals used in research is paramount, not only for animal welfare but also for the quality and reliability of research. It is well recognised that pain may affect the outcome of studies, as it can affect the functionality of various organ systems, including the immune system (National Committee for the protection of animals used for scientific purposes, 2016; Poole, 1997). The European Directive emphasises the need to reduce pain during research procedures as a part of refinement (European Commission, 2010a).

Unfortunately, pain assessment is not always straightforward, as animals cannot verbally communicate whether and in how much pain they are, and may hide their pain (in particular prey animals; Short, 1998) and/or show signs of pain that might be too subtle or with which we might not be adequately familiar to recognise these as deviations from normal physiology and behaviour (Mayer, 2007). Therefore, the analogy principle is used, which states that, unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals (Dawkins, 1980; Sherwin, 2001). If the analogy principle is insufficient, the precautionary principle should be used. This principle states that, in the absence of any scientific consensus as to the presence of pain, it will be assumed that the animal has pain (National Committee for the protection of animals used for scientific purposes, 2016). However, species specific indicators of pain should still be identified as these principles cannot always be applied (e.g. animals might experience unexpected pain that is not caused by a procedure or medical condition). Additionally, species differences in pain perception have been found, i.e. animals recover quicker and seem to tolerate some disease conditions better than humans (Morton and Griffiths, 1985).

Different species-specific pain scales have been developed, which generally list changes in the animal's activity pattern, appearance, temperament, vocalisations, ingestion and physiology as indicative of pain (Carstens and Moberg, 2000; Flecknell, 1994; Stasiak et al., 2003). However, the assessment of pain using these scales is highly subjective and suffers from high inter- and intra-observer variation (Flecknell, 1994; Weary et al., 2006). This highlights the importance of species-specific knowledge on pain behaviour. Unfortunately, signs of pain and other forms of discomfort in ferrets that are mentioned in the literature are nonspecific, inconsistent, subjective and sometimes even contradictory (see reviews van Oostrom et al., 2011 and Chapter 6). This lack of knowledge and unreliability of species specific pain scales additionally underlines the urgent necessity for a new reliable and easy to use pain assessment tool to ensure further refinement in experimental methods for ferrets. A survey amongst ferret owners provides some indication on where to focus our efforts (Chapter 7). In a first attempt to develop a new tool in the toolbox for pain recognition in ferrets, we have developed and evaluated a Ferret Grimace Scale (Chapter 8).

Aim and scope of the thesis

There is a large gap in the knowledge on how to refine the care and use of laboratory ferrets. Therefore, the studies presented in this thesis are performed with the aim of giving insight into possible ways to refine the care and use of laboratory ferrets, focusing on I) the value and effect of environmental enrichment, and II) the recognition of pain and other forms of discomfort in ferrets.

Part I starts with **Chapter 2**, that deals with the practical considerations regarding the design of a consumer demand study for ferrets. This 2-chamber consumer demand study set-up was the first attempt at measuring the motivation for different enrichment items in ferrets. Using this set-up, three prerequisites were tested: 1) whether the maximum price the ferrets paid for food reflected their maximum push capacity; 2) whether the task was strenuous; and 3) whether the maximum price the ferrets paid for an empty chamber was low and sufficiently distinctive from the maximum price they paid for food, to enable detecting differences in MPP between the different resources presented in the enrichment chamber. Though the first two prerequisites were met, the third was not, rendering the 2-chamber set-up unsuitable for further research. Some adaptations to the regular 2-chamber consumer demand study set-up were assessed, in an (unfruitful) attempt to find a suitable set-up to assess the ferrets' motivation for different enrichments.

To find a suitable consumer demand study set-up for ferrets and to assess how robust the results of different consumer demand study set-ups are, the effect of two adaptations to a 3-chamber consumer demand study in ferrets were analysed in **Chapter 3**. The maximum price paid, mean visit number, mean visit duration and mean enrichment interaction time the ferrets would show in a 3-chamber consumer demand set-up were compared to the result of the 7-chamber consumer demand set-up and an "all-but-one" 3-chamber set-up. First, the multi-chamber set-up was hypothesised to be a more suitable set-up than a 3-chamber set-up for ferrets. Second, as the 2-chamber set-up revealed that there might be an influence of the items in the home chamber on the maximum price the ferret will pay, it was interesting to investigate whether adding preferred items to the home chamber in the "all-but-one" 3-chamber set-up would yield different results than a "regular" 3-chamber set-up.

Subsequently, the ferrets' preferences and behavioural priorities were determined in a 7-chamber consumer demand study, as this proved to be the optimal set-up for ferrets (**Chapter 4**). The maximum price paid for, number of visits to, average duration per visit to and interaction time with the different enrichment categories (i.e. foraging enrichment, sleeping enrichment, tunnels, balls, social enrichment and water bowls) were analysed. With these results, a first advice regarding the enrichment items that might be suitable to refine research using ferrets as an animal model could be formulated.

Finally, the differences in behaviour and physiology of ferrets that were provided with enrichment were compared to the differences in behaviour and physiology of ferrets housed in standard conditions for eight weeks (**Chapter 5**). Additionally, differences in the effect of preferred enrichment (i.e. highly valued items as determined in the consumer demand set-up) and non-preferred enrichment (i.e. low valued items as determined in the consumer demand set-up) were assessed, where it was hypothesised that preferred enrichment would result in more positive changes in the behaviour and physiology of the ferrets than the provision of non-preferred enrichment.

Part II focuses on refinement through the minimisation of pain, by developing methods to recognise pain in ferrets. To this end, a review on the evaluation of pain in laboratory ferrets was written (**Chapter 6**). This review first describes physiological changes, behavioural changes, and changes in facial expression that are generally observed in animals that are experiencing pain. Second, the changes that are reported to be useful for the recognition of pain in ferrets are discussed, followed by an overview of the limitations that are encountered when using these parameters in laboratory ferrets.

This review is followed by **Chapter 7**, where the results of an international survey amongst ferret owners on how they recognise pain in their ferrets are presented, again divided in physiological changes, behavioural changes and changes in facial expression. Both chapter 6 and 7 are used to identify gaps in the current knowledge on pain recognition in ferrets and to identify possibilities for future research on how to improve this.

As the possibility of using changes in facial expressions for the recognition of pain in ferrets were under-exploited, the final chapter of part II, **Chapter 8**, discusses the composition and evaluation of a Ferret Grimace Scale. Five Action Units (i.e. *orbital tightening*, *nose bulging*, *cheek bulging*, *ear changes* and *whisker retraction*) were identified from lateral pictures ferrets' faces before and after telemetry probe implantation (a potentially painful procedure) without additional analgesics. The Ferret Grimace Scale was subsequently evaluated using scores assigned to the pictures by observers that were blinded to the timing of the pictures and objective of the study.

In the final chapter of this thesis, the identified possibilities for refinement of the care and use of ferrets by providing effective environmental enrichment and early and adequate recognition of pain and remaining questions and opportunities for further research are discussed (**Chapter 9**).

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PART I

Value and effect of environmental enrichment for laboratory ferrets

Chapter 2

Workaholic ferrets: does a two-chamber consumer demand study give insight in the preferences of laboratory ferrets (*Mustela putorius furo*)?

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Abstract

Although provision of environmental enrichment is an effective tool to refine animal experiments, it is currently unknown which enrichments ferrets prefer. This study aimed to assess the suitability of a closed economy, two-chamber consumer demand set-up to determine ferrets" preferences for selected enrichments. Twelve female ferrets were housed in a set-up consisting of a home and enrichment chamber (EC) connected by a weighted door. The maximum weights the ferrets pushed for food (MPP_{food}) and an empty chamber (MPP_{empty}) were determined to evaluate the maximum push capacity of the animals and as a control. Although the ferrets pushed significantly more for food (1325±213 g) than for the empty chamber (1169±193 g), the weight difference was minor (MPP_{empty} was 89±13% of MPP_{food}). To evaluate the ferrets" underlying motivation to push for the empty chamber, a second study was performed in which MPP_{empty} was tested in seven alternative set-ups. The first three set-ups included adapted versions of the standard design (set-up A_1, A_2 and A_3), intended to determine the functional value of the empty chamber. The four other set-ups (set-up B_0, B_1, B_2, B_3) aimed to evaluate the attractiveness of the door elements by allowing the ferrets to choose whether or not to use the weighted door to enter EC. Results demonstrated no significant differences in MPP_{empty} between the A-set-ups, indicating that the value of the empty chamber could not be reduced by adapting the set-up. MPP_{empty} reduced when allowing the ferrets free access to EC, demonstrating that the empty chamber had reinforcing properties. Nevertheless, the ferrets were still motivated to use the weighted door despite being granted free access to EC, indicating that the door also has reinforcing properties. The ferrets decreased the use of the weighted door most when, in a set-up with free access to EC, the nest box in the home cage (53±22% of MPP_{food}) was replaced by a manipulable plastic bucket (26±13% of MPP_{food}). These results indicate that availability of items in the home chamber may influence the results, which should be taken into account when designing motivation studies similar to the one performed in this study. The lack of differences between MPP_{food} and MPP_{empty} furthermore demonstrates that the two-chamber set-up is not suitable for evaluating the ferrets" motivation for enrichments, thus necessitating other alternatives, such as a three- or multi-chamber consumer demand study, to be explored.

Introduction

Ferrets (*Mustela putorius furo*) are commonly used for research purposes (e.g. influenza research, Boyce et al., 2001). To ensure humane use of laboratory animals, the principles of reduction, replacement and refinement (the three R's) are employed (Russell et al., 1959). Refinement includes the optimisation of the animal's housing conditions, e.g. by providing environmental enrichment (Russell et al., 1959). Environmental enrichment can improve animal welfare at least to a certain degree by providing some of the appropriate stimuli needed to perform species-specific behaviour (Newberry, 1995).

Traditionally, the preference for alternative resources is assessed in preference tests, in which the animal is given a two-way unweighted choice between alternative resources. One preference test with ferrets indicated that these animals prefer an enriched over a barren cage, but did not specify which enrichments were preferred (Cruden, 2011). Unfortunately, these preference tests only address the relative rather than the actual value of the enrichment (Kirkden and Pajor, 2006). In other words, they assess whether animals "like" (affective consequence) a specific enrichment item and not whether they "want" (are motivated to access) this item (Berridge et al., 2003).

To address the actual value of a specific enrichment item, so-called consumer demand studies may be used. Such studies have been used extensively in various laboratory and production animals, including mice (Sherwin, 1996), rats (Manser et al., 1998; Manser et al., 1996; Patterson-Kane et al., 2002), silver foxes (Hovland et al., 2006), and mink (Mason et al., 2001). A consumer demand study comprises a set-up in which increasing costs are imposed on the animal in order for it to gain access to a specific resource.

The value of the enrichment can be expressed using various indices, including the price elasticity of demand index, the consumer surplus index and the maximum price paid index (Kirkden et al., 2003). The maximum price paid (MPP) index indicates the "breakpoint" at which the animal is no longer willing to pay the price for the resource. This index has several advantages over the other indices, including a) its relative insensitivity to external cues (Warburton and Mason, 2003); b) its usefulness for the testing of "all-or-none" goods (Olsson et al., 2002); and c) the possibility to use qualitative rather than quantitative increases in price, thereby omitting the need to make assumptions about the subjective value of a task (Cooper, 2004). As a result, the MPP index is generally preferred for analysing the value of resources.

To infer information on the actual value of a resource, the MPP is tested consecutively for various resources, including food (MPP_{food}, which is considered to reflect the maximum amount of weight an animal is able to push, i.e. the maximum push capacity), an empty chamber (MPP_{empty}, which serves as a control) and the different enrichment items (e.g. Asher et al., 2009). Most commonly, testing takes place in a closed economy two-chamber set-up, i.e. the animals are housed permanently in the experimental set-up. This prevents the animals from becoming less motivated to work for a resource because of its (free and/or unlimited) access to the item outside of the experimental set-up (Jensen and Pedersen, 2008; Ladewig et al., 2002). Animals have also been tested in three- or multi-chamber designs, in which they can simultaneously gain access to one or multiple resources and a control (e.g. Hovland et al., 2006; Mason et al., 2001; Seaman et al., 2008). These set-ups, however, introduce an extra variable, as the animal only has limited income (i.e. the time and energy available per day) which it then needs to divide between the different resources, thereby potentially yielding lower MPP values for resources that are less important.

As no consumer demand studies have been performed in ferrets thus far, the current study focused on establishing the functionality of a two-chamber consumer demand study in ferrets using the MPP index. Similar to the consumer demand study with mink (Mason et al., 2001), a weighted door was used. To be considered suitable for testing the ferrets' motivation for resources, the experimental set-up needs to meet three prerequisites: 1) MPP_{food} should reflect the maximum push capacity; 2) the task should be perceived as strenuous; and 3) MPP_{empty} should be low and sufficiently distinctive from MPP_{food}. To assess whether these prerequisites were met, MPP_{food}, MPP_{empty} and duration and number of visits to the chamber with food and to an empty chamber were measured. As ferrets were found to push excessively for an empty room (i.e. the third prerequisite was not met), two subsequent studies were performed to assess how and which features of the design may have affected MPP_{empty}.

Animals, materials and methods

Ethical approval

This study was ethically approved by the Institutional Animal Care and Use Committee of Intravacc (DEC 201300057) and Utrecht University (DEC 2013.I.09.073).

Animals

For study 1, twelve female neutered ferrets from Schimmel B.V. were used that weighed 1000±200 g (665-1145 g). Six of these ferrets were four years old and chemically neutered using a hormonal implant (Suprelorin®, Virbac, the Netherlands); the other six ferrets were five months old and surgically neutered (ovariectomised). For study 2, five of the four-year-old female ferrets from study 1 were used. These ferrets weighed 838±113 g (665-938 g).

Housing and nutrition

Ferrets were housed indoors in a room that was kept at a temperature between 18°C and 22°C. They were exposed to a 8:16 h light:dark schedule using artificial lighting (light bulbs) that switched on at 9:00 h and off at 17:00 h. In addition, auditory stimulation was available in the form of a radio, which automatically switched on and off concurrent with the light. The ferrets were provided water and food (Hill's M/D® for the four-year old ferrets, Hope Farms® ferret balance pellets for the five-month-old ferrets) *ad libitum*. Refreshing of the food and water, as well as cleaning of the cages took place daily at 10:00 h. The ferrets' health and overall condition were monitored prior to and throughout the study.

Experimental housing

Throughout the experiments, the ferrets were individually housed in a closed economy two-chamber set-up consisting of a phenolic faced plywood floor pen with solid floors and walls that measured 1.6 m^2 (design A_0, Figure 2.1a). The pens were divided in two equal spaces by means of a 70 cm high, 6 mm thick phenolic faced plywood divider equipped with a non-transparent one-way cat flap and a one-way horizontal swinging weighted door (Tecnilab-BMI, Someren, The Netherlands; Figure 2.1b). Under the door, a wire mesh strip was mounted to provide a traction surface for the ferrets to facilitate them to apply force to the door. The door allowed the ferrets to move from a home chamber (HC) equipped with a resting area (i.e. nest box or plastic sleeping bucket), food (except when testing MPP_{food}) and water (provided via a nipple) to the enrichment chamber (EC) where the resource to be tested (e.g. food, enrichment) was placed. The one-way unweighted cat flap could subsequently be used by the ferrets to return to HC.

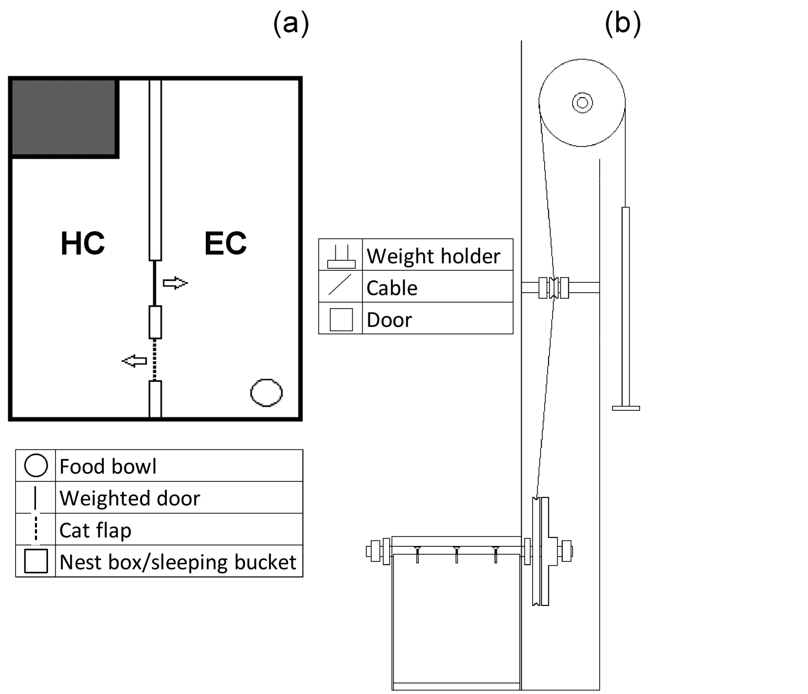

Figure 2.1 (a) Layout of design A_0 (study 1, *N*=12). HC = home chamber; EC = enrichment chamber; arrows indicate the direction in which the ferrets could pass through the cat flap or weighted door. (b) Drawing of the door mechanism. Weights could be added to a cable that was connected to the door via three pullies, thereby increasing the effort needed to open the door to gain access to EC.

Task

Similar to mink (Cooper and Mason, 2001), ferrets had to push a weighted door, which is considered to be a naturalistic task for ferrets that requires little training and is less prone to operant-reinforcer biases than unnatural tasks (Dawkins, 1990). To open the unweighted door, ferrets needed to exert a force of 200 g. Similar to mink, the force needed to push open the door was gradually increased by adding 250 g of weight to the door mechanism on each consecutive day (Mason et al., 2001). The mechanism would transfer 50% of the added weight to the actual force needed for the ferret to open the door. Thus a weight of 250 g translated to a push force of 325 g (200+50% of 250 g), a weight of 500 g to a push force of 450 g (200 +50% of 500 g), etc.

Acclimatisation and training

The ferrets were allowed to acclimatise to the experimental housing for 14 days prior to initiating the training. During this period, the animals were encouraged to visit both chambers by placing food in EC and providing free access to both rooms by leaving the cat flap and door open. In the third week, the ferrets were trained to use the door and the cat flap by gradually closing both doors over a period of four days, necessitating the ferrets to push the door open further each day to gain access to the other room. As the ferrets readily used the door and the cat flap, no additional training was required. Prior to commencing the experiments, the ferrets were allowed free access (i.e. closed, but unweighted door) to both sides for another three days. Once they had used both the door and the cat flap and accessed both rooms for three consecutive days, the experiment commenced.

Study 1 – Suitability of the design

Study 1 was designed to test whether the standard design (A_0) met the three prerequisites needed to demonstrate suitability of the design to evaluate motivation for different resources (i.e. MPP_{food}=MPC; the task is strenuous; MPP_{empty}<MPP_{food}).

The first prerequisite (MPP_{food}=MPC) was tested by having the ferrets work for food in the standard set-up (A_0). Since food passage time in ferrets is approximately 3 hours (Bleavins et al., 1981), the ferrets were expected to be motivated to gain access to the food in EC at least once per 24 hours. Ferrets were considered to have reached their maximum price paid for food (MPP_{food}) when they did not visit EC for 24 hours. MPP_{food} was subsequently recorded as the last weight the ferrets successfully pushed to gain access to food. The ferrets were monitored through 24-hour video recordings to see whether they attempted to push the door above their MPP_{food}, but were unable to succeed or stopped pushing the door altogether, thereby confirming that MPP_{food} indeed equalled MPC.

The second prerequisite (pushing the door is a strenuous task) was tested by evaluating whether changes in the number and duration of visits occurred upon increasing the effort to gain access to EC. The task should be demanding as a task that is fun or easy will result in ferrets pushing the door merely just for the act of pushing rather than gain access to the resources provided in EC. If the task is demanding, the number of visits should decline and the duration of visits is expected to increase with increasing weights to keep consumption of the resource constant at the lowest possible daily total price, as has been observed in other studies (Cooper and Mason, 2000; Sherwin and Nicol, 1996).

The third prerequisite (MPP_{empty}<MPP_{food}) was tested by determining the weight the ferrets were willing to push to gain access to an empty chamber (MPP_{empty}). For this purpose, the food was moved back to HC after which 250 g of weight was added to the door on a daily basis. Similar to MPP_{food}, MPP_{empty} was determined as the last recorded weight at which the ferrets pushed the weighted door to gain access to EC within a 24-h time frame.

Study 2 - Exploring the motivational background for pushing for the empty chamber

Prior to initiating study 2, ferrets were allowed to acclimatise to the new set-up for 7 days. Similarly, an acclimatisation period of 7 days was implemented between testing of the different set-ups. These set-ups were designed to assess whether the empty chamber had a functional value (exploring, patrolling, defecating; study 2a) and whether the door design had reinforcing values (pushing the door, tunnel around the door; study 2b).

Study 2a – Testing the functional value of the empty chamber

The ferrets (*N*=3 for each set-up) were randomly tested in three alternatives to set-up A_0 (A_1, A_2 and A_3) to assess how the changes to the design would influence MPP_{empty} (Figure 2.2). Set-up A_1 comprised an extra compartment compared to set-up A_0. This extra compartment allowed evaluation of the ferret's motivation to push for the empty compartment to access a remote area to defecate and urinate (i.e. away from their sleeping box). Set-up A_2 comprised a see-through divider between EC and HC compared to the wooden divider in study A_0 to evaluate whether ferrets valued the opportunity to visually inspect EC. Set-up A_3 comprised a mesh tunnel around the perimeter of EC that could be freely accessed. This tunnel allowed the ferrets to freely perform territorial patrolling around the perimeter of EC, which is indicated as an important species-typical behaviour for ferrets (Moors and Lavers, 1981; Powell, 1979).

Study 2b – Identifying potential reinforcing values of the door design

To evaluate whether the door in itself had an intrinsic reinforcing value for the ferrets, resulting in them pushing for the empty compartment, MPP_{empty} was tested in a set-up where the ferrets (*N*=6) were given a choice between accessing the empty compartment through the weighted door or freely accessing this compartment through a hole in the partition, which was realised by removing the cat flap (design B_0). Subsequently, MPP_{empty} was tested in three alternative set-ups to identify whether and which features of the door design may have influenced the ferrets' motivation to push the weighted door (design B_1-B_3, *N*=3; Figure 2.3). In set-up B_1 the (non-manipulable) nest box in HC was replaced by a manipulable sleeping bucket. Both the nest box and the sleeping box served the function of a secluded resting area, but the bucket could serve more functions as the ferret was able to play with it. In set-up B_2 the cat flap could swing both ways, thereby allowing the ferrets to visit EC and play with the cat flap without having to push the weighted door. Set-up B_3 had a small tunnel placed around the cat flap that could swing both ways to test whether the tunnel-like design of the weighted door affected its use.

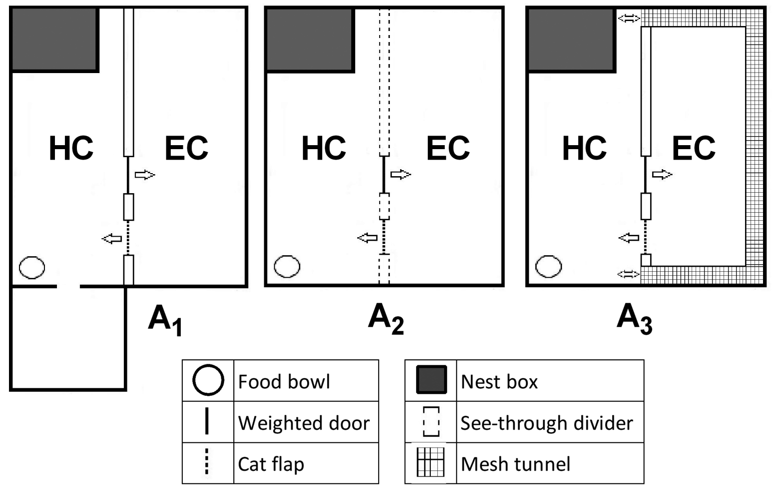

Figure 2.2 The three set-ups that were used in study 2a. Set-ups were similar to the original design in study 1, but included (A_1) an extra chamber, (A_2) a see-through divider rather than a wooden divider, and (A_3) a mesh tunnel that allowed the ferret to explore the perimeters of the empty chamber without it having to push the door. Arrows indicate the way the ferrets can pass through the cat flap or weighted door.

Measurements

MPPfood and MPP_{empty} in study 1 were evaluated for each individual ferret and recorded in absolute weight (g) and in percentage relative to the ferret's bodyweight. MPP_{empty} was furthermore recorded as a percentage relative to MPPfood. Similarly, MPP_{empty} was evaluated separately for each ferret for each of the different set-ups in study 2. Similar to study 1, these values were recorded both in absolute value (g) as well as expressed in relative value (%) compared to MPPfood as determined in study 1. Behaviour of the ferrets was continuously recorded using infra-red surveillance cameras. Using these videos, the number, mean and total duration of visits to EC were analysed.

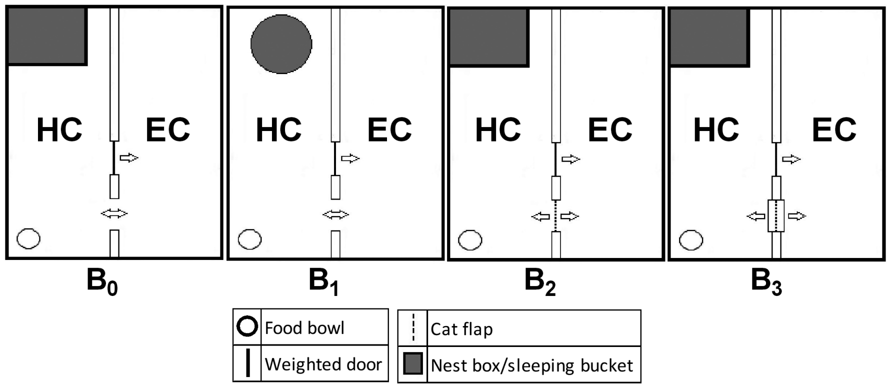

Figure 2.3 The four set-ups that were used in study 2b. In these set-ups ferrets were given a free choice to gain access to EC (i.e. cat flap was removed and/or could be opened in two directions). B_0 = Standard set-up without cat flap to allow free access to EC; B_1 = set-up similar to B_0 but including a manipulable bucket instead of a nest box; B2 = set-up similar to B_0 but with a 2-way cat flap; B_3= set-up similar to B2 but with an extra tunnel around the cat flap.

Statistical analysis

Analyses were performed using IBM SPSS software (version 22.0). Data were expressed as mean ± SD; the probability level accepted for statistical significance was $P<0.05$ unless stated otherwise. Normality of distribution of the residuals was determined with a Kolmogorov-Smirnov test and homogeneity of variances was analysed with a Levene's test for equality of variances.

Differences between MPP_{food} and MPP_{empty} and changes in visit number and duration to food and an empty compartment in study 1 were analysed using a repeated measures ANOVA. Initial visit number and duration for food and an empty chamber were compared using a paired T-test. MPP_{empty} in the different set-ups of study 2a (A_1, A_2, A_3) were compared to MPP_{empty} from study 1 (set-up A_0). Results were analysed using a repeated measures ANOVA. MPP_{empty} in set-up A_0 and B_0 and MPP_{empty} as identified in set-up B_0 was compared to set-up B_1, B_2 and B_3 using descriptive statistics due to a large number of missing values and the small sample size (N=3).

Results

Study 1 - Suitability of the design

MPP_{food} was 1325±213 g (950-1575 g; N=12), which equalled 143±31% (101-200%) of the ferrets' bodyweight. Ferrets did attempt to push the weighted door above MPP_{food}, but were unable to do so. MPP_{emty} was 1169±193 g (850-1425 g; Figure 2.4). Although a statistically significant difference was present between MPP_{food} and MPP_{empty} (P=0.024, F=6.818), this difference was very small: ferrets pushed 89±13% (60-100%) of MPP_{food} for the empty chamber.

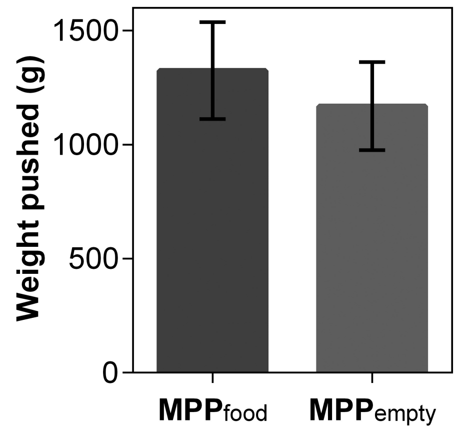

Figure 2.4 Maximum price paid for food (MPP_{food}) and the empty chamber (MPP_{empty}) by the ferrets in study 1, set-up A_0 (N=12).

When the doors were unweighted, ferrets visited EC 32±18 times per day for 5±6 minutes each time to gain access to food, compared to 16±9 times per day for 2±1 minutes each time when EC was empty (P=0.019 for visit number and P=0.095 for visit duration). The number of visits to both the empty chamber and the chamber with food decreased with increasing weights on the door (P<0.001 in both situations). The number of visits to EC remained higher when food was present than when EC was empty (P=0.001; Figure 2.5a). In addition, the ferrets paid longer visits to EC with increasing weights when food was present in this chamber (P=0.043), whereas visits to EC shortened when EC was empty (P=0.018; Figure 2.5b). Total daily visit duration remained constant when food was placed in EC (P=0.192) whereas total visit duration decreased when EC was empty (P<0.001; Figure 2.5c).

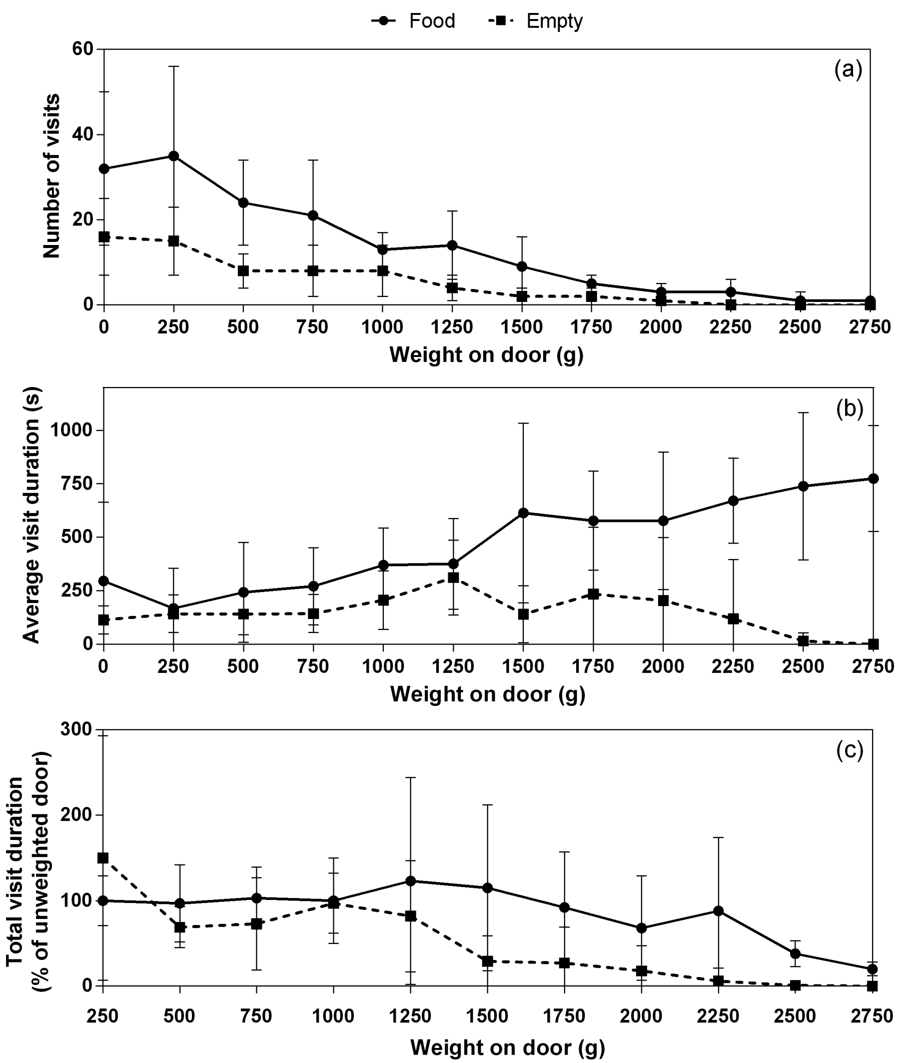

Figure 2.5 (a) Number of visits, (b) average visit duration and (c) total visit duration of ferrets to EC (containing food; solid line, and an empty room; dashed line), tested in set-up A_0 (N=12).

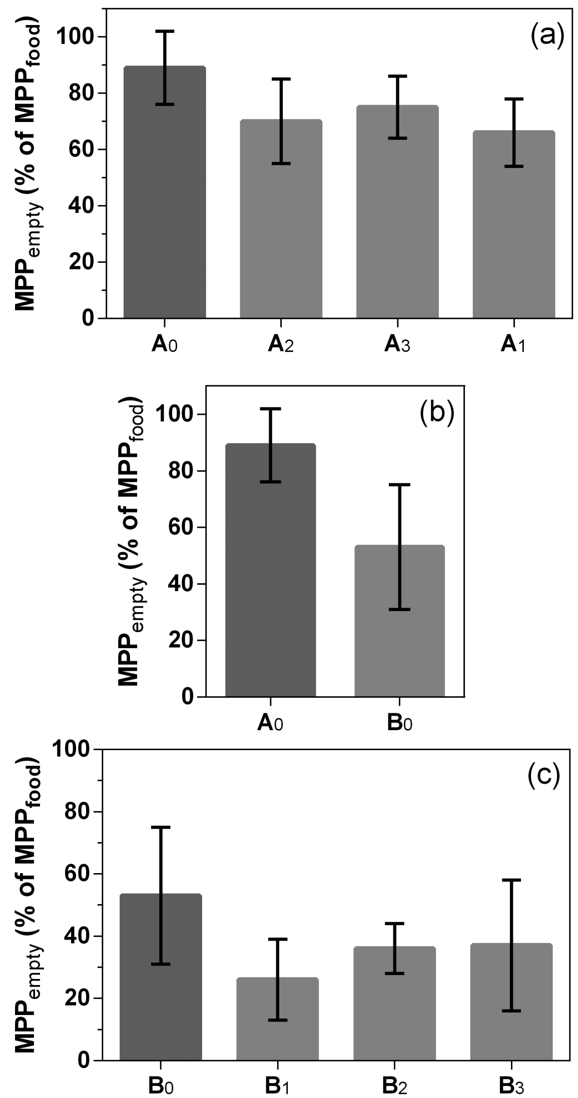

Figure 2.6 Bar chart demonstrating the ferrets" (a) MPPempty for design A_0-A_4, (b) for design A_0 versus B_0 and (c) for design B_0-B_3. A_0= standard set-up (N=12), A1= standard with an extra chamber (N=3), A_2= standard with a see-through divider (N=3), A_3= standard with a mesh tunnel around the perimeter (N=3), B_0=standard without a cat flap (free access to EC) (N=6), B_1= design B_0 with a manipulable sleeping bucket instead of a nest box (N=6), B2= design B_0 including a 2-way cat flap, B_3= design B2 with a tunnel surrounding the 2-way cat flap (N=3).

Study 2a - Testing the functional value of the empty chamber

Ferrets pushed 70±15% of MPP_{food} (60-87%; *N*=6) for the empty chamber in the design with the extra chamber (A_1) versus 75±11% (52-74%) in the design with a see-through divider (A_2) and 66±12% (69-87%) in the design with the mesh tunnel (A_3). None of these values were statistically different from the standard design from study 1 (A_0: 89±13%) (PA_0-A_1=0.267; PA_0-A_2=0.297; PA_0-A_3=0.071; Figure 2.6a).

Study 2b- Identifying potential reinforcing values of the door design

MPP_{empty} was lower when the ferrets did not have to push the weighted door to reach EC (B: 53±22%) compared to the situation in which they were required to work to gain access to EC (A_0: 89±13%) (Figure 2.6b). In the three alternative set-ups, the ferrets pushed less than in set-up B_0 (26±14% (B_1), 36±8% (B_2) and 37±21% (B_3) of MPP_{food}). Set-up B_1 (in which a manipulable sleeping bucket was provided) resulted in the lowest MPP_{empty} (Figure 2.6c).

Discussion

In this study, a two-chamber closed economy consumer demand set-up using a weighted door was tested for its suitability to determine the ferrets' motivation for specific resources by evaluating whether it fulfilled the following three prerequisites: 1) MPP_{food} should reflect MPC; 2) the task should be perceived as strenuous, as indicated by a decrease in number of visits and an increase in the duration of visits upon increasing the effort to gain access to EC; and 3) MPP_{empty} should be low and sufficiently distinctive from MPP_{food} to enable detecting differences in MPP between the different resources presented in EC.

The first prerequisite was most likely met, as the ferrets attempted to push the door above MPP_{food}, but were unable to do so. MPP_{food} of the ferrets (143±31% of bodyweight) was similar to the MPC of mink (147±43% of bodyweight, Cooper and Mason, 2001) and both Silver foxes (Hovland et al., 2006) and rabbits (Seaman et al., 2008) have shown to pay the highest price for food. Therefore, it is considered likely that the MPP_{food} found in this study reflects the true maximum push capacity of these ferrets, thereby enabling this value to be used as a benchmark for comparison of other resources and establishing their value.

The second prerequisite was also met, as the number of visits declined and the duration of visits to EC increased with increasing effort to gain access to food. A similar reorganisation of behaviour has been observed in other consumer demand studies (Cooper and Mason, 2000; Sherwin and Nicol, 1996). Based on these results, a weighted door seems a suitable task to use in a consumer demand study for ferrets.

In contrast to the first two prerequisites, the third prerequisite was not met during this study, as the ferrets were found to push almost 90% of their maximum push capacity (MPP_{food}) to gain access to an empty chamber. This is considerably higher than results found in other animal species. Mink, for example, were found to push 67% of their MPC (which was determined as the MPP for a water pool) for an empty chamber (Mason et al., 2001). Although the difference between MPP_{empty} and MPP_{food} was found to be statistically significant, the relative difference between the two was deemed too small to be able to assess and distinguish between the values of different resources. As a result, the third prerequisite was deemed not to have been met with the set-up used in this study.

To examine whether the third prerequisite could be met by adapting the design and to assess why the ferrets in our study pushed almost as hard for food as for an empty chamber, study 2 was performed during which we tested whether and which aspects of the design (e.g. functionality of the empty chamber [set-up A_1-A_3] or reinforcing value of elements of the door [set-up B_0-B_3]) could have resulted in the relative high MPP_{empty}.

None of the three set-ups of design A (with extra compartment, a see-through divider or a mesh tunnel) yielded a significantly lower MPP_{empty} compared to study 1. In other words, the hypothesised motivational backgrounds for pushing for access to the empty chamber could not be confirmed and we were not able to adapt the design in such a way that the functional value of EC was significantly decreased.

Comparison between set-up A_0 and B_0 (in which ferrets were allowed a choice between free access or pushing the weighted door to gain access to EC) showed that ferrets pushed less when they could freely access EC through a hole in the partition. MPP_{empty} decreased to 53±22% of MPC, which is comparable to that of mink (67%, Mason et al., 2001). This indicates that the empty room does have a value for the ferrets. Additionally, one or more elements of the door design may also have intrinsic reinforcing value for the ferrets, as they remained motivated to some extent to push the weighted door. Therefore, another study was performed to test the hypothesised effects of reinforcing elements of the weighted door on MPP_{empty} (e.g. the tunnel around the door, manipulating the door/cat flap).

In all of the set-ups of design B, the ferrets used the weighted door regularly. A possible explanation for this seemingly unnecessary pushing behaviour could be that ferrets need a high level of stimulation and that, even though it was actually designed as a task, the weighted door evokes object manipulation in a stimulus poor environment. Support for this explanation comes from the observation that pet ferrets show high levels of exploration and are focused on tactile stimulation (Fisher, 2006) and ferrets in barren cages were found to be distressed, showing signs of stereotypic behaviour (e.g. bar chewing and head swaying) and/or becoming lethargic (Cruden, 2011).

The small difference in MPP_{empty} when allowed free access to EC in set-up B_2 and B_3 indicates that neither the action of pushing the door nor the tunnel around the weighted door could account for the attractiveness of the door. The ability to manipulate the sleeping bucket in HC (set-up B_1) lowered MPP_{empty} the most. Thus, the ability to manipulate an object may be an attractive element of the door and the ferrets apparently valued the ability to manipulate a mounted door or cat flap lower than the ability to play with a manipulable item such as the plastic bucket. Pet ferrets are often found dragging their toys to secluded locations (Fisher, 2006). It is possible that moving the sleeping bucket (which could be moved) approximates this species-typical behaviour better than the ability to play with the mounted door or cat flap (which cannot be dragged around), thereby explaining the ferret's overall higher motivation to play with the bucket.

Although results from study 2 provide some insight into the underlying motivation for the ferrets to gain access to the empty compartment and use the door, results of this study did not reveal a solution to ensure that the third prerequisite would be met without compromising the set-up of the consumer demand study. Thus, alternative methods should be considered in order to reliably establish the value of resources for ferrets.

One option to overcome the issue of the high MPP_{empty} would be to use an alternative index for determining the value of the resources. For example, the price elasticity of demand index uses the rate of decline in consumption per unit increase in cost (Houston, 1997). This index makes it possible to distinguish resources for which the number and duration of visits remain relatively constant with increasing prices (such as food) from resources for which the number and duration of visits decline more rapidly (such as an empty chamber). Price elasticity of demand, however, has several disadvantages including a) the possibility for a resource to have more than one elasticity value (Dawkins, 1983; Houston, 1997); b) overestimation of the value for resources that quickly lead to satiation, as these are associated with low price elasticity (Kirkden et al., 2003; Kirkden and Pajor, 2006); c) underestimation of the value of resources that are consumed in great amounts in the initial phases (Kirkden et al., 2003; Kirkden and Pajor, 2006; Seaman et al., 2008; Warburton and Mason, 2003); d) difficulties to detect elasticity of demand in an experiment as the demand can be relatively equal over a large price range (Kirkden et al., 2003); e) difficulties to determine the correct size of the price unit rise, as it is essential that each unit rise in price is regarded as equal by the animal (Cooper, 2004); and f) the necessity to keep the reward size constant, which might devalue all-or-none goods (Mason et al., 1998). Alternative to the maximum price paid and price elasticity of demand index, the consumer surplus index can be used. This index measures the area under the demand curve (Mason et al., 2001; Seaman et al., 2008). Although this index is deemed as more valid than the price elasticity of demand index due to its omission of disadvantages a-c, it does use the same curve as the price elasticity of demand index, thereby sharing disadvantages d-f (Houston, 1997; Kirkden et al., 2003). These disadvantages render both indices unsuitable for our goal and are therefore not considered a viable solution.

The second option would be to use a set-up with more than two chambers. Although such set-ups have the disadvantage that they require the animals to divide their time and energy between multiple resources (thereby potentially yielding a lower MPP for less preferred resources), such a set-up may provide the ferrets with a larger choice in activities thereby potentially lowering the ferrets' motivation to push the weighted door for the empty chamber. Thus, a three- or multi-chamber set-up (e.g. (Hovland et al., 2006; Mason et al., 2001; Seaman et al., 2008) may be suitable to overcome the issues regarding the high motivation to push for the empty door, thereby allowing us to test the ferrets' motivation for resources and rank these according to their value. Further research is, however, necessary to determine whether this indeed is a feasible and suitable alternative to test the value of resources for ferrets.

Conclusion

The current study shows that the closed-economy two-chamber set-up is not suitable for testing the ferrets" motivation for different resources as MPP_{empty} and MPP_{food} were relatively similar, thereby leaving little room for distinction between the value of different resources. The ferrets showed high motivation to push a weighted door to gain access to an empty room, even when they could freely access this room through a two-way cat flap or a hole. In the latter situation, motivation to push the weighted door did decrease, however, indicating that motivation to push for the empty room cannot solely be attributed to reinforcing properties of the door design, but most likely also to functional aspects of the empty room. It appears most likely that the ferrets' high MPP_{empty} originates from a high desire for (tactile) exploration, which renders this an important factor to consider when designing a consumer demand study for ferrets. To determine the value of enrichments for ferrets, other set-ups, such as a three- or multi-chamber set-up, should be considered.

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Chapter 3

Are all motivation tests the same? The effect of two adaptations to a 3-chamber consumer demand study in ferrets

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Abstract

Ferrets are increasingly used in infectious disease studies, particularly in influenza research. Which specific housing conditions and environmental enrichment are of particular importance for ferrets have not been part of a systematic evaluation. The motivation ferrets showed to reach different enrichments was assessed in multiple consumer demand study set-ups. To address the question whether these consumer demand set-ups give similar results, we assessed the effects of two ways of offering enrichments concurrently instead of consecutively. Six ovariectomised female ferrets were successively tested in a 7-chamber (7Ch), 3-chamber (3Ch) and 3-chamber "all-but-one" (ABO) set-up. We compared the maximum price paid, visit number, visit duration and interaction time with the enrichments in the 3Ch versus the 7Ch and ABO set-up, respectively. Compared to the 3Ch set-up, the ferrets in the ABO and 7Ch set-up showed a lower motivation to access, paid less and shorter visits to and interacted less with the enrichments. In the 7Ch, the ferrets especially showed a lower motivation for the less preferred enrichments and the empty chamber. These findings indicate that testing all the enrichments concurrently in the 7Ch set-up forced the ferrets to make more economic decisions, thereby providing more valuable information on how different enrichments are valued relative to each other. Adding preferred enrichment items to the home chamber as was done in the ABO set-up, might have reduced the motivation to access or look for additional enrichment items. However, this set-up might not have a closed economy, making the ABO set-up unsuitable. Based on these findings, we advise testing all the enrichment categories concurrently instead of consecutively and keeping the number of items in the home cage to a minimum when performing a consumer demand study, as this appears the most optimal set-up to determine motivational priorities for resources in ferrets.

Introduction

Consumer demand studies are commonly used and regarded as a valid method to assess an animals' motivation for different types of environmental enrichment. This technique has been derived from human micro-economics and has been proposed as a tool to identify behavioural needs (Dawkins, 1983) as it can aid in understanding what animals "want" (i.e. are motivated to access, Berridge and Robinson, 2003). A consumer demand study uses an operant conditioning task in which the "price" (e.g. number of responses) to gain access to a resource is increased or the available "income" (e.g. available time to respond) is decreased (Cooper and Mason, 2001; Dawkins, 1990). Ultimately, the "price" an animal is willing to pay for access to an enrichment indicates the motivation an animal has to reach it, and how important it is for that animal to have access to that specific enrichment (Kirkden and Pajor, 2006). Consumer demand studies are therefore often used to substantiate which changes to an animals' housing should be made to improve their welfare (e.g. Mason et al., 2001; Seaman et al., 2008).

A 2-chamber consumer demand study was designed with the aim to identify enrichments that could be provided to ferrets to refine studies using these animals. This set-up proved to be unsuitable to test ferrets as they would push to their maximum ability to reach an empty chamber (Reijgwart et al., 2015). This study further indicated that the items in the home chamber influenced the ferrets' motivation to enter an empty chamber (Reijgwart et al., 2015). The subsequent search for a suitable consumer demand set-up for ferrets resulted in two possible alternative set-ups: a 3-chamber and a multi-chamber set-up (e.g. Hovland et al., 2006; Mason et al., 2001; Seaman et al., 2008). It was not clear, however, whether these set-ups would give similar results when applied in ferrets.

In the literature, there are many issues discussed regarding how a consumer demand study can optimally be designed and interpreted. First, an animal should be tested in a closed economy (i.e. only providing access to the enrichment during the experiment), as testing in an open economy (i.e. giving the animals access to the enrichments outside of the test environment) might lower the motivation for enrichments in the study (Ladewig et al., 2002; Mason et al., 1998). Secondly, a naturalistic operant task (such as a push door or a narrow gap) should be used, as it requires little training and is least prone to operant-reinforcer bias (Cooper, 2004; Kirkden et al., 2003; Mason et al., 1998). Thirdly, the MPP or reservation price index, the price at which the animal is no longer willing to perform the task, should be used to calculate the animals' motivation as it is considered the most appropriate for the assessment of unsubstitutable, discrete and nondivisable resources such as enrichments (Cooper, 2004; Cooper and Mason, 2001; Jensen and Pedersen, 2008; Mason et al., 1998). Fourthly, to be able to interpret the price the animals pay for resources correctly, it is vital to determine the negative control (i.e. the minimum price the animal will pay) and positive control (i.e. the maximum price the animal is able to pay) (Dawkins, 1983). As a negative control, the animals' motivation for an empty chamber is often measured in order to quantify how motivated the animal is to reach additional space and/or to perform the task itself. As a positive control, the animals' maximum push capacity (MPC), i.e. the maximum effort that the animal is able to exert to accomplish the task, is established. This parameter can be used as a yardstick to relate the findings to. The animals' motivation for food or water are often used as MPC as these are essential needs, for which the animal will presumably perform to its maximum ability (Dawkins, 1983; Matthews and Ladewig, 1994). These minimum and maximum motivation levels can subsequently be used to determine where on the motivational scale the value of different enrichments lie.

To the authors' knowledge, it has not yet been studied whether offering enrichments concurrently or consecutively affects the results obtained in consumer demand studies. Therefore, we compared the results of a 3-chamber (3Ch) consumer demand set-up with a 7-chamber (7Ch) set-up (comparison 1). Additionally, we compared the results of the 3Ch set-up with the results of a 3-chamber "all-but-one" (ABO) set-up (comparison 2) to assess the effect of adding freely available enrichment items to the home chamber. In the 7Ch set-up, the ferrets are expected to make more economic choices than in the 3Ch set-up, in view of the limited time (and energy) available to work for and visit the concurrently provided enrichment items. In the ABO set-up, the ferrets are expected to be less motivated to access additional enrichment items than in the 3Ch set-up, as there are highly valued items freely available in the home chamber already fulfilling some motivations. We therefore predict that offering the enrichments concurrently in the 7Ch set-up and adding enrichment items to the home chamber in the ABO set-up will result in a lower MPP, visit number, visit duration and interaction time with the enrichments than in the 3Ch set-up.

Animals, materials and methods

Ethical note

This study was ethically approved by the Animal Care and Use Committee of the Institute for Translational Vaccinology (Intravacc, DEC 201400137). As this was a study into the enrichment priorities of ferrets, there were very little welfare implications of this study. The ferrets were housed solitary when they were being tested, which might have caused some distress. However, as it is not yet determined whether ferrets are a truly social species (i.e. whether they suffer when housed solitary), this might not have been the case. In the 7-chamber set-up, the ferrets could always push a door to gain access to conspecifics.

Animals, housing and husbandry

For the study, six female surgically neutered (ovariectomised) ferrets were used that were obtained from Schimmel B.V., weighed 1035±131 grams and were approximately 1 year old at the start of the experiment. The ferrets were ovariectomised to prevent the ferrets from going intro oestrus, which could affect the ferrets' behaviour and might lead to anaemia, weight loss, alopecia and even bone marrow depression if she is not bred (Fox et al., 2014). The ferrets were housed under the conditions as described in Reijgwart et al. (2016), namely indoors, between 19°C and 23°C, in a 8:16 h light:dark schedule with auditory stimulation. The ferrets were group housed between experiments in floor pens (163x94 cm) with sawdust, a flexible plastic bucket and *ad libitum* water (from a nipple) and food (Hope Farms® ferret balance pellets in a stoneware bowl). The ferrets' health and overall condition were monitored prior to and throughout the study.

Enrichments

Enrichments from six different enrichment categories were tested, i.e. sleeping enrichment, water enrichment, foraging enrichment, social enrichment, tunnels and balls; Table 3.1). These enrichment categories were chosen to accommodate behaviours seen in feral ferrets, where each category represents a different behavioural motivation (see Reijgwart et al. 2016). For each enrichment category two or three items with different characteristics were offered in one enrichment chamber to increase the chance of testing the motivation for the preferred item within the category. These items were chosen based on variability within a category and practicality in a laboratory setting. Additionally, an empty chamber was tested to control for the value of extra space, patrolling and the rewarding properties of the task itself (i.e. negative control). Food was used as a resource to serve as a positive control, i.e. to determine the MPC of the ferrets (see Reijgwart et al., 2015).

Table 3.1 Overview of the enrichments in each enrichment category. Numbers indicate the supplier of the enrichment. 1 = van der Neut, Groenekan, The Netherlands, 2 = onlinedierenspeciaalzaak.com, 3 = zooplus.nl, 4 = Tecnilab-BMI, Someren, The Netherlands, 5 = Schimmel BV, The Netherlands.

Enrichment category	Enrichments	Specifications
Sleeping	Bucket	Flexible plastic bucket[1]
	Savic Cocoon	Savic Cocoon®, 34.5 x 26.5 x 16.0 cm[1]
	Hammock	Adori® hammock, 50 x 45 cm[1]
Water	Large bowl	Marchioro® kitten litterbox filled with water, 26 x 36 x 9 cm[1]
	Small bowl	Adori® stoneware food bowl filled with water, ø18 cm, 5 cm high[1]
Foraging	Foraging ball	Happy Pet® tumble 'n treat, ø6 cm[1]
	Tumbler	Nina Ottoson Cat pyramid®, 9.5 cm high[2]
Social	Conspecifics	Two familiar female ferrets[5]
Tunnels	Rigid tunnel	Ferplast® tunnel FPI 4840, ø10.5 cm, length 29 cm[1]
	Flexible tunnel	Zooplus® 260697.0, ø10 cm, length 19-75 cm[3]
Balls	Ball with bell	Cat play ball[1]
	Golf ball	ø4 cm
	Ferret ball	Ferret ball, ø25 cm, 4 holes ø10.2 cm[4]

Experimental housing

During the experiment, the ferrets were subsequently individually housed in three types of closed economy consumer demand studies. These were all closed-economy set-ups consisting of one home chamber, an empty control chamber and one or six enrichment chambers for the 3- or 7-chamber set-up, respectively.

General

Between the chambers there was a 70 cm high, 6 mm thick phenolic faced plywood divider. The divider was equipped with a mesh window through which the ferrets could see whether and which items were present in the chamber. This was done to ensure that the ferrets were at all times aware of the items present in each enrichment chamber rather than them having to learn and memorise the locations of each of these, which might have been more difficult in the 7-chamber set-up than in the 3-chamber set-up. In addition, a non-transparent one-way cat flap and a one-way horizontal swinging weighted door (Tecnilab-BMI, Someren, The Netherlands) were mounted in the divider. Under the doors, a wire mesh strip was mounted to provide a traction surface for the ferrets to facilitate them to apply force to the doors. The doors allowed the ferrets to move from the home chamber/corridor to the empty control chamber and the enrichment chamber(s), whereas the one-way unweighted cat flap could be used by the ferrets to return to the home chamber/corridor.

Operant task

The naturalistic operant task chosen for this study included pushing open a weighted door. Opening the unweighted door required the ferrets to exert a force of 200 g. Upon adding weights to the door mechanisms, the effort needed to open the doors could be gradually increased. The mechanism was designed in such a way that 50% of the added weight was transferred to the actual force needed for the ferret to open the door. Thus, a weight of 500 g translated to a push force of 450 g (200+50% of 500 g), a weight of 1000 g to a push force of 700 g (200 +50% of 1000 g), etc. For all set-ups, the weight added to the mechanism was increased daily with 500 g/day up to 3000 g (i.e. a push force of 1700 g), which was established to be just above the maximum push capacity of the ferrets (1450±144 g, Reijgwart et al., 2015). Ferrets were considered to have reached their maximum price paid (MPP, i.e. the maximum weight the animal was prepared to push) for a particular enrichment category or the empty chamber when they did not visit the chamber of that category for 24 hours. MPP was recorded as the last weight successfully pushed to gain access to the chamber.

Acclimatisation and training

The ferrets were allowed to acclimatise to the experimental housing for seven days prior to commencing each experiment. During this period, all doors were closed but unweighted. The ferrets were trained in using the weighted door using food as a reinforcer in a previous experiment (Reijgwart et al., 2015) and no additional training was required.

Study design

Ferrets were tested in three different set-ups, i.e. a 7-chamber (7Ch) set-up, a 3-chamber (3Ch) set-up and a 3-chamber all-but-one (ABO) set-up. The set-ups were kept as similar as possible (except for the simultaneous provision of enrichment). For logistical reasons, the order in which the set-ups were tested was the same for each ferret.

Experimental set-up 1: 7-chamber set-up (comparison 1)

The 7-chamber set-up was used to determine the motivation of ferrets for enrichments that were concurrently presented in adjacent chambers. This set-up consisted of one home corridor (692 cm long, 54 cm wide) connected to seven phenolic faced plywood floor pens (ground surface 107×94 cm): one empty control chamber (CC) and six enrichment chambers (ECs). The home corridor was equipped with *ad libitum* food (Hope Farms® ferret balance pellets) in a bowl and water from a nipple. In each of the six ECs, items from one of the enrichment categories (i.e. sleeping enrichment, water enrichment, foraging toys, social contact, tunnels and balls) were placed. The location of CC and the different ECs were randomised between ferrets. (Figure 3.1a, Reijgwart et al., 2016)

Experimental set-up 2: 3-chamber set-up (comparison 1 and 2)

The 3-chamber set-up was used to determine the motivation of ferrets for enrichments that were consecutively presented. This set-up was used as a comparison for both the 7-chamber and the ABO set-up and consisted of one home chamber, one empty control chamber and one enrichment chamber that each measured 95x80 cm. The home chamber had *ad libitum* food (Hope Farms® ferret balance pellets) in a bowl, water from a nipple and a sleeping bucket (except when testing the sleeping enrichment). Items from one of the enrichment categories that were also tested in the 7Ch (i.e. foraging toys, social contact, sleeping, water, tunnels, and balls) were placed in EC in random order. The location of EC and CC and the order in which the enrichment categories were tested were randomised between enrichment categories and between ferrets. (Figure 3.1b)

<u>*Experimental set-up 3: all-but-one (ABO) set-up (comparison 2)*</u>

The ABO set-up was used to determine the motivation of ferrets for enrichments when there were also freely available enrichments in the home chamber. Based on the findings of the 7Ch set-up, the enrichments from three enrichment categories for which the ferrets were most motivated for and interacted most with were selected to be tested in comparison 2 (sleeping enrichment, water enrichment and foraging enrichment). The ABO set-up was identical to the 3Ch set-up described above, with the only difference being that the home chamber was not only equipped with food, water and a sleeping bucket (except when testing the sleeping enrichment), but also contained enrichments from two of three enrichment categories (sleeping, water bowls, foraging,). Items from the third enrichment category (not present in the home chamber) were placed in the EC. Similar to the 3Ch set-up, the location of EC and CC and the order in which the enrichment categories were tested were randomised between enrichment categories and between ferrets. (Figure 3.1c)

Measurements

Four different parameters were recorded in the three consumer demand set-ups: maximum price paid (MPP), number and duration of visits to the enrichment chamber(s) and interaction with enrichments. The MPP was defined as the maximum weight the ferret pushed to gain access to a resource (in grams). The total visit number, visit duration and enrichment interaction time for each weight (i.e. per 24 h) were calculated from the videotaped behaviour in the enriched chambers. For social contact and the empty control chamber, only MPP, visit number and duration were noted. When a ferret made no visits to a resource at a given weight, visit number, visit duration and enrichment interaction time were noted as missing values.

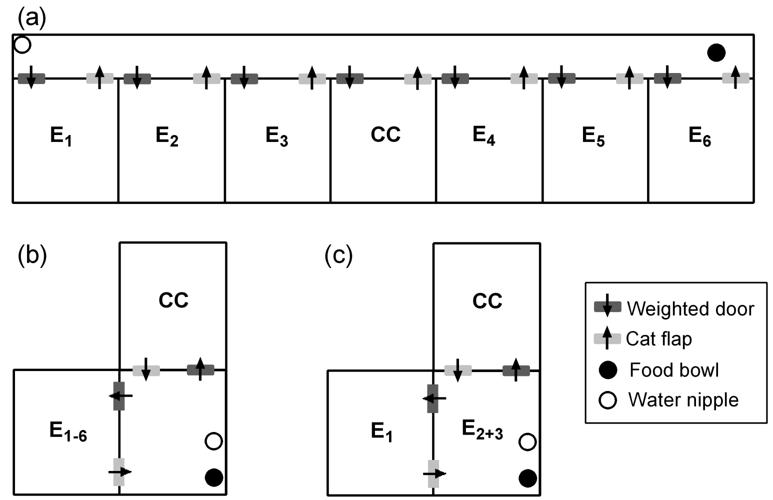

Figure 3.1 Design of the (a) 7-chamber, (b) 3-chamber and (c) all-but one consumer demand set-up. E1-6=enrichment 1-6; CC=empty control chamber.

Statistical analysis

Analyses were performed using IBM SPSS software (version 24.0). Data were expressed as mean ± SD and duration of visits was log transformed to ensure normality of data distribution. The probability level accepted for statistical significance (P<0.05) was corrected with the False Discovery Rate (FDR, Benjamini et al., 2001) to guard against type I errors in case of multiple comparisons.

Differences in MPP, visit number, visit duration and enrichment interaction between the 3Ch vs the 7Ch set-up and the 3Ch versus the ABO set-up were analysed with a Linear Mixed Model (LMM) using a compound symmetry structure, with ferrets as subject and set-up, enrichment, order and weight (not for MPP) as repeated factors. In the model, MPP, visit number, visit duration, and enrichment interaction were entered as a dependent variable, set-up, enrichment, set-up*enrichment and weight (not for MPP) as fixed factor, and order of enrichments (nested within ferret) and ferret as random factors. Sum contrasts were used for the fixed factors. Post hoc comparisons were made for the set-up*enrichment effects when there was an overall interaction effect.

Results

Comparison 1: 3-chamber versus 7-chamber set-up

Linear Mixed Model analysis revealed that the 7Ch set-up yielded lower MPPs than the 3Ch set-up ($F_{1,\ 19.433}$=36.210, P<0.001). There was also an interaction effect between set-up and enrichment on MPP ($F_{6,\ 98.375}$=4.705, P<0.001), indicating that the difference in MPP was most pronounced for foraging enrichment, balls and empty (P=0.010, P<0.001 and P<0.001, respectively, Figure 3.2).

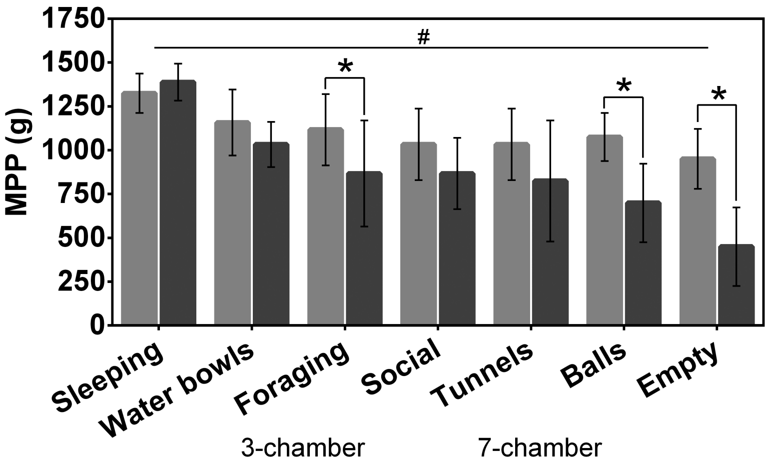

Figure 3.2 The maximum price the ferrets paid for the different enrichment chambers - with sleeping enrichment, water enrichment, foraging enrichment, social enrichment, tunnels, balls and an empty chamber - in the 3-chamber versus the 7-chamber set-up of the consumer demand study (mean±SD; N=6, N=6; # indicates an overall difference between the set-ups with P<0.014, * indicates a significant interaction effect between set-up and enrichment with P<0.014).

The ferrets paid less visits to EC in the 7Ch than in the 3Ch set-up (LMM: $F_{1,20.369}$=128.633, P<0.001). There were no interaction effects between set-up and enrichment on visit number (LMM: $F_{6,\ 394,468}$=0.843, P=0.537, Figure 3.3).

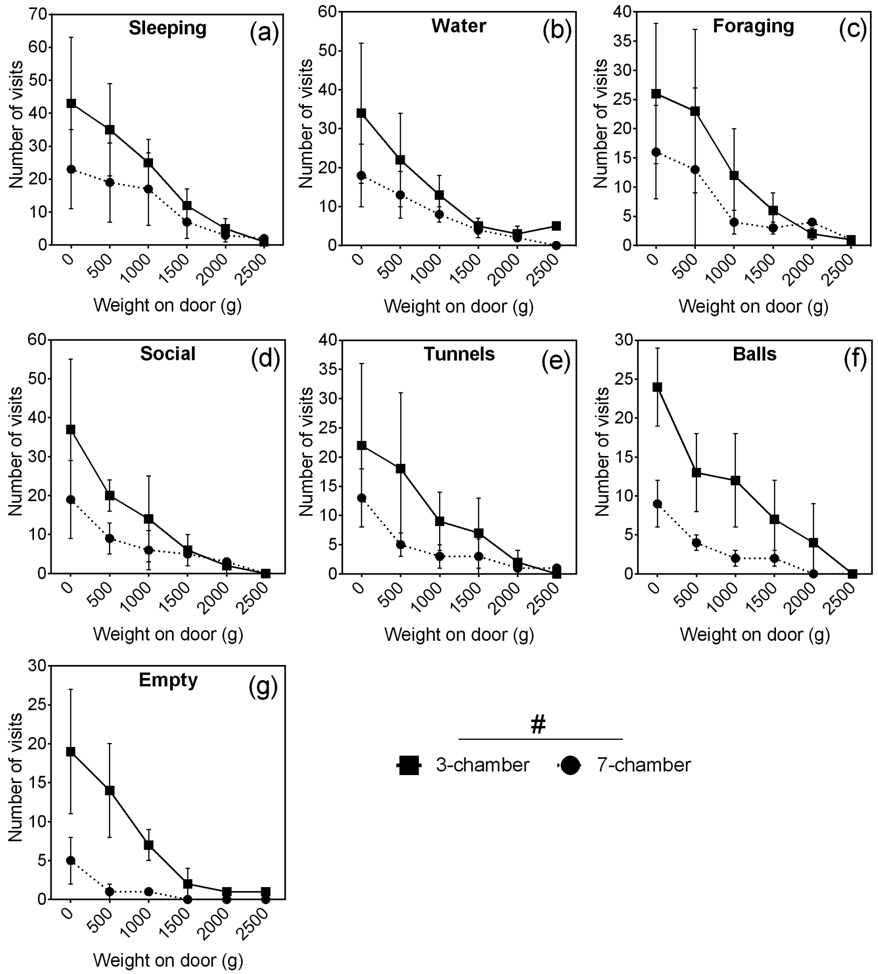

Figure 3.3 The number of visits the ferrets made to the different enrichment chambers - with (a) sleeping enrichment, (b) water enrichment, (c) foraging enrichment, (d) social enrichment, (e) tunnels, (f) balls and (g) an empty chamber - in the 3-chamber versus the 7-chamber set-up of the consumer demand study (mean±SD; N=6, N=6; # indicates an overall difference between the set-ups with P<0.014).

The ferrets paid shorter visits to the enrichment chambers in the 7Ch than in the 3Ch set-up (LMM: $F_{1,21.765}$=30.670, P<0.001). There was also an interaction effect between set-up and enrichment on visit duration (LMM: $F_{6,389.265}$=6.782, P=0.005), indicating that the difference in visit duration was most pronounced for balls and empty (P=0.001 and P<0.001, respectively, Figure 3.4).

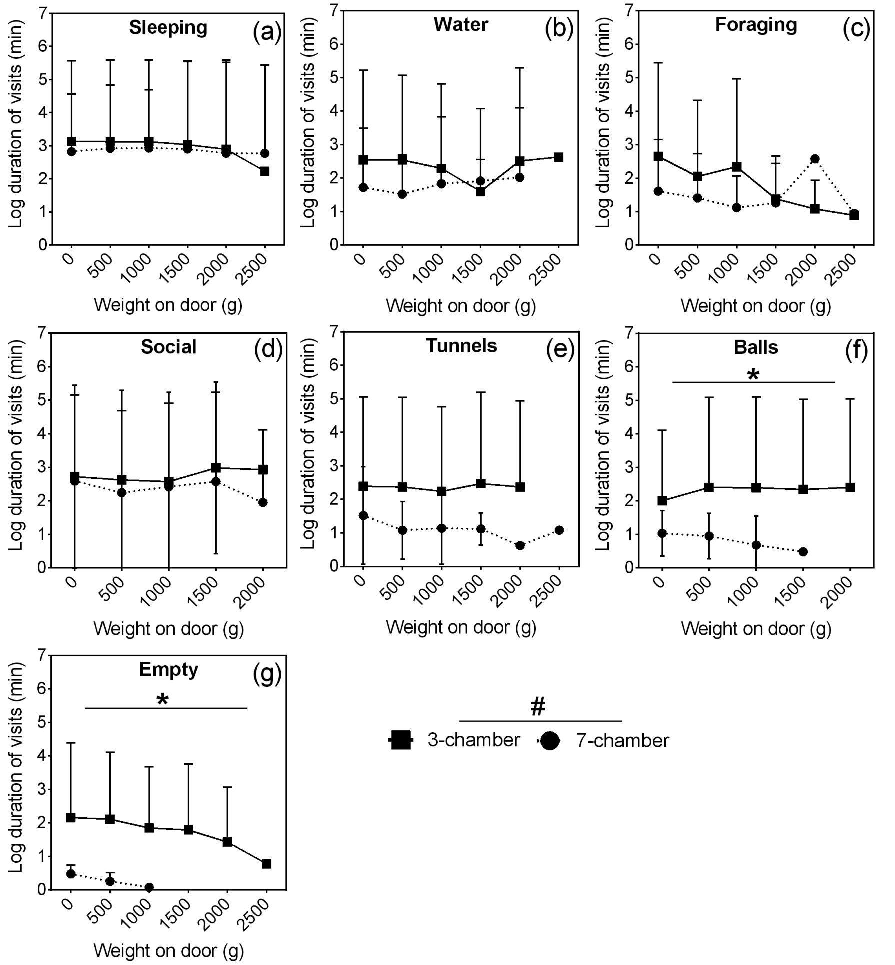

Figure 3.4 The log transformed duration of visits the ferrets made to the different enrichment chambers - with (a) sleeping enrichment, (b) water enrichment, (c) foraging enrichment, (d) social enrichment, (e) tunnels, (f) balls and (g) an empty chamber - in the 3-chamber versus the 7-chamber set-up of the consumer demand study (mean±SD; N=6, N=6; # indicates an overall difference between the set-ups with P<0.014, * indicates a significant interaction effect between set-up and enrichment with P<0.014).

The ferrets interacted shorter with the enrichments in the 7Ch than in the 3Ch set-up (LMM: $F_{1,1907.918}$=128.566, P<0.001). There was also an interaction effect between set-up and enrichment for interaction time (LMM: $F_{11,1809.668}$=8.030, P<0.001), indicating that the difference in interaction was most pronounced for the Savic Cocoon®, small water bowl, tumbler, flexible and rigid tunnel, ferret ball, golf ball and ball with bell (P<0.001 for all, Figure 3.5).

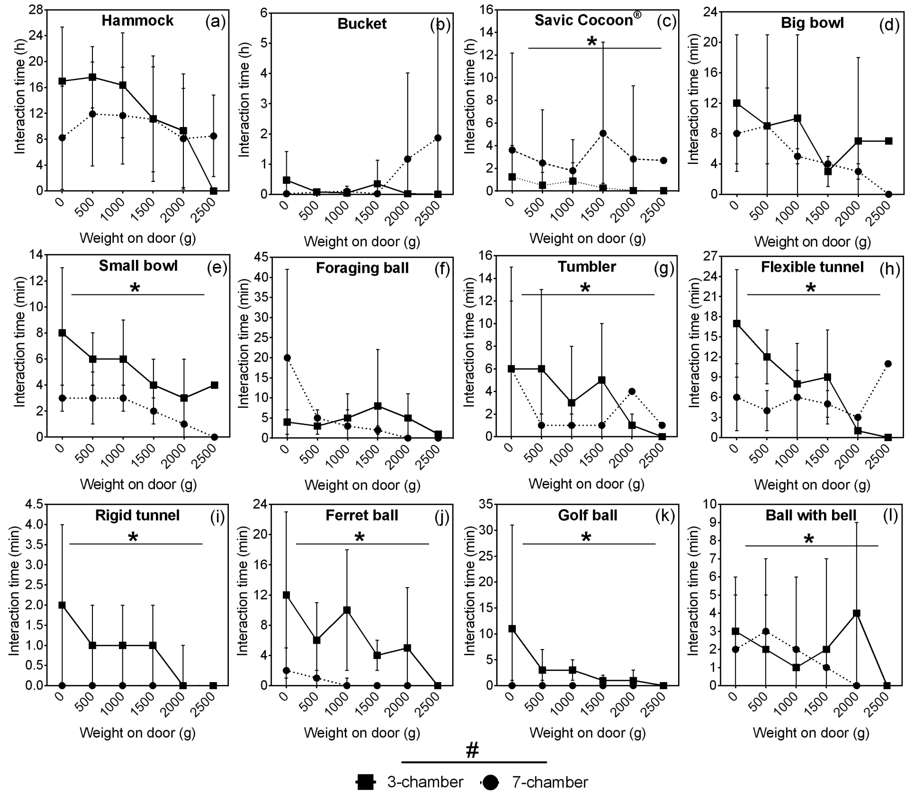

Figure 3.5 The time the ferrets interacted with the enrichments - a (a) hammock, (b) bucket, (c) Savic Cocoon®, (d) big water bowl, (e) small water bowl, (f) foraging ball, (g) tumbler, (h) flexible tunnel, (i) rigid tunnel, (j) ferret ball, (k) golf ball, and (l) ball with bell - in the 3-chamber versus the 7-chamber set-up of the consumer demand study (mean±SD; N=6, N=6; # indicates an overall difference between the set-ups with P<0.014, * indicates a significant interaction effect between set-up and enrichment with P<0.014).

Comparison 2: 3-chamber versus ABO set-up

Based on the findings of the 7Ch, three enrichment items for which the ferrets were most motivated were selected to be tested in comparison 2. During the ABO set-up, one of the ferrets learned to circumvent pushing the door, therefore her results were removed from the statistical analysis.

Linear Mixed Model analysis revealed that overall, the MPPs were lower in the ABO than in the 3Ch set-up (LMM: $F_{1,78.592}$=11.235, P=0.001). There were no interaction effects between set-up and enrichment on MPP ($F_{3,54.291}$=0.166, P=0.919, Figure 3.6).

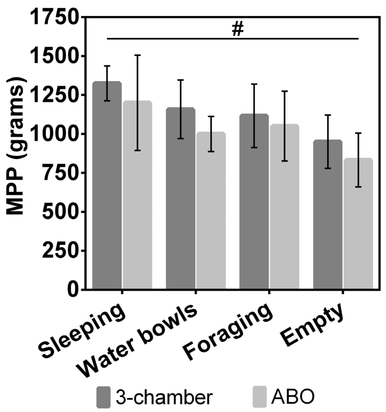

Figure 3.6 The maximum price the ferrets paid for the different enrichment chambers - with sleeping enrichment, water enrichment, foraging enrichment, and an empty chamber - in the 3-chamber versus the ABO set-up of the consumer demand study (mean±SD; N=6, N=5; # indicates an overall difference between the set-ups with P<0.014).

The visit numbers were lower in the ABO set-up than in the 3Ch set-up (LMM: $F_{1,295.370}$=9.867, P=0.002). There were no interaction effects on visit number between the 3Ch and ABO set-up (LMM: $F_{3,290.371}$=2.222, P=0.086, Figure 3.7).

Overall, the visit durations were lower in the ABO than in the 3Ch set-up (LMM: $F_{1,83.470}$=10.593, P=0.002). There were no interaction effects between set-up and enrichment on visit duration (LMM: $F_{3,112.805}$=2.730, P=0.047, Figure 3.8).

Overall, the ferrets interacted less with the enrichments in the ABO than in the 3Ch set-up (LMM: $F_{1,318.277}$=10.549, P=0.001). There were no interaction effects between set-up and enrichment on enrichment interaction (LMM: $F_{6,319.398}$=0.397, P=0.881, Figure 3.9).

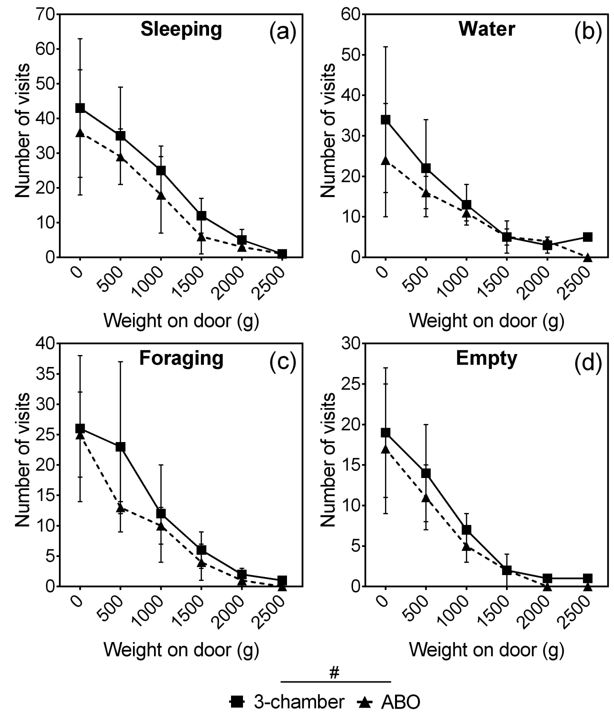

Figure 3.7 The number of visits the ferrets made to the different enrichment chambers - with (a) sleeping enrichment, (b) water enrichment, (c) foraging enrichment, and (d) an empty chamber - in the 3-chamber versus the ABO set-up of the consumer demand study (mean±SD; N=6, N=5; # indicates an overall difference between the set-ups with P<0.014).

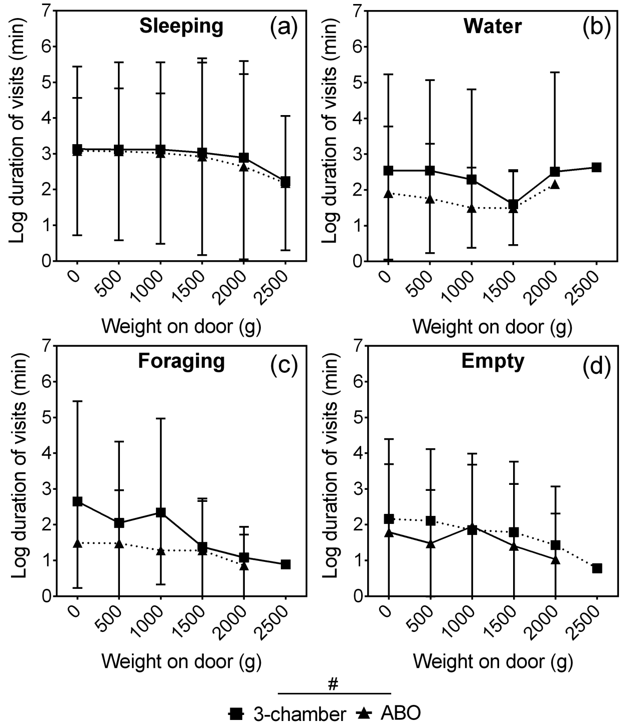

Figure 3.8 The duration of the visits the ferrets made to the different enrichment chambers - with (a) sleeping enrichment, (b) water enrichment, (c) foraging enrichment, and (d) an empty chamber - in the 3-chamber versus the ABO set-up of the consumer demand study (mean±SD; *N*=6, *N*=5; # indicates an overall difference between the set-ups with *P*<0.014).

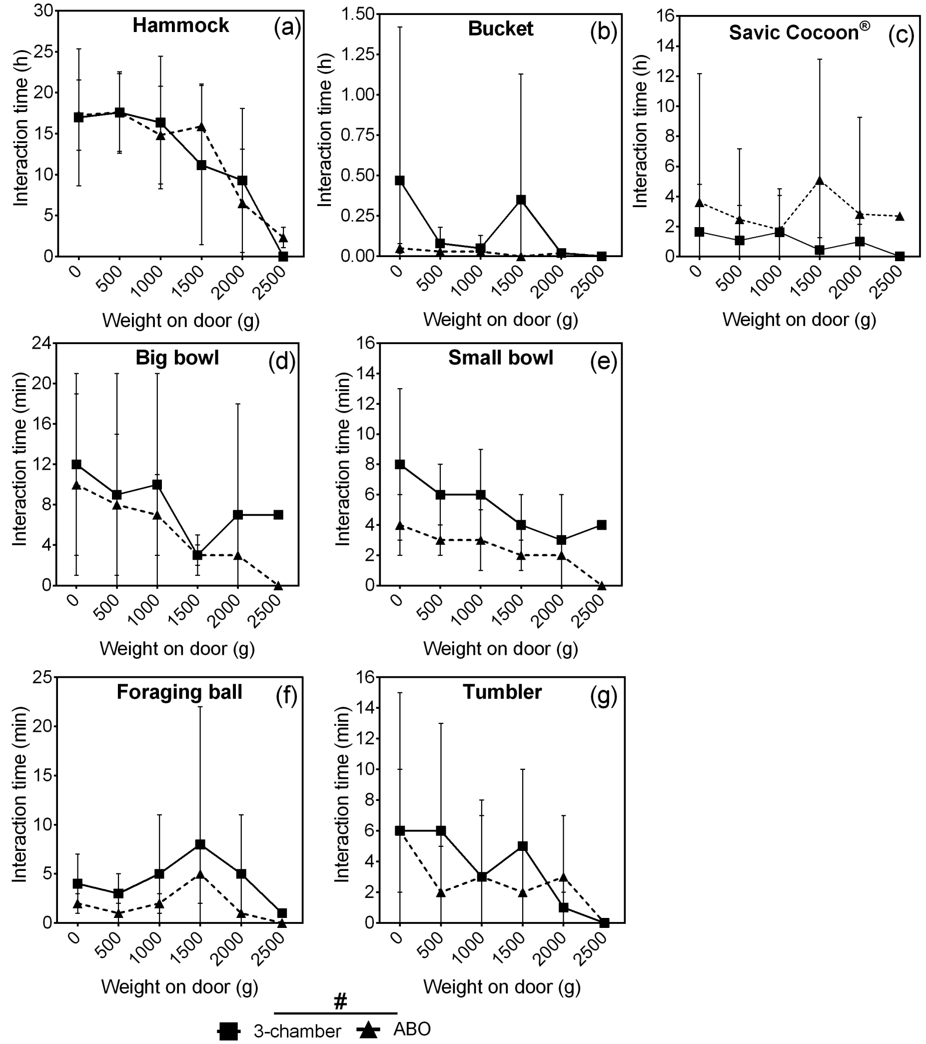

Figure 3.9 The time the ferrets interacted with the enrichments - a (a) hammock, (b) bucket, (c) Savic Cocoon®, (d) big water bowl, (e) small water bowl, (f) foraging ball, and (g) tumbler - in the 3-chamber versus the ABO set-up of the consumer demand study (mean±SD; *N*=6, *N*=5; # indicates an overall difference between the set-ups with *P*<0.014).

Discussion

This study investigated whether testing enrichments concurrently instead of consecutively (comparison 1) and providing freely available enrichment item in the home chamber (comparison 2) influenced the results of a consumer demand study.

Comparison 1: 3-chamber versus 7-chamber set-up

As expected, the ferrets showed a lower motivation to enter and interacted less with the enrichments and paid less and shorter visits to the enrichment chambers when they were tested concurrently compared to consecutively, especially for the less preferred enrichments (tunnels and balls) and the empty chamber.

There are three possible explanations for these findings. First, in the 7Ch set-up, the ferrets had a higher number of enrichment chambers to choose from than in the 3Ch set-up. In this way, the 7Ch set-up limited the available time for each chamber/enrichment category. These time constraints have been shown to differentially affect time spent on activities in dairy cows (Munksgaard et al., 2005). Likewise, the time constraints in the 7-chamber set-up might have forced the ferrets to make more economic choices. Essentially both the animals' income (i.e. time per enrichment) and the price of the enrichments (i.e. weight of the door) were manipulated in the 7Ch set-up, whereas only the latter was increased in the 3Ch set-up. This might have caused the ferrets to reduce "luxury behaviour" such as interacting with a ball and exploring the empty chamber in the 7Ch set-up to defend the time and energy needed for essential behaviours (as suggested by Houston and McFarland, 1980).

Alternatively, it might be possible that the provision of multiple choices in itself had enriching capacities as suggested by (Hutchinson, 2005). For example, giant pandas and polar bears that were given the choice to access an (less enriched) alternative space showed signs of improved welfare (Owen et al., 2005; Ross, 2006). The increased choices offered in the 7-chamber might therefore have resulted in a loss of interest and lower motivation for less valued enrichments and the empty chamber. This might especially be true for enrichment options that are – in part – substitutes and allow for performance of similar behaviours (Kirkden and Pajor, 2006). For example, the foraging ball provided the ferrets with similar opportunities as regular balls, next to the opportunity to forage. Alternatively, choice aversion, i.e. a decline in choice behaviour due to too much choice, might explain the lower motivation in the 7-chamber set-up (Hutchinson, 2005). However, this has not likely occurred, as in that case interest and motivation for all enrichment categories would have declined equally and to a greater extent.

Finally, it is possible that the ferrets experienced too little stimulation in the 3-chamber set-up compared to the more complex 7-chamber set-up. Similar to rats that explored more when housed in unenriched cages (Abou-Ismail et al., 2010), the impoverished 3-chamber set-up might have resulted in more novelty-seeking visits to the empty control chamber compared to the 7-chamber set-up (Abou-Ismail et al., 2010).

These explanations all favour the 7Ch set-up as the most useful design to assess the value of enrichment items have for ferrets, as we want the animals to make economic choices, show a low motivation for the least valued enrichments and prevent novelty-seeking in a consumer demand study.

However, it should be taken into account that in the 3-chamber set-up, the location of the enrichment chamber and empty control chamber were randomised between enrichment categories, which was not possible in the 7-chamber set-up as all the enrichment chambers and the empty control chamber remained at the same location during the whole test per ferret and could only be randomised between ferrets. In the 3-chamber set-up, this might have made the environment in both chambers unpredictable, which has been suggested to increase exploration (with the aim to learn about the environment and thereby reducing unpredictability, Doya, 2008; Inglis, 2000). The more unpredictable 3-chamber set-up might have therefore motivated the ferrets to push harder for and make more and longer visits to both chambers and interact more with the enrichments in the chambers. In this case, the results would not reflect the motivation the ferrets have to access the items in the chamber, but would reflect a more general motivation to explore.

Comparison 2: 3-chamber versus "all-but-one" set-up

The ferrets, as expected, showed a lower motivation to access and interact with the enrichments and paid less and shorter visits to the enrichment chambers when enrichments were provided in the home chamber compared to a home chamber with only bare necessities.

One explanation for these findings is that, in the ABO set-up, the ferrets might have experienced more stimulation and/or satisfaction of their behavioural needs by the preferred enrichments that were freely available in the home chamber. In rats, the provision of enrichment in their home cage lowered exploration behaviour compared to non-enriched rats (Abou-Ismail et al., 2010). The lower motivation for both the enrichment and the empty chamber in the ABO set-up, might be a similar effect of the provided enrichment in the home chamber lowering the motivation to explore the environment in search of novelty.

Additionally, in the 3Ch there was no reason to return to the home chamber except for essential food and water intake, while there was in the ABO-set-up. These decreased opportunities for transition between behaviours might have resulted in a higher visit duration in the 3Ch set-up.

All enrichment categories consisted of alternate (more preferred) ways of presenting resources (i.e. water form a bowl instead of the nipple; food from a foraging ball instead of a bowl; and the opportunity to sleep in a hammock instead of the sawdust) that were already available in the home chamber in both set-ups. Ferrets need to eat and drink multiple times per day, as they have a short intestinal tract (i.e. their gastrointestinal transit time is approximately three hours, Bleavins and Aulerich, 1981; Kaufman, 1980) and ferrets need a place to sleep multiple times per day, as they are polycyclic sleepers with sleep cycles of two to four hours (Marks and Shaffery, 1996). Therefore, when water and foraging enrichments were tested in the ABO set-up, the ferrets would frequently return to the home chamber to sleep in the hammock (which was determined to be the most valued enrichment, Reijgwart et al., 2016) after visiting one of the other chambers. As it is suggested that these short behaviours with a high consumption level (number of visits) are differentially affected by increasing price than longer lasting behaviours (such as sleeping, Kirkden et al., 2003), they might have been undervalued in the ABO set-up. Additionally, the foraging enrichment could be taken back to the home chamber (which the ferrets did every day) as it was small enough to fit through the cat flap and therefore only had to be worked for once daily, which might have influenced the ferrets' choice in favour of the this enrichment.

It should be taken into account that the items from three enrichment categories were being rotated in the ABO set-up: the items from two of the enrichment categories were freely available in the home chamber while the ferret was working to gain access to the items from the third enrichment category. This means that the ferrets had free access to the tested enrichment items before and after the 8-day session in which they were asked to work for that specific enrichment category. Therefore, there is a possibility that the ABO set-up cannot be regarded as a closed economy, but as an open economy, lowering the motivation to work for access to the enrichments and violating one of the basic requirements for a reliable consumer demand study (Ladewig et al., 2002; Mason et al., 1998).

Potential confounding factors

There were three unavoidable temporal confounding factors that should be kept in mind when interpreting the results. First, all set-ups were tested in the same sequence, so habituation or deprivation effects could have occurred. If habituation effects took place, the ferrets would show a lower motivation for the enrichments in subsequent set-ups. Whereas if a deprivation effect took place, the ferrets would show a higher motivation for the enrichments in subsequent set-ups (e.g. Latham and Mason, 2010). We saw the latter pattern in the 3Ch (i.e. second) set-up compared to the 7Ch set-up, but the first pattern for the ABO (i.e. third) set-up compared to the 3Ch set-up. These opposite temporal patterns make it difficult to determine whether a habituation and/or deprivation effect were indeed confounding factors in this study. However, these effects could not be avoided, as the three set-ups were built from each other and switching between the 7Ch and 3Ch set-ups was not only very difficult, but also a great disturbance to the ferrets as it included using powertools and hammers inside the animal room. Additionally, we wanted to provide the ferrets with preferred enrichment items in the ABO set-up, for which we needed the results of the 7Ch and 3Ch set-up.

Secondly, the price increased continuously within each set-up and for each enrichment, which could affect the animals' motivation as cost and familiarity are increased simultaneously (Seaman et al., 2008). However, presenting costs in a random order has its own disadvantages, as the time at which different costs are imposed and the contrast with previously presented costs both influence the demand (Asher et al., 2009). As the disadvantages of random costs seem to outweigh those of using ascending costs (Asher et al., 2009), we have chosen to use the latter approach.

Thirdly, the experiments differed in respect to their duration, as all enrichment categories were tested concurrently in the 7Ch set-up, while this was done consecutively in the 3Ch set-up. The only way to create an equal duration for the 7Ch and 3Ch set-up would be to use six times smaller weight increases (i.e. 40 g instead of 250 g daily increase in push force) in the 7Ch set-up, which also would have been a confounding factor as it has been shown that the contrast between costs influences an animals' motivation (Asher et al., 2009).

Conclusion

The results of this study indicate that both ways of providing enrichments concurrently instead of consecutively affect the results of a consumer demand study. In the 7Ch set-up, the animals seemed to consider the resources and time available to them and abandon luxury behaviour and novelty seeking when appropriate. The ABO set-up on the other hand, could be regarded as an open instead of a closed economy. Overall, using a multichamber set-up (i.e. testing all the enrichment categories concurrently) and keeping the number of items in the home cage to a minimum seem to aid in measuring an animals' motivation for enrichments in a consumer demand study and might, after replication, be added to the best practice guidelines.

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Chapter 4

Ferrets' (*Mustela putorius furo*) enrichment priorities and preferences as determined in a seven-chamber consumer demand study

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Abstract

Knowledge of species-specific motivation and preferences for enrichment options is necessary to put in place an appropriate enrichment plan. This knowledge is currently lacking for ferrets. Therefore, seven female ferrets were consecutively housed in a seven-chamber closed economy consumer demand set-up consisting of a corridor that was connected to six enrichment chambers (EC) and an empty control chamber (CC) via weighted doors. In each EC, enrichments from the categories tunnels, balls, water bowls, foraging, sleeping and social enrichment were placed in random order. Motivation to reach EC was measured by daily increasing the doors" weight until the ferret no longer entered EC (the maximum price paid, MPP). Preferences within a category were evaluated by comparing interaction times with the enrichments. Ferrets pushed the highest weights for sleeping enrichment (MPP 1450±120 g). MPPs for water bowls (1075±153 g), social enrichment (995±267 g), foraging enrichment (950±228 g) and tunnels (940±393 g) were also significantly higher than for CC. Compared to other enrichments, inter-individual variation in motivation for access to tunnels was very high. Ferrets preferred the hammock (9.2±5.9 hours) over the Savic Cocoon® (0.6±0.8 hours; P=0.011) within the category sleeping enrichment; the large (5.8±1.7 min) over the small water bowl (3.1±0.8 min; P=0.014) within the category water bowls; the flexible (6.1±2.6 min) over the rigid tunnel (0.3±0.2; P<0.001) within the category tunnels; and the ferret ball (0.9±0.5 min) over the golf ball (0.3±0.3 min, P<0.001) within the category balls. Within the category foraging enrichment, no preference for one over the other item was found (P=0.144). Results of this study show that a hammock, conspecifics, foraging enrichment and a large water bowl are preferred enrichment options for ferrets.

Introduction

Ferrets (*Mustela putorius furo*) are commonly used for research purposes (e.g. influenza research) (Boyce et al., 2001) and are kept as pets, but research on behaviour and behavioural priorities of these animals is scarce (for a review, see Vinke and Schoemaker, 2012). It is believed that ferrets could benefit greatly from environmental enrichment (Fisher, 2006), which is demonstrated by their use of three-dimensional environments containing toys and multilevel shelves (Wolfensohn and Lloyd, 2003). In addition, ferrets used a barren cage 6-12 times less than any of three enriched cages in a preference test (Cruden, 2011). Moreover, ferrets in barren cages showed signs of stereotypic behaviour (bar chewing and head swaying) and quickly became lethargic, whereas the ferrets in an enriched isolation cage were active and curious and remained so throughout the study (Cruden, 2011).

The aforementioned studies did not investigate the preference and/or motivation for specific enrichment options. However, enrichments for which ferrets show a high motivation could possibly allow for performance of behavioural priorities and a lack of opportunity to do so could lead to the development of abnormal behaviour and stress (Jensen and Pedersen, 2008). This in turn is detrimental to animal welfare as well as the reliability of study results, as inter-individual variation might increase due to stress (e.g. Verwer et al, 2009). A validated method to assess the motivational strength and value of resources is measuring the price an animal is prepared to "pay" for (unlimited) access to these resources (Cooper, 2004; Cooper and Mason, 2001; Mason et al., 1998). Such studies, referred to as consumer demand studies, involve imposing a strenuous task on the animal in order for it to gain access to a specific resource in a closed economy set-up. The task that the animal has to perform to gain access to the resource preferably involves an action that is considered a naturalistic task for the animal. Such a task requires the least amount of training and is also less prone to operant-reinforcer biases than unnatural tasks (Dawkins, 1990). In mink, a weighted door has been used for this purpose with success (Cooper and Mason, 2001).

By gradually increasing the effort that is needed to gain access to the resource, the maximum price paid (MPP) can be determined: the price at which the animal is no longer willing or able to perform the task. Compared to other indices used for measuring motivational strength, the MPP-index is believed to have the greatest internal validity (Houston, 1997), because 1) it is relatively insensitive to external cues (Warburton and Mason, 2003); 2) it can be applied to "all-or-none" goods (Jensen and Pedersen, 2008; Olsson et al., 2002); and 3) an increase in price only has to be qualitative, so no assumptions about the subjective value of a task have to be made (Cooper, 2004).

Consumer demand studies often involve two-chamber set-ups that consist of a home chamber and one enrichment chamber in which the enrichments and an empty control are tested consecutively, as opposed to a three- or multi-chamber set-up, in which one or multiple resources and a control are tested concurrently. However, a recent study showed that a two-chamber set-up using a push door was unsuitable for ferrets, as they would push almost to their maximum push capacity for an empty compartment (Reijgwart et al., 2015). Thus, alternative set-ups (three- or multi-chamber) needed to be considered (e.g. Hovland et al., 2006; Mason et al., 2001; Seaman et al., 2008).

In a three-chamber set-up, however, the enrichments are still tested consecutively, which might not solve the problems encountered in the two-chamber set-up. Therefore, a seven-chamber consumer demand study using a push door was used in this study to determine the maximum price ferrets paid for six enrichment categories (with different options per category) and one control chamber.

Animals and methods

Ethical note

This study was ethically approved by the Animal Care and Use Committee of Intravacc, Bilthoven, the Netherlands (DEC 201400137).

Animals, housing and husbandry

For the study, seven female, approximately 1 year old (range: 8-15 months), ferrets were used. Ferrets were obtained from Schimmel B.V., were surgically neutered (ovariectomized) at an age of 5 months and weighed 1011±137 grams at the moment of testing. Throughout the study, the ferrets were housed indoors in a room that was kept at a temperature between 19˚C and 25˚C. They were exposed to a 8:16 h light:dark schedule using artificial lighting (light bulbs) that switched on at 9:00 h and off at 17:00 h. In addition, auditory stimulation was available in the form of a radio to mask environmental noises, which automatically switched on and off concurrent with the light phase. Before and after the experiment, the ferrets were group-housed in phenolic faced plywood floor pens of 163x94 cm. In this pen, ferrets were provided with sawdust, a hiding place in the form of a flexible plastic bucket and *ad libitum* water (from a nipple) and food (Hope Farms® ferret balance pellets, Hope Farms, Woerden, the Netherlands). Refreshing of the food and water, as well as cleaning of the cages, took place daily at 9:30 am. Prior to and throughout the study, the ferrets' health and overall condition were monitored on a daily basis.

Experimental housing

During the experiment, the ferrets were successively individually housed (24 hours per day for a total of 26 days) in a closed economy, seven-chamber set-up consisting of one long corridor (692 cm long, 54 cm wide) connected to seven phenolic faced plywood floor pens (ground surface 107x94 cm; Figure 4.1) with sawdust bedding. Between the corridor and each chamber, a 70 cm high, 6 mm thick phenolic faced plywood divider was present. The divider contained a wire mesh window through which the ferrets could see what was in the chamber, a non-transparent one-way cat flap (Petsafe® 4 Way Locking Deluxe Cat Flap, PetSafe, Ochten, The Netherlands); and a one-way horizontally hinged weighted door (Tecnilab-BMI, Someren, The Netherlands), similar to those used in the two-chamber study (Reijgwart et al., 2015).

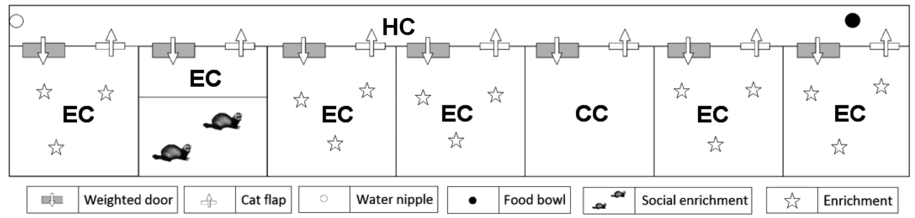

Figure 4.1 Schematic representation of the experimental housing (HC=home corridor with a water nipple and food bowl, CC=control chamber, EC=enrichment chambers with, in randomised order, foraging enrichment, sleeping enrichment, tunnels, balls, conspecifics and water bowls).

The weighted doors allowed the ferrets to move from the corridor, where *ad libitum* food and drinking water (via a nipple drinker) were provided, to the control chamber (CC), with only sawdust bedding, or the enrichment chambers (EC), where options from one of the enrichment categories (foraging toys, social contact, sleeping enrichment, water bowls, tunnels, balls) were placed in random order for each ferret. The one-way unweighted cat flaps, in contrast, could be used to return to the corridor. To push open the unweighted door, ferrets needed to exert a force of 200 g. Similar to the two-chamber study (Reijgwart et al., 2015), weights were added to the doors on a daily basis to gradually increase the effort needed to open the doors, starting with 250 grams/day up to 1500 grams, following which weights were increased with 125 grams/day. The mechanism would transfer 50% of the added weight to the actual force needed for the ferret to open the door. Thus, a weight of 250 g translated to a push force of 325 g (200+50% of 250 g), a weight of 500 g to a push force of 450 g (200+50% of 500 g), etc. Under the doors, a wire mesh strip was mounted to provide a traction surface for the ferrets to facilitate them to apply force to the doors.

Enrichments

Because environmental enrichment provides best results in terms of improvement of welfare if it is biologically relevant (i.e. improves biological functioning of the animal, Newberry, 1995), enrichment categories were selected based on their relevance for enabling species-specific behaviours to be performed such as those seen in feral ferrets. i.e. foraging , sleeping, exploring, hunting, social interaction, drinking (Clapperton, 2001). In total, six enrichment categories were selected: 1) foraging enrichment, which enabled the animals to perform appetitive and consummatory behaviours ; 2) sleeping enrichment, which provided the ferrets with a safe, dark place to sleep, similar to a burrow; 3) rigid and flexible tunnels, which provided the ferrets with play and exploration opportunities (Lloyd, 1999; Tynes, 2010); 4) balls, that were provided to stimulate the ferrets' hunting and capture behaviours, as well as play behaviour (Fisher, 2006); 5) social enrichment in the form of companionship of two familiar conspecifics; and 6) water bowls, which the ferrets could play in/with and allowed the ferrets to drink in a more natural way than from a nipple. (Table 4.1)

For each enrichment category, multiple options with different characteristics were offered to increase the likelihood of at least one of the options from the category being favoured by the ferrets. All ferrets were presented with the same range of enrichment options and the EC in which the options from each category were placed (cage order) was randomised for each ferret. With the exception of the sleeping bucket and conspecifics, none of the options had been previously provided to the ferrets. The conspecifics that were used as enrichment were randomly selected from the six ferrets that were not being tested at that moment and were housed behind mesh wire on sawdust, with a sleeping bucket, *ad libitum* food and a drinking bottle. Therefore, the ferrets could smell and hear each other, but had only limited opportunity for physical contact. In addition to the six enrichment categories, an empty cage was added as a seventh chamber that enabled to control for value of extra space, patrolling and the rewarding properties of interacting with the weighted door itself (Hansen et al., 2002). The six enrichment categories and the control chamber were allocated at random and the order was randomised for each ferret to control for chamber preferences.

Acclimatisation and training

The ferrets were already housed in the testing room and trained in using the weighted door for a previous experiment, in which their maximum push capacity (MPC) was determined using food as a reinforcer (1450±144 g; Reijgwart et al., 2015). Each ferret was allowed to habituate to the experimental housing for seven days prior to commencing the experiment. In this period, all ferrets visited every chamber at least once and were therefore familiar with the enrichments in each chamber. During this period, doors were left unweighted and the enrichments were placed in EC to allow ferrets to enter and explore the enriched chambers without having to put in extra effort. After the acclimatisation period, weights were added to the doors on a daily basis over a period of 19 days until a weight of 3000 grams was reached.

Maximum Price Paid

The maximum weight the ferrets were prepared to push (i.e. maximum price paid) for each of the enrichment categories and a control chamber were determined by daily increasing the effort needed to open the doors, while ensuring that all doors had similar weights added to the doors at all times. Ferrets were considered to have reached their maximum price paid (MPP) for a particular enrichment category when they did not visit the chamber containing that specific category for 24 hours. MPP was subsequently determined as the last weight successfully pushed to gain access to EC and recorded in grams for each individual ferret and enrichment category. To identify which enrichment categories are most likely important to all ferrets and in which categories there are more individual preferences, standard deviations from the mean MPP and individual ranking of the MPPs are discussed.

Behavioural analyses

In addition to MPP, video recordings were made of the ferrets' activities in the experimental set-up. These recordings were used to score the number of times the ferrets entered the different chambers for each weight, as well as the time spent in each of the chambers. This was done by continuously scoring the videos and noting the time of day the ferrets entered a chamber through the weighted door and exited through the weighted cat flap in. For this purpose, the time of day that was displayed on the video footage (hh:mm:ss) was used. These data were used to validate the increasing weight as a cost. In addition, the ferrets' interactions with each of the individual options (except for the social enrichment) were evaluated in order to determine the ferrets' preferences for the options within an enrichment category and to investigate possible underlying motivations for the priorities and preferences of the ferrets. When a ferret made no visits to a resource at a specific weight, number of visits and mean visit duration were noted as missing data.

Statistical analysis

Analyses were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, N.Y., USA). Data are expressed as mean±SD and the probability level accepted for statistical significance was $P<0.05$ unless stated otherwise. Differences in MPP, order effects and changes in number and average duration of visits to the enrichment chambers with increasing weights were analysed using a Linear Mixed Model with enrichment category and weight on door as fixed effects and ferret ID as a random factor. Prior to running the analysis, normality of residuals and random intercept were tested to ensure that all required assumptions were met. A Paired Samples T-test (for the enrichment categories with two options) or 1-way ANOVA (for the enrichment categories with three options) were used to analyse differences in time spent with the enrichment options within an enrichment category. Normality of data and homogeneity of variances were tested and where appropriate, a nonparametric analysis was used. Individual ranking of the enrichment categories was done by assigning ranks 1-7 for the highest to the lowest MPP per individual ferret. When enrichment categories had the same MPP, they were assigned the same rank. The total, and average of these individual rankings were calculated.

Results

Maximum price paid

The maximum price paid by the ferrets to gain access to the different chambers differed significantly (LMM: $F_{36.000}$=10.030, *P*<0.001; Figure 4.2). The chamber in which the enrichments were placed was not found to affect MPP for the different EC (LMM: $F_{36.000}$=0.827, *P*=0.556).

Findings demonstrated that the MPP for sleeping enrichment (1450±120 g) was similar to the ferrets' MPC (1450±144 g; *P*=0.720). Additionally, the MPP for sleeping enrichment was significantly higher than the MPP for all other enrichments and the CC (*P*=0.008 for water bowls; *P*=0.001 for social enrichment; *P*<0.001 for all other comparisons). For four of the enrichment categories (water bowls: 1075±153 g; social enrichment: 995±267 g; foraging enrichment: 950±228 g; tunnels: 940±393 g), the MPPs were significantly higher than that for CC (539±187 g; *P*=0.002; 0.017; 0.005; 0.020, respectively). Only for the balls (754±215 g) no significant differences were found when comparing its MPP with that for the CC (*P*=0.175).

Individual differences in MPP

The SD in MPP was lowest for sleeping (120 g) and highest for tunnels (292 g, Figure 4.2). Additionally, sleeping enrichment was ranked the highest in all but one ferret and CC was ranked lowest or next-to-lowest in 6 out of 7 ferrets (Table 4.2). All other enrichments were ranked more variable by the individual ferrets (Table 4.2).

Table 4.1 Overview of the enrichments in each enrichment category. Numbers indicate the supplier of the enrichment, 1=Van der Neut, Groenekan, The Netherlands, 2=onlinedierenspeciaalzaak.com, 3=Zooplus.nl, 4=Tecnilab-BMI, Someren, The Netherlands 5=Schimmel BV, The Netherlands.

Enrichment category	Enrichments	Specifications
Foraging enrichment	Foraging ball	Happy Pet® tumble 'n treat, ø6 cm[1]
	Tumbler	Nina Ottoson Cat pyramid®, 9.5 cm high[2]
Sleeping enrichment	Bucket	Flexible plastic bucket[1]
	Savic Cocoon	Savic Cocoon®, 34.5 x 26.5 x 16.0 cm[1]
	Hammock	Adori® hammock, 50 x 45 cm[1]
Tunnels	Rigid tunnel	Ferplast® tunnel FPI 4840, ø10.5 cm, length 29 cm[1]
	Flexible tunnel	Zooplus® 260697.0, ø10 cm, length 19-75 cm[3]
Balls	Ball with bell	Cat play ball[1]
	Golf ball	ø4 cm
	Ferret ball	Ferret ball, ø25 cm, 4 holes ø10.2 cm[4]
Social enrichment	Conspecifics	Two familiar female ferrets[5]
Water bowls	Large bowl	Marchioro® kitten litterbox filled with water, 26 x 36 x 9 cm[1]
	Small bowl	Adori® stoneware food bowl filled with water, ø18 cm, 5 cm high[1]

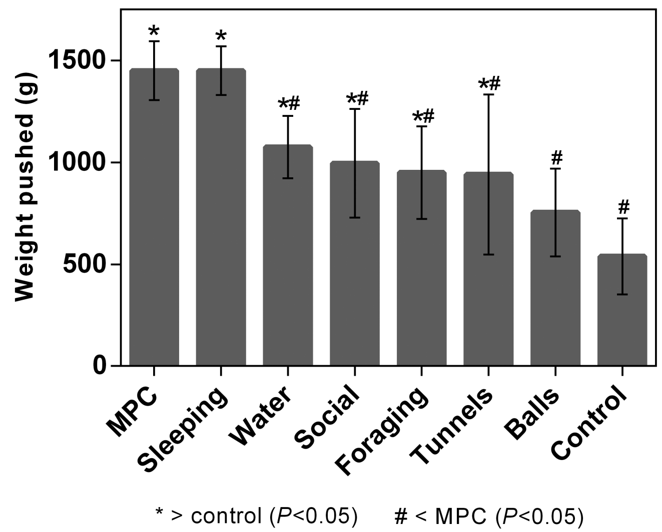

Figure 4.2 Maximum price paid by neutered female ferrets (*N*=7, mean±SD) for the six different enrichment categories and a control.

Validation of costs

The number of visits to the enrichment chambers declined upon increasing the weights on the door (LMM: $F_{917.000}$=43.722, P<0.001; Figure 4.3a), while weight had no significant effect on the average visit duration (LMM: $F_{917.000}$=3.590, P=0.058; Figure 4.3b).

Interaction with enrichment options

In addition to using the sleeping enrichment for its intended purpose (sleeping), the hammock and Savic Cocoon® were also used as tugging toy and hiding location for food and foraging enrichment, respectively. When comparing the different enrichments within this category, significant differences were observed in interaction times (Friedman df=2, P<0.05, Figure 4.4), with interaction times with the hammock (9.2±5.9 hours) being significantly higher than that for the Savic Cocoon® (0.6±0.8 hours, P=0.011).

The ferrets played with all types of balls, interaction with the balls consisted of pushing the balls forward, running or hopping after the balls and pouncing the balls. Occasionally, the ferrets were also observed to crawl through the holes in the ferret ball. Upon comparison of the interaction times with the different balls, significant differences were observed (Friedman: df=2, P=0.021, Figure 4.4), with interaction times with the ferret ball (0.9±0.5 min) being significantly higher than that with the golf ball (0.3±0.3 min, P=0.003).

The tunnels were mainly used to walk through. In addition, ferrets also used the tunnels as toys to push around. When comparing the different tunnels, interaction time with the flexible tunnel (6.1±2.6 min) was higher than interaction time with the rigid tunnel (0.3±0.2 min; Paired Samples T-Test: df=6, P<0.001, Figure 4.4).

Most of the times, the ferrets used the foraging enrichment to directly obtain food from, which they achieved by pushing the items around. However, ferrets were also frequently observed to drag the enrichments out of EC and stash them elsewhere, most often the EC with sleeping enrichment and more specifically, the Savic Cocoon®. Upon comparing the different foraging enrichments, no significant differences were observed between the items (foraging ball: 4.2±2.3 min; tumbler: 2.1±1.6 min; Paired Samples T-Test: df=4, P=0.114, Figure 4.4).

Table 4.2 Total number of neutered female ferrets that ranked the seven categories 1-7 and average ranking of the enrichment categories.

Enrichment Category	Ranking							Average
	1	2	3	4	5	6	7	
Sleeping enrichment	6	1	-	-	-	-	-	1
Water bowls	-	4	1	1	1	-	-	3
Social	1	1	2	2	1	-	-	3
Foraging enrichment	1	-	2	3	1	-	-	3
Tunnels	1	-	1	3	1	1	-	4
Balls	-	-	1	-	2	2	2	6
Control	-	-	-	-	1	2	4	6

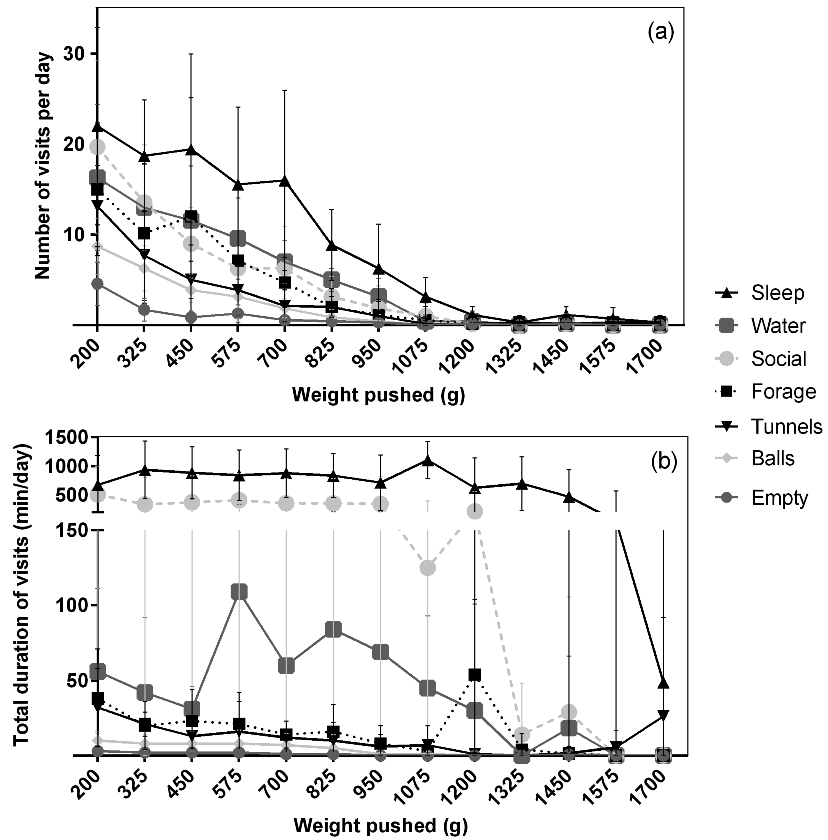

Figure 4.3 Number of (a) and average duration per (b) (mean±SD) visit to six different enrichment chambers (sleeping, water bowls, social, foraging, tunnels, balls) and a control chamber upon increasing the weight on the door by neutered female ferrets (N=7).

Despite the ferrets having free access to a drink nipple in the corridor, they were often found to use the water bowls as an alternative water source. In fact, as long as they gained access to the water bowls, they fully ceased using the drink nipple. Occasionally, the ferrets were found to play and display digging behaviours in the water bowls. Comparison of the different water bowls revealed interaction times with the large water bowl (5.8±1.7 min) to be significantly higher than with the small water bowl (3.1±0.8 min; Paired Samples T-Test: df=6, *P*=0.014, Figure 4.4).

Evaluation of the type of interactions between the ferrets in the social enrichment revealed that, despite the inability to have direct contact, the ferrets would interact with the familiar conspecifics. The type of interaction seen mostly involved sleeping next to the mesh that separated them from their conspecifics. Occasionally, the ferrets would also perform short bouts of social play behaviour (weasel war dance, play invitation) towards the other ferrets. No aggressive or marking behaviour was seen. Upon the door weights exceeding 1 kilogram, a marked difference was observed in the ferrets' behaviour. Instead of going into the EC with social enrichment, they would sleep in the corridor next to the EC containing the familiar conspecifics, thereby allowing them direct visual contact with the other ferrets.

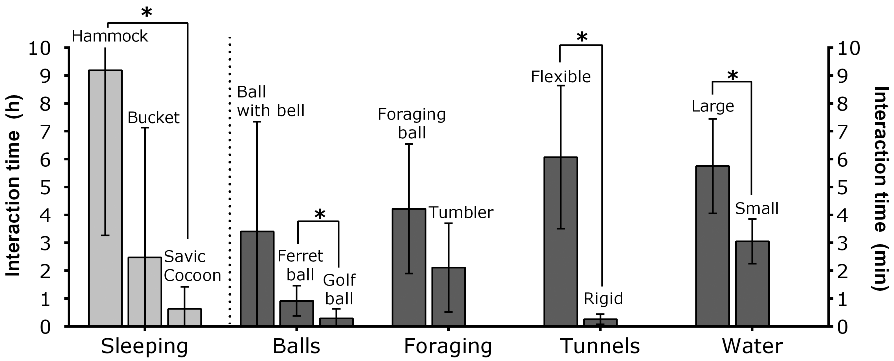

Figure 4.4 Average interaction time (mean±sd) with the enrichments in the five different categories (sleeping enrichment, balls , tunnels, foraging enrichment and water bowls) by neutered female ferrets (*N*=7).

Discussion

The aim of this study was to evaluate the preferences and motivational strengths of ferrets for specific enrichments in a seven-chamber consumer demand study using a weighted push door. Based on the results for MPP, ferrets were found to work harder to gain access to sleeping enrichment than for any of the other enrichment categories.

In addition, inter-individual differences in motivation for sleeping enrichment were the smallest, with all but one ferret being motivated the most to gain access to the sleeping enrichments. Ferrets furthermore put significantly more effort in gaining access to social enrichment, water bowls, foraging enrichment and tunnels compared to an empty control chamber. However, inter-individual differences in motivation for these enrichments were higher than for sleeping enrichment (i.e. some ferrets were highly motivated to gain access to these enrichment categories, while others were not), with inter-individual variation being highest for tunnels. Of the six enrichment categories provided, ferrets showed the least motivation to gain access to the balls, with no significant differences found between this enrichment category and the control chamber.

Sleeping enrichment

The high motivation for the sleeping enrichments were expected, as laboratory ferrets sleep 60-70% of the time (Jha et al., 2006; Marks and Shaffery, 1996). The distinct preference for the hammock compared to the other sleeping enrichments was surprising, however, as biological data suggests that feral ferrets in the wild are mostly nocturnal and sleep in areas with cover during the day (Clapperton, 2001). Similarly, others have reported ferrets to prefer sleeping in dark, enclosed areas (Lloyd, 1999). However, other authors have anecdotally suggested a preference for hammocks as a resting site in pet ferrets (Tynes, 2010), as was demonstrated in our study. Possibly, the soft lining of the hammock mimics physical contact with conspecifics, which was another enrichment category the ferrets showed high motivation for and where the ferrets spent a large proportion of the time sleeping (as reflected in the long visit duration). This warrants further investigation, however, as there might be other explanations for the preference for the hammock, such as the rocking motion, warmth due to the soft material, space (the hammock is a "second floor") or the snugness of the hammock.

Social enrichment

The high motivation for social interaction is in accordance with the observation that domesticated ferrets are gregarious and will sleep together, share food and water and play vigorously (Fisher, 2006; Lloyd, 1999). In addition, Warburton and Mason (2003) found that mink (*Mustela vison*), a close relative of the ferret with similar biologic characteristics, worked to gain access to conspecifics from which they were separated by a wired fence. An alternative hypothesis might be that the ferrets were highly motivated to visit the social chamber in order to gather information on the potential competitors in their territory (Chang et al., 2000; Takahashi, 1991). This explanation seems less likely, however, given the fact that the ferrets had been housed together previously with no fights occurring during this time. In addition, territorial behaviour might have been reduced by the ovariectomy (Takahashi, 1991). Moreover, if territoriality would play a role, interaction with the other ferrets would likely only involve awake and marking behaviours rather than sleeping in proximity of the other ferrets, which all ferrets did. Therefore, it is more likely that the visits to the conspecifics were social behaviour instead of territorial behaviour.

Water bowls

During our study, we found ferrets to be highly motivated to gain access to water bowls, a motivation they share with mink, which showed a preference for access to a swimming bath that was 60 cm deep (Mason et al., 2001). This is surprising, however, as the two species have different hunting territories. Whereas semi-aquatic mink hunt predominantly in the water (Dunstone and Davies, 1993), ferrets are primarily known as ground hunters that are rarely seen swimming (Clapperton, 2001). The ferrets in our study mainly used the water bowls as an alternative source of drink water, but as the water in the bowls in our study was too shallow to swim in, it is impossible to determine whether swimming was not observed as a result of the study design or whether ferrets really lack motivation to swim. Despite this limitation, we can conclude that ferrets prefer drinking from a bowl opposed to a nipple, as they were motivated to push to gain access to the water bowls even though the water nipple was freely available. Similar results have been found in other animal species, potentially because the water bowl allows for a more natural way of drinking (e.g. Tschudin et al., 2011). However, the water bowls also induced play behaviour (scratching) and may even be used for thermoregulation, therefore warranting further investigation in order to draw definitive conclusions about the underlying reasons for the high motivation for water containers. Similarly, the study design does not allow for conclusions to be drawn regarding the underlying reason for the preference for the larger, plastic litter box over the smaller, stoneware bowl, as both size and type of material differed between the two bowls. However, despite the small difference in interaction times being statistically significant, the biological relevance of this difference appears to be questionable given its small size.

Foraging enrichment

Aside from the sleeping enrichment, social contact and water bowls, ferrets also showed high motivation to gain access to foraging enrichment. This finding in itself is not surprising as many animals are observed to be willing to put in extra effort to obtain food. The observation that the ferrets worked (pushed the heavy door) to work (roll the ball or tumbler) for food, while freely accessible food was available in the home corridor, can be explained by a concept called contrafreeloading, which states that working for food can be inherently enriching (Inglis et al., 1997). Foraging enrichment is considered to have great value to an animal, as it enables the animal to perform its natural foraging and feeding behaviours, one of the most essential behavioural patterns for survival (Newberry, 1995). The ferrets could perform natural hunting behaviour (i.e. stalking, catching and eating) using the foraging ball and tumbler, which also were small enough to pick up and drag elsewhere. Mustelids have been reported to cache their food (Macdonald, 1976) and ferret owners report food and item caching, so these foraging enrichments might also satisfy the tendency to cache food in a secluded area (in our study the Savic Cocoon®). Since both items were made of similar materials, contained the same reward and were light enough for the ferrets to carry around, both may have been equally attractive to the ferrets, thereby explaining the lack of a preference for one over the other.

Balls

As the golf ball, ferret ball and the ball with bell could only fulfil the appetitive part of the hunting sequence and because these enrichments were tested concurrently with foraging enrichment (which could fulfil the appetitive as well as the consummatory part of the hunting sequence and allowed for stacking food away from the food bowl), it is not surprising that the ferrets would stop visiting the EC with balls at low weights. In line with this, the ferrets preferred the ball with the bell within the category balls, which had a novel feature (the sound of the bell) and was the only ball could be picked up with the mouth. The differences in interaction time with the three types of balls were small, however, so the found differences, despite being statistically significant, may not be biologically relevant. Interestingly, none of the ferrets ever carried the ball with the bell out of the EC, as they did do with the foraging enrichments. Possibly, the need for caching food/items was already met using the foraging enrichments, which made it less worth wile to put in the effort to stash the ball.

Tunnels

Tunnels were also among the options for which ferrets were willing to put in effort to gain access to, even though individual variation in motivation was high for this enrichment. Considering the fact that feral ferrets' sleep in rabbit burrows and their main hunting grounds consists of rabbit tunnels (Clapperton, 2001), the high motivation for tunnels is not surprising as walking through a tunnel could readily help to mimic this behaviour. The preference for the opaque flexible tunnel over the rigid see-through tunnel is in line with the ferrets' preference for enrichment that mimics more natural circumstances, as rabbit holes are dark and usually do not follow a straight line. Moreover, the lighter, flexible tunnel provided an opportunity for ferrets to manipulate, drag and play with the tunnel, behaviours they were found to display also with the other items.

Limitations of the study

Unfortunately, the fundamental reasons behind the preferences for certain options within the categories can only be guessed, as this study was not designed to investigate which characteristics of the enrichments are most appealing to ferrets. Moreover, enrichment options will rarely serve a single function, which makes it difficult to choose options that only fit in one single category. However, the behavioural observations performed during this study may provide some indication regarding the enrichments' functionalities for the ferrets. Similarly, these limitations highlight the importance of testing multiple options within an enrichment category to obtain more reliable results regarding the value of a specific enrichment category. Choosing a single option to represent a specific category may pose a risk of drawing incorrect conclusions regarding the value of this category when an option is chosen that is not of interest to the ferret. In light of this, inter-individual preferences or aversion for specific options or conspecifics should also be taken into consideration, especially for categories for which a large inter-individual variation in motivation was found. Inter-individual variation can arise due to sex, life history, age and reproductive state, e.g. Vasconcellos et al., 2009. In this study, the sex, reproductive state and life histories of the ferrets were equal, but only one ferret could be tested at a time, which caused an age difference of 5.5 months between the first and the last ferret that was tested. However, the largest differences in motivation were not always found in the ferrets with the largest age differences, thus rendering it less likely for age to be a (sole) contributing factor in the animals' motivation for a resource. A large inter-individual variation may just as well indicate that those options are less important for the ferrets in general, therefore being more prone to individual preferences, whereas essential behavioural priorities are equally important to all ferrets, therefore resulting in high MPPs with little inter-individual variation.

Only female ferrets were used in this study, as male ferrets are less commonly used in animal studies. This comes with the advantage of less variability due to sex (as stated in the previous paragraph), but has the disadvantage that the results are not generalisable to male ferrets.

Test conditions may also be of influence on the results that were found. For example, motivation for and interaction with enrichments has been found to change when animals are tested when housed socially in groups (Elmore et al., 2011; Mench and Stricklin, 1990; Pedersen et al., 2002; Sherwin and Nicol, 1998). In addition, motivation to work for full social contact may be different from that for limited (vocal and visual) contact, as we evaluated in our study. In further studies, it would therefore be of interest to investigate the effects of social housing and access to full physical contact on the ferrets' preferences for different enrichments. The priorities and preferences of the ferrets were furthermore tested in a multi-chamber (seven-chamber) set-up, as a two-chamber set-up proved to be unsuitable for ferrets. However, a multi-chamber set-up can lead to interpretative problems due to animals abandoning resources at a different rate (Kirkden and Pajor, 2006). If one resource is abandoned, the value of the remaining resources might change because of the unavailability of the abandoned resource. In addition, an animal only has limited income (i.e. the time and energy available per day) which it needs to divide between the different resources if offered simultaneously as is the case in a multi-chamber consumer demand study, thereby potentially yielding lower MPP values for resources that are deemed less important to the animal. Especially if enrichment options are – in part – substitutes and allow for performance of similar behaviours to be performed (e.g. balls and foraging enrichment both allow appetitive behaviour), animals may choose the option that is most preferred, despite the other option being of interest to the animal as well. Thus, concurrent testing of (substitutable) resources might have caused erroneously low MPPs for some of the (less important) enrichment categories. It would therefore be important to also test the ferrets in a (3-chamber) set-up where they would not be offered the different options simultaneously to prevent them from having to choose and divide their time.

Conclusion

This study showed that, in a closed economy, seven-chamber consumer demand set-up, ferrets were motivated highest to gain access to a chamber containing sleeping enrichment, as represented by the high MPP, and long, frequent visits to this chamber. In addition, ferrets' motivation for social enrichment, water bowls, foraging enrichment and tunnels was also higher than for the chamber containing balls or the control chamber. For these enrichments, inter-individual variation was higher than for sleeping enrichment and inter-individual variation for tunnels was highest, indicating that ferrets may value these resources differently. Within the highest ranking categories (sleeping enrichment, social contact, water bowls, foraging enrichment and tunnels), ferrets were found to prefer the hammock, large water bowl and flexible tunnel, whereas no clear preference was found for either of the foraging enrichments. These preferences should be taken into account so that a captive living environment can be created that is optimally adjusted to the ferrets' preferences and behavioural priorities.

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Chapter 5

The effect of provision of preferred and non-preferred enrichment on behavioural and physiological parameters in laboratory ferrets (*Mustela putorius furo*)

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Abstract

Environmental enrichment is often advocated to refine animal studies. Despite the increasing use of ferrets as an animal model in biomedical research, the knowledge on effects of the provision of enrichment on these animals is limited. Additionally, it is unknown whether varying types of enrichment (i.e. preferred and non-preferred) have a different effect. Therefore, to investigate the behavioural and physiological effects of providing (differently valued) enrichment to ferrets, three groups of six female ferrets were housed in standard conditions (with bedding, a flexible bucket, a food bowl and a water nipple), with additional non-preferred enrichment (with two ferret balls, a golf ball and an extra food bowl) and with additional preferred enrichment (with two hammocks, a foraging ball and a water bowl) for eight weeks. At the beginning and end of this period, behavioural (i.e. time spent on food and water intake, elimination, maintenance, inactivity, enrichment interaction, exploration, play, and agonistic behaviour) and physiological (i.e. bodyweight and Neutrophil/Lymphocyte ratio [N/L ratio]) parameters were recorded and compared. Results showed that agonistic behaviour increased in the ferrets housed in standard conditions, which was not observed in ferrets that were provided with preferred or non-preferred enrichment. In addition, the ferrets housed with preferred enrichment showed an increase in social play behaviour and a decrease in rearing behaviour (as part of the exploratory behaviours) which were not observed in the ferrets housed in standard conditions or with non-preferred enrichment. Moreover, the ferrets housed with preferred enrichment showed a clear preference for being inactive in the hammock and drinking from the water bowl. These results indicate that providing laboratory ferrets with preferred enrichment has positive effects on their behaviour that are not observed in ferrets provided with non-preferred enrichment or housed in standard conditions. Therefore, we recommend to house laboratory ferrets with a hammock, foraging ball and water bowl as these enrichments might help to refine studies using these animals.

Introduction

The environment of laboratory animals is closely-managed, standardised and controlled by human agency (Ohl and Putman, 2014). Often, these animals have limited living space and lack of opportunities to exert control and perform species specific behaviour, thereby restricting the animals' ability to adapt and potentially leading to impaired health and welfare (Sambrook and Buchanan-Smith, 1997). To prevent this, the provision of environmental enrichment is advocated. However, to exert the desired positive effects on animal welfare, the enrichment must have features that are functionally and biologically relevant to the animals (Newberry, 1995). As such, sufficient knowledge of the behavioural and physiological needs of the species in question is required. For commonly used laboratory animals such as rats and mice, the requirements and effects of housing and husbandry conditions are well-documented (e.g. Bayne and Turner, 2013; Gonder and Laber, 2007). Unfortunately, for less common laboratory species, such as ferrets, this information is largely lacking. As a result, these animals may be housed under suboptimal conditions, which may subsequently affect the results of experimental studies (e.g. Verwer et al., 2009).

An online survey showed that, according to their owners, pet ferrets that were confined for long periods of time and were provided with more enrichment items were less aggressive and performed more play behaviour than ferrets that were given less enrichment items (Talbot et al., 2014). This study unfortunately did not specify the types of enrichments that were provided, thereby preventing specific recommendations to be made regarding the provision of enrichment to ferrets. A prior consumer demand study performed with laboratory ferrets categorised enrichments as "preferred" (hammock, water bowl and foraging ball) and "non-preferred" (ferret ball and golf ball) (Reijgwart et al., 2016). However, it is currently unknown whether and what effects provision of (non-)preferred enrichment has on the behaviour and physiology of ferrets. This study therefore aimed to evaluate these parameters in ferrets that were housed in standard conditions, with non-preferred enrichment or with preferred enrichment for eight weeks. In mink, it was shown that access to a swimming bath (highly preferred enrichment, Mason et al., 2001) resulted in more play behaviour compared to provision of a cylinder and platform (less preferred enrichment) (Vinke et al., 2005). It is therefore expected that preferred enrichment will have a greater (positive) effect on the behaviour and physiology in ferrets than non-preferred enrichment. In addition to increased play behaviour, we expect to see (greater) positive effects on other behavioural (e.g. a decrease in inactivity, agonistic behaviour and exploration behaviour) and physiological (e.g. Neutrophil/Leukocyte ratio) parameters in the ferrets that are provided with (preferred) enrichment (Baumans, 2005; Broom, 1986; Davis et al., 2008; Yeates and Main, 2008).

Animals, material and methods

Ethical approval

This study was ethically approved by the Animal Care and Use Committee of Intravacc (DEC 201300161).

Animals

The study was performed with 18, six-month old ovariectomised female wildtype (sable) ferrets (*Mustela putorius furo*) from Marshall BioResources, USA. At the breeding facility, the ferrets were group-housed (3 ferrets per cage) in 2- or 3-tiers of mesh cages. Each cage had a dropped nesting pan with bedding in it. Prior to shipping, all ferrets were health checked and vaccinated for distemper and rabies. Upon arrival in the research facility, the ferrets were again health checked and weighed prior to being included in the study. The ferrets weighed 812±83 grams (min: 660 g, max: 945 g) at the start of the experiment.

Housing conditions prior to the experiment

Following arrival in the research facility, the ferrets were randomly divided over three groups of six individuals that were housed in phenolic faced plywood floor pens (1.8 m^2, 150l x 120w x 70h cm) with sawdust bedding (JRS LIGNOCEL® Hygienic Animal Bedding). The room in which the ferrets were placed had an ambient temperature of 20-22 °C, a relative humidity of 50-70% and a light-dark cycle of 8:16 with artificial lighting (lights turned on at 8:00h). Radio music was continuously played to provide auditory stimulation to the ferrets. Cages were cleaned daily and all ferrets were weighed weekly. The ferrets had *ad libitum* access to water (1L drinking bottle), food (Hope Farms Ferret Balance® in a stoneware bowl) and a flexible plastic bucket that was placed on its side to provide a hiding/sleeping opportunity.

Experimental design

The ferrets were allowed to acclimatise for two weeks. At the end of the acclimatisation phase, baseline behavioural and physiological measures were taken (T_0). After that, one group of ferrets remained housed in the same standard conditions (SC), whereas the other groups were provided with either non-preferred enrichment (npEC) or preferred enrichment (pEC) for a period of eight weeks (see below for further details). At the end of this period, a second series of behavioural and physiological measures was taken (T_1).

Table 5.1 Overview of the provided enrichments in each condition. SC = standard conditions, pEC = preferred enrichment condition, npEC = non-preferred enrichment condition. Numbers indicate the supplier of the enrichment. 1 = Van der Neut, Groenekan, The Netherlands, 2 = Tecnilab-BMI, Someren, The Netherlands.

Condition	Enrichment	Specifications
SC	Bucket	Flexible plastic bucket[1] on its side
	Food bowl	Adori® stoneware bowl[1] (ø18 cm, 5 cm high) filled with Hope Farms Ferret Balance® pellets
pEC	Water bowl	Adori® stoneware bowl[1] (ø18 cm, 5 cm high) filled with tap water
	2x Hammock	Adori® hammock[1] (50 x 45 cm) attached to cage walls at 3 points
	Foraging ball	Happy Pet® tumble 'n treat[1] (ø6 cm) filled with Hope Farms Ferret Balance® pellets
npEC	Extra food bowl	Adori® stoneware bowl[1] (ø18 cm, 5 cm high) filled with Hope Farms Ferret Balance® pellets
	2x Ferret ball	Ferret ball[2] (ø25 cm) with 4 holes (ø10.2 cm)
	Golf ball	ø4 cm

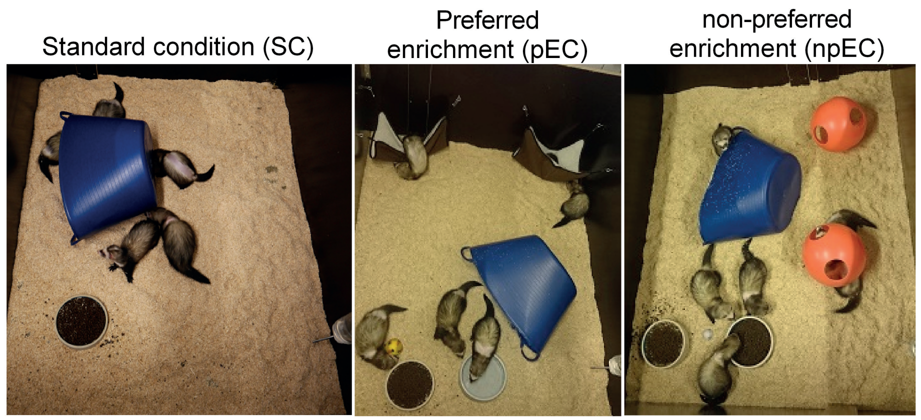

Figure 5.1 Pictures of the three housing conditions used in this experiment. Left: standard housing condition (SC), middle: preferred enrichment (pEC), right: non-preferred enrichment (npEC).

Experimental housing conditions

In the standard housing condition (SC), the ferrets had *ad libitum* access to water (1L drinking bottle), food (Hope Farms Ferret Balance® in a stoneware bowl) and a flexible plastic bucket that was placed on its side to provide a hiding/sleeping opportunity. In the preferred enrichment housing condition (pEC), ferrets were provided with two hammocks, one foraging ball and one water bowl in addition to the enrichment provided in SC. In the non-preferred enrichment housing condition (npEC), ferrets were provided with two ferret balls, one golf ball and an extra food bowl in addition to the enrichment provided in SC (see Table 5.1 and Figure 5.1 for details on the enrichment items).

Behavioural observations

At T_0 and T_1, behavioural observations were made over a 24-hour period. To prevent the observer from influencing the ferrets' behaviour and enable detailed analysis of their activity pattern, infra-red surveillance cameras were installed above the enclosures to record the behaviour. Offline analysis subsequently took place using a focal-animal sampling method, recording the behaviour of all animals in a group. To enable individual recognition of the ferrets, a different part of each ferrets' fur was shaven off (see Figure 5.1). The fifteen minutes before someone entered the animal room, the thirty minutes during cleaning and feeding of the animals and the fifteen minutes after all persons had left the animal room (one hour in total, from 9:30-10:30) were excluded from the analysis. During the analysis, the duration of the following behaviours was recorded: elimination behaviour (urinating/defecating), maintenance behaviours (self-grooming/scratching), inactive behaviour (sleeping/lying still/ resting), enrichment interaction, food and water consumption (eating/drinking), play behaviour (object and social play), agonistic interactions and exploration (rearing, scratching and tunnelling; see Table 5.2 for the complete ethogram).

First, a general time budget for a ferret in standard housing conditions was calculated using the average total duration for elimination, maintenance, inactivity, eating, drinking, play, agonistic and exploration behaviour from all three housing conditions at T_0. Second, for each individual ferret, the time spent on each type of behaviour at T_0 was subtracted from the time spent on this behaviour at T_1, resulting in a difference score (Δ T_1-T_0) per ferret for each behaviour. For each behaviour, these difference scores were subsequently compared between the housing conditions. Third, the total durations for inactivity in the bucket, sawdust, ferret ball and hammock; eating from the bowl, sawdust and foraging ball; and drinking from the bottle and bowl at T_1 were calculated per ferret and compared between the housing conditions.

Physiological data

At T_0 and T_1, on the day following the behavioural observations, physiological measures were taken. In addition to recording the bodyweight, blood was taken for a total white blood cell count and differentiation. To facilitate blood collection the ferrets were lightly anaesthetised using medetomidine (Domitor®; 0.1 mL [T_0=66-95 µg/kg, T_1=74-113 µg/kg] IM). One hour prior to injecting the anaesthetic agent, food was removed from the enclosures to limit the risk of food regurgitation and aspiration pneumonia. Once the ferrets were sufficiently anesthetised (as evaluated by the absence of a response to toe pinching), two mL of blood was collected from the vena cava cranialis and placed in a 2 mL Vacuette® blood collection tube with EDTA as an anticoagulant. The EDTA-tubes were immediately swerved by hand, checked for blood clots and placed on a roller shaker (30 rpm) to prevent blood clot formation. If a clot was detected at this stage, another sample was taken from the ferret while it was still anesthetised. Following collection of the blood, the ferrets were antagonised using atipamezole (Antisedan®; 0.1 mL [T_0=330-473 µg/kg, T_1=370-563 µg/kg] IM) and returned to their enclosures (where food was placed back) once they were deemed sufficiently awake. The blood samples were stored at +4°C and a complete white blood cell count (CBC) including white blood cell differentiation (Diff) was performed within 24 hours using a hematology analyser (ADVIA® 120, Perox Method). The N/L-ratio was calculated by dividing the number of neutrophils*103/µL blood by the number of lymphocytes*103/µL blood.

Statistical analysis

Statistical analyses were performed using IBM SPSS software (version 24.0). Data in the text are durations at T_0 and T_1 expressed as mean±SD, data in the figures are differences in duration (Δ T_1-T_0) expressed as median±IQR. Normality of distribution of the residuals was determined with an exact one-sample Kolmogorov–Smirnov test and homogeneity of variances was analysed with a Levene's test for equality of variances. Differences between the housing conditions in Δ T_1-T_0 of all parameters were analysed using a one-way ANOVA. Differences between the housing conditions and preferences within ferrets in time spent inactive in the bucket, sawdust, ferret ball and hammock; time spent eating from the bowl, sawdust and foraging bowl; and time spent drinking from the bottle and bowl at T_1 were also analysed using a one-way ANOVA. The probability level accepted for statistical significance was P<0.05. The p-values were corrected for multiple comparisons using the False Discovery Rate (FDR, Benjamini et al., 2001).

Results

Time budget at (T_0)

On average, the ferrets spent 3±1 min eliminating, 31±23 min on maintenance, 20.4±0.7 h inactive, 50±21 min eating, 24±17 min drinking, 2±2 min on play, 1±1 min on agonistic behaviour, 6±7 min on exploration and 1 min doing other things at T_0 (Figure 5.2).

Behavioural observations

Elimination

The ferrets in SC, npEC and pEC showed similar changes (ΔT_0 to T_1) in elimination behaviour (3±1 min to 2±1 min, F_2=0.058, P=0.943; Figure 5.3a).

Maintenance

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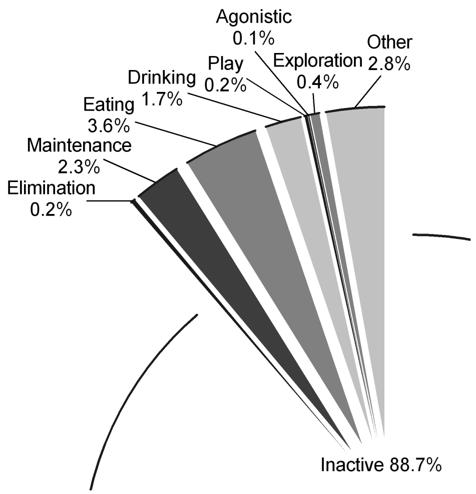

Figure 5.2 The average behavioural time budget of the ferrets in this study (average % of T_0, N=18).

The ferrets in SC, npEC and pEC showed similar changes (ΔT_0 to T_1) in maintenance behaviour (31±23 min to 48±23 min, F_2=1.524, P=0.250; Figure 5.3b).

Inactivity

The ferrets in SC, npEC and pEC showed similar changes (ΔT_0 to T_1) in inactivity (20.4±0.7 h to 20.2±0.8 h, F_2=0.165, P=0.850; Figure 5.3c).

There were differences in the location where the ferrets spent their inactive time at T_1. The ferrets in SC, npEC and pEC spent equal amounts of time inactive in the sawdust (2±3 h, F_2=1.430, P=0.270; Figure 5.3d), but not all groups of ferrets spent equal amounts of time inactive in the bucket (F_2=69.158, P<0.001; Figure 5.3d). More specifically, the ferrets in SC spent more inactive time in the bucket (17±4 h), than the ferrets in npEC (0±0 h, P<0.001) and pEC (3±1 h, P<0.001). This is also reflected in the preferences that were observed in each group of ferrets. The ferrets in SC showed a preference for being inactive in the bucket (17.1±4.4 h) over the sawdust (2.8±4.4 h; F_1=87.314, P<0.001). The ferrets in npEC showed a preference for being inactive in the ferret ball (18.1±2.0 h) over the sawdust (2.0±1.7, P<0.001) and the bucket (0.3±0.3, P<0.001)(F_2=239.737). The ferrets in pEC showed a preference for being inactive in the hammock (17.3±2.0 h) over the bucket (2.8±1.3 h, P<0.001) or the sawdust (0.2±0.4 h, P<0.001)(F_2=51.666).

Table 5.2 Ethogram used for the observation of the ferrets

Behaviour pattern	Contains	Description
Elimination	Urinating & defecating	Ferret backs up (often into the corner), squats with rear legs spread slightly apart, back slightly arched and tail raised over the back and urinates or defecates. Urine and faeces are not buried
Ingestion	Eating from bowl	Ferret takes pellet from bowl in her mouth and ingests it
	Eating from foraging ball	Ferret pushes foraging ball, causing pellets to fall out, she then takes a pellet from the sawdust in her mouth and ingests it
	Drinking from bowl	Ferret touches the water with her mouth and drinks from the water bowl
	Drinking from bottle	Ferret touches the nipple from the bottle with her mouth and drinks from the bottle
Maintenance	Scratching self	Ferret quickly moves the paw from her hind leg over her body
	Grooming	Ferret licks and gently nibbles her fur
Enrichment interaction		Ferret is in interaction with the bucket, ferret ball, golf ball, hammock, foraging ball or water bowl
Inactive		Ferret sits or lies still in sawdust, bucket, ferret ball or hammock
Play	Object play	Ferret pounces, shakes, nuzzles, chews, chases, picks up or drags an object around
	Social play	Ferret runs towards or away from another ferret with a jerking, bouncing gait; biting is short and inhibited; performed with an open mouth play face; reciprocal in nature
Agonistic	Dragging	Ferret drags another ferret around while holding the neck region of the other ferret with her mouth
	Shaking	Ferret bites another ferret and holds on while shaking her head
	Neck bite	Ferret bites another ferret in the neck region
	Chase	Ferret pursues another ferret that is actively trying to move away from it
	Aggressive "play"	Ferret performs high intensity social play behaviour, characterised by pronounced biting, little reciprocity and little bouncing
	Lunge attack	Ferret lunges towards the neck of another ferret with its mouth open
Exploration	Rearing	Ferret stands upright on her hind legs, either without support or against a wall or enrichment item
	Scratching	The ferret scratches with its front paws in the sawdust or against the wall, another ferret or enrichment object
	Tunnelling	Ferret puts its head down in sawdust and pushes forward using its hind legs, creating a tunnel in the sawdust

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Enrichment interaction

The ferrets in SC, npEC and pEC showed different changes in enrichment interaction (F_2=47.875, P<0.001; Figure 5.3e) from T_0 to T_1. More specifically, the ferrets in SC increased their time spent interacting with enrichment (3±2 h to 18±5 h) more than the ferrets in npEC (14±0 h to 19±2 h, P<0.001) and pEC (20±1 h to 21±1 h, P<0.001). Additionally, the ferrets in npEC showed a greater increase in enrichment interaction than the ferrets in pEC (P=0.010).

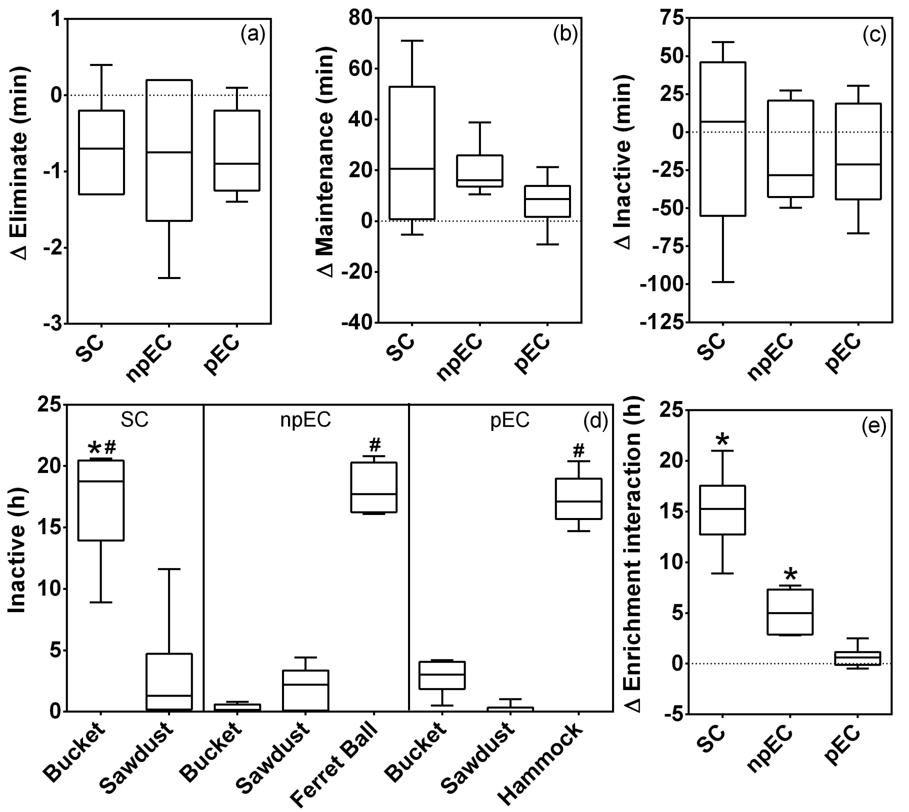

Figure 5.3 The change in time the ferrets in standard conditions (SC), with non-preferred enrichment (npEC) and with preferred enrichment (pEC) spent (a) eliminating, (b) on maintenance, (c) inactive and (e) on enrichment interaction from T_0 to T_1; (d) the absolute time the ferrets spent inactive in the bucket, sawdust, ferret ball and/or hammock (median±IQR, N=6, * indicates a significant difference from all other groups with P<0.05, # indicates a preference within a group).

Eating

The ferrets in SC, npEC and pEC showed a similar change in eating behaviour (50±21 min to 37±17 min, F_2=1.981, P=0.172; Figure 5.4a).

The ferrets in SC, npEC and pEC showed equal amounts of time eating from the bowl at T_1 (26±15 min, F_2=0.274, P=0.764, Figure 5.4b). However, there was a difference between the groups in the time the ferrets spent eating food from the sawdust (F_2=4.508, P=0.029, Figure 5.4b). More specifically, the ferrets in npEC (22±20 min) spent more time eating from the sawdust than the ferrets in pEC (1±2 min, P=0.010). This is also reflected in the preferences that were observed. The ferrets in npEC had no preference for eating from the bowl or the sawdust (bowl: 23±10 min, sawdust: 22±20 min, F_1=0.008, P=0.930), while the ferrets in SC showed a preference for eating from the bowl over the sawdust (bowl: 26±15 min, sawdust: 8±6 min; F_1=7.662, P=0.020). The ferrets in pEC showed a preference for eating from the bowl over the sawdust and the foraging ball (bowl: 30±20 min, sawdust: 1±2 min; foraging ball=2±3 min F_2=11.089, P=0.001 for both).

Drinking

The ferrets in SC, npEC and pEC showed different changes in drinking behaviour (F_2=20.038, P<0.001; Figure 5.4c) from T_0 to T_1. More specifically, the ferrets in SC greatly decreased their time spent on drinking (44±15 min to 11±3 min), while the ferrets in npEC did not change their time spent on drinking (17±5 min to 18±5 min, P<0.001). Also, the ferrets in pEC decreased their time spent on drinking less than the ferrets in SC (12±3 min to 5±3, P<0.001).

The ferrets also spent different amounts of time drinking from the bottle at T_1 (F_2=40.477, P<0.001, Figure 5.4d). More specifically, the ferrets in pEC (0±1 min) drank less from the bottle than the ferrets in SC (11±3 min, P<0.001) and npEC (18±5 min, P<0.001). Additionally, the ferrets in SC drank less from the bottle than the ferrets in npEC (P=0.003). This is also reflected in the preferences that were observed for drinking location. The ferrets in pEC preferred drinking from the bowl (5±2 min) over the bottle (0±1 min, F_1=19.322, P=0.001).

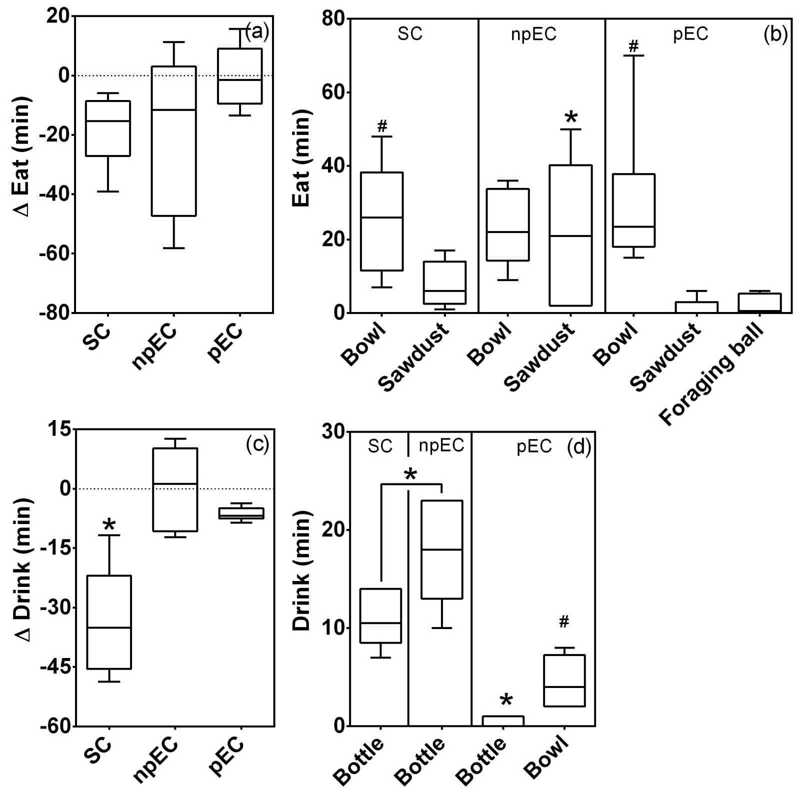

Figure 5.4 The change in time the ferrets in standard conditions (SC), with non-preferred enrichment (npEC) and with preferred enrichment (pEC) spent (a) eating and (c) drinking from T_0 to T_1; the absolute time the ferrets spent (b) eating from the bowl, sawdust and foraging ball and (d) drinking from the bottle or bowl (median±IQR, *N*=6, * indicates a significant difference from all other groups with *P*<0.05, # indicates a preference within a group).

Play

The ferrets in SC, npEC and pEC showed similar increases in play behaviour (2±2 min to 5±6 min, F_2=2.562, *P*=0.110; Figure 5.5a).

When play behaviour is split up in object and social play, all groups showed an equal increase in object play behaviour (2±2 min to 3±3 min, F_2=0.314, *P*=0.735; Figure 5.5b), but showed different changes in social play behaviour (F_2=3.585, *P*=0.021; Figure 5.5c). More specifically, the ferrets in pEC showed an increase in social play behaviour from T_0 to T_1 (0±0 min to 5±6 min), while the ferrets in SC (0±0 min to 1±0 min, *P*=0.0.031) and npEC (0±0 min to 0±0 min, *P*=0.040) did not.

Agonistic

The ferrets in SC, npEC and pEC showed a different change in agonistic behaviour (F_2=3.107, *P*=0.042; Figure 5.5d) from T_0 to T_1. More specifically, the ferrets in SC showed an increase in agonistic behaviour (from 2±2 min to 10±9 min, Figure 5.5d), which the ferrets npEC (0±0 min to 1±1 min, *P*=0.040) and pEC (0±0 min to 1±2 min, *P*=0.044) did not show.

Exploration

The ferrets in SC, npEC and pEC showed similar changes in exploration from T_0 to T_1 (6±7 min to 4±2 min, F_2=3.019, *P*=0.079; Figure 5.5e). When exploration is split up in rearing, scratching and tunnelling, the ferrets showed different changes in rearing (F_2=6.085, *P*=0.012; Figure 5.5f), but not in scratching (3±6 min to 3±2 min, F_2=2.719, *P*=0.098; Figure 5.5g) and tunnelling (1±1 min to 1±1 min, F_2=3.180, *P*=0.071; Figure 5.5h). More specifically, the ferrets in pEC showed a decrease in rearing from T_0 to T_1 (3±2 min to 0±0 min) that the ferrets in npEC (0±1 min to 1±1 min, *P*=0.003) and SC (1±2 min to 1±0 min, *P*=0.066) did not show.

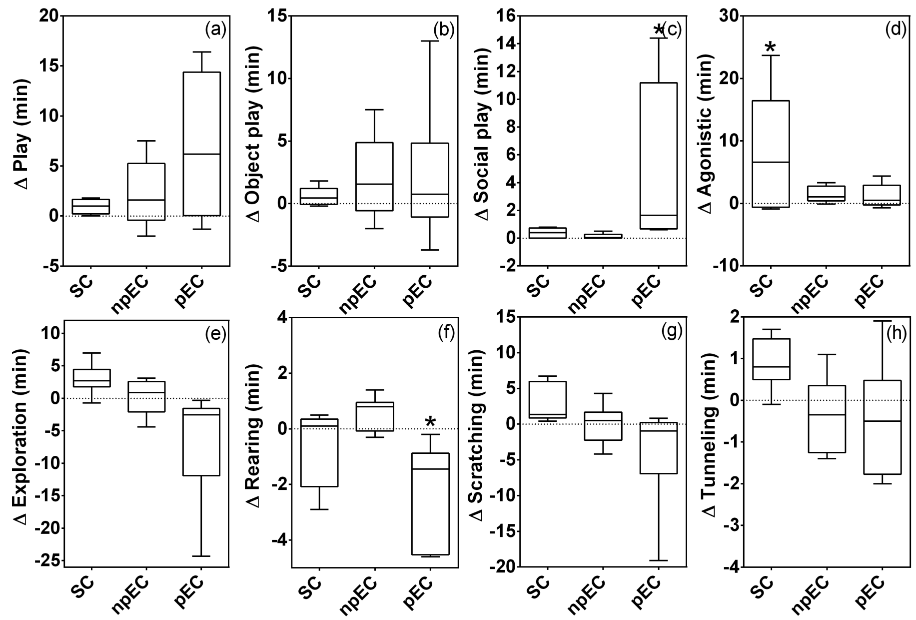

Figure 5.5 The change in time the ferrets in standard conditions (SC), with non-preferred enrichment (npEC) and with preferred enrichment (pEC) spent (a) playing, (b) playing with an object, (c) on social play (d) on agonistic behaviour, (e) exploring, (f) rearing, (g) scratching, and (h) tunnelling from T_0 to T_1; (median±IQR, *N*=6, * indicates a significant difference from all other groups with *P*<0.05).

Physiological parameters

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Weight

The ferrets in SC, npEC and pEC showed similar changes in weight (812±83 g to 923±78 g, F_2=2.327, P=0.132; Figure 5.6a)

N/L-ratio

The ferrets in SC, npEC and pEC showed similar changes in N/L-ratio (0.54±0.14 to 0.52±0.16, F_2=2.371, P=0.127; Figure 5.6b) from T_0 to T_1.

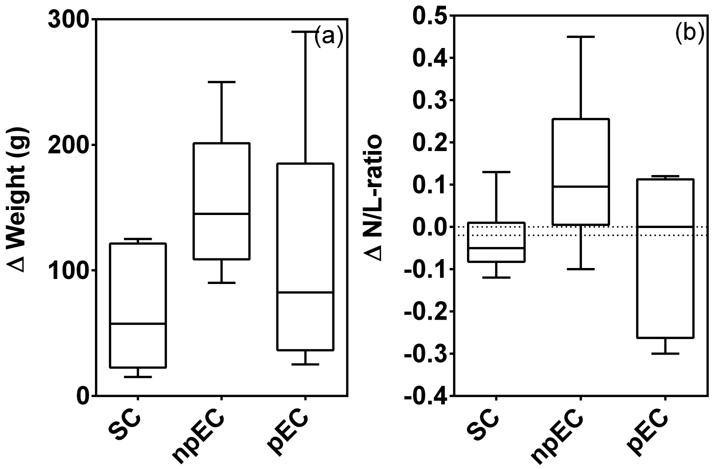

Figure 5.6 The change in (a) weight and (b) N/L-ratio from T0-T1 (median±IQR) of ferrets in standard conditions (SC), with non-preferred enrichment (npEC) and with preferred enrichment (pEC) (median±IQR, N=6).

Discussion

The objective of this study was to investigate the effects of the provision of preferred and non-preferred enrichment items on behavioural and physiological parameters in ferrets. The ferrets that were provided with (preferred) enrichment showed different changes in play, agonistic and exploration behaviour, but showed similar or very small changes in time spent eliminating, maintenance, inactive behaviour, enrichment interaction, eating and drinking, weight and N/L ratio, compared to ferrets housed in standard conditions.

Conform expectations, the ferrets provided with preferred enrichment showed the largest increase in social play behaviour. Play behaviour is regarded as a positive welfare indicator as it does not occur under stress, it acts as a reward, brings psychological benefits and is contagious (Held and Špinka, 2011; Oliveira et al., 2010). Also conform expectations, the ferrets in standard conditions showed an increase in agonistic behaviour that was not observed in either enriched group. A general increase in agonistic behaviour in maturing group-housed ferrets is not surprising, as feral ferrets show intrasexual territoriality (Clapperton, 1985). Agonistic behaviour is regarded as a negative welfare indicator as it is a major cause of social stress (Blanchard et al., 2001). Our results on play and agonistic behaviour are in agreement with a study in mink, where the occurrence of play behaviour was increased and the incidence of agonistic behaviour was reduced by the provision of cage enrichment (Meagher et al., 2014). After eight weeks of standard housing conditions, unreciprocated play invitations often escalated in aggression in these ferrets. Potentially, the preferred enrichment reduced play frustration in the ferrets, by providing more choice in activity and/or providing an alternative outlet for frustration, which may have allowed for more positive social interaction between the ferrets. Additionally, it is possible that the enrichments provided compartmentalisation of the environment, thereby reducing agonistic encounters (Desforges et al., 2016). An alternative explanation might be that the hammocks and ferret balls (both preferred resting places) allowed the ferrets to choose whether they wanted to sleep together and with whom, without having to give up their preferred resting place, possibly reducing frustration and therefore reducing aggression (Arnone and Dantzer, 1980). This could be further investigated by comparing the aggression showed by ferrets that are provided with only hammock/ferret ball versus ferrets that are provided with two hammocks/ferret balls.

Aside from changes in social interaction, the ferrets with preferred enrichment showed very little rearing behaviour at T_1, whereas this was observed more in the other two groups at this time point. This reduction in exploration time is in line with findings in rats, which reduced exploration behaviour directed at the outside of the cage when presented with enrichment in their cages (Abou-Ismail et al., 2010). As this effect was not seen in the ferrets that were given non-preferred enrichment, and the number of enrichment items were the same in preferred and non-preferred enrichment conditions, the effects of the enrichment on rearing behaviour might be due to the value of the provided enrichment and not due to an increase in environmental complexity (Reijgwart et al., 2016). However, it should be taken into account that rearing behaviour was performed for very short periods of time and the differences between the groups were therefore also very small albeit statistically significant. This raises questions on the biological relevance of this difference in rearing behaviour between the groups. Additionally, the two other recorded exploratory behaviours (i.e. scratching and tunnelling) did not show the expected reduction after provision of preferred enrichment, as was seen in mink and rats (Dallaire et al., 2012, Abou-Ismail et al., 2010). In mink, repetitive and intensive scratching at the cage wire mesh with the front paws is a commonly observed repetitive behaviour (Hansen, 1993; Mason, 1993) and is proposed to be indicative of frustration (Mason, 1991). The behavioural motivation behind tunnelling is unknown, i.e. whether it was part of exploratory behaviour or whether it was performed as a part of play or scent-marking behaviour is unknown to the authors, possibly resulting in misclassification of tunnelling behaviour as exploratory behaviour.

The lack of difference in change in inactivity between the groups of ferrets is in agreement with a study in mink (Vinke et al., 2005). Additionally, the ferrets were as inactive as was expected based on the available values of inactivity reported in pet ferrets (Fisher, 2006). However, it should be noted that we were unable to separate time spent sleeping from time spent inactive awake, as the ferrets were not always visible, and when they were, the video footage was not detailed enough to allow us to accurately identify whether the ferrets had their eyes open (i.e. being inactive awake) or closed (i.e. sleeping). In a study in mink where inactive behaviour was studied in more detail, sleeping, the time spent inactive in the nest box (fear or anxiety induced hiding) and the time spent lying awake (a boredom-like state) were hypothesised to represent different motivations (Meagher and Mason, 2012; Meagher et al., 2013). Therefore, it is recommended to separate these three types of inactivity in future studies. Possibly, these differences in valence of inactivity can also be identified in ferrets. Possibly only one of these subtypes of inactivity was reduced in the enriched ferrets in our study. We did notice a clear preference for sleeping in the ferret ball (npEC) or hammock (pEC) over the bucket, which was expected based on the results of the consumer demand study (Reijgwart et al., 2016), possibly indicating that the standard housing conditions do not provide the ferrets with a suitable resting place.

It is not surprising that the ferrets' weight and N/L ratio reacted similarly to the ferrets' inactivity, as these parameters are closely linked (Mormède et al., 2007). For example, the HPA-axis is not only influenced by the provision of enrichment (e.g. Hansen et al., 2007), but can also be activated in response to increased activity (e.g. Girard & Garland 2002). In turn, HPA-axis activity can affect the N/L ratio (Davis et al., 2008; Hansen and Damgaard, 1991) and the weight of an animal (e.g. (Hansen et al., 2007). Ultimately, there is a great lack of consistency in the effects of enrichment on the HPA-axis and these physiological data should be viewed in light of the limitations that come with the interpretation of HPA-axis activity (Rushen, 1991). Nonetheless, it is possible that spikes in N/L ratio were missed due to the eight-week sampling interval, if the cortisol response (and, with some delay, subsequent leukocyte response) had adapted within this period, which has been suggested to occur in chickens and dogs (Hennessy et al., 2001; McFarlane and Curtis, 1989; Romero, 2004).

The ferrets' increased interaction with enrichment in standard conditions and with non-preferred enrichment were largely due to the ferrets choosing to sleep in the bucket or the ferret ball, respectively, instead of the sawdust. As a result, these changes in enrichment interaction times are unlikely to provide any information on the affective state of the animals and are probably an effect of group dynamics (i.e. the first ferret randomly choosing a resting place, where the rest joins). Likewise, no conclusions can be drawn from the observations on eating and drinking behaviour. The ferrets with non-preferred enrichment most likely ate longer from the sawdust than the other groups of ferrets because a ferret in this group dug in the food bowl, resulting in more pellets that could be eaten from the sawdust. Similarly, the foraging ball could only hold a limited amount of pellets, making it impossible for the ferrets to forage enough food from this ball. Nonetheless, the ferrets emptied the foraging ball (which was refilled) daily. For drinking behaviour, the actual water consumption was not measured, which prevented us from determining whether the reduced drinking time of ferrets in standard conditions was a result of more efficient drinking or reduced water consumption. However, when provided a choice, as was the case for ferrets in pEC, a clear preference was observed for drinking from the bowl, while drinking from the bottle reduced to a minimum. A similar preference has been observed in mink and rabbits, possibly because a water bath/bowl allows for a more natural way of drinking (Cooper and Mason, 2000; Tschudin et al., 2011).

There were some methodological limitations to this study. Performing 24-hour behavioural observations was the best method for this species, but is also very labour-intensive, therefore choices had to be made regarding the times at which the observations were made (e.g. no observations were done right after provision of the enrichment, making it impossible to assess the short-term effects) and how many days per observation point were sampled (i.e. one 24-hour period per observation period is not optimal). Additionally, for each housing condition only one group of ferrets was observed due to time, physical and ethical restraints. It is preferable to observe more groups of ferrets per housing conditions as the ferrets within a group may influence each other's behaviour. Finally, the effects of our enrichments on the behaviour and physiology of the ferrets were not always as marked as those found in other studies and some behaviours were performed for very short periods of time, raising questions on the biological relevance of the small differences in expression for these parameters. These limitations highlight the necessity for further research to identify enrichments that can have a positive effect on behavioural and physiological parameters in laboratory ferrets. Future research should therefore focus on replicating the results found in this study, potentially also exploring the effects of other enrichments, as well as the effects of removing enrichment, group size and husbandry conditions. Additionally, other measures that might give an indication of the welfare state of the animals, such as measuring "judgement biases" (Mendl et al., 2009) could be considered.

Conclusion

Overall, the ferrets that were provided with (non-)preferred enrichment showed no increase in agonistic behaviour, whereas the ferrets that were housed in standard conditions did. Additionally, the provision of preferred enrichment (hammocks, water bowl and foraging ball) to laboratory ferrets resulted in an increase in social play behaviour and a reduction in rearing behaviour, changes which were not observed in standard housing conditions or when non-preferred enrichment was provided. Since these changes have been linked to positive welfare changes in other species, provision of these preferred enrichments (hammocks, a water bowl and foraging ball) should be considered as these may help to refine studies using laboratory ferrets.

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PART II

Recognition of pain and discomfort in laboratory ferrets





Chapter 6

A review on the evaluation of pain and other forms of discomfort in laboratory ferrets

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Abstract

Ferrets (*Mustela putorius furo*) are regularly used as models in biomedical research. They are ideal to model human respiratory diseases such as influenza, in particular because of similarities in lung morphology and (patho)physiology. However, this type of research poses a considerable risk of causing pain and other forms of discomfort to the animal. Parameters that might be used for evaluation of pain in general and in laboratory ferrets specifically are identified based on a review of existing literature. The limitations of the identified parameters are discussed, revealing the need for specific, consistent and practically applicable parameters. A promising new addition to the toolbox could be the identification of facial expressions that occur when a ferret is experiencing pain. Until then, weight loss, changes in respiration and decreased activity, play and ingestion are the most promising parameters to evaluate discomfort in laboratory ferrets.

Introduction

Ferrets (*Mustela putorius furo*) are used as models in biomedical research as the morphology and (patho)physiology of their respiratory system are remarkably similar to that of humans, making ferrets ideal to model several human respiratory diseases such as cystic fibrosis (Sun et al., 2010), *Coronavirus*-associated severe acute respiratory syndrome (Roberts et al., 2008) and influenza (Belser et al., 2011; Enkirch and von Messling, 2015; Tripp and Tompkins, 2009). In 2011, 91.7% of laboratory ferrets used in the European Union (2331 out of 2540 animals) were used for research into human diseases (European Commission, 2013). Studies on diseases have the potential of causing severe discomfort, as the animals have to undergo a disease process. Despite the ferrets' suitability as an animal model, there is a shortage of studies specifically pertaining to the welfare of laboratory ferrets. Consequently, ferrets receive little mentioning in guidelines on the use of laboratory animals (European Commission, 2010; National Research Council, 2011).

Timely recognition and treatment of discomfort in animals used in research is paramount, not only for animal welfare but also for the quality and reliability of research. It is well recognised that discomfort may affect the outcome of studies, as it can affect the functionality of various organ systems, including the immune system (National Committee for the protection of animals used for scientific purposes, 2016; Poole, 1997). Thus, it is important to guard the welfare of laboratory animals and recognise and reduce the amount of discomfort experienced. The 3R's principle (Replacement, Reduction, Refinement) is utilised to focus our efforts on protecting the welfare of laboratory animals (Russell and Burch, 1959). While replacement and reduction of the use of ferrets as a research model are clearly important, they pertain more to the indirect improvement of animal welfare through their phasing out from experimentation. Refinement, on the other hand, directly impacts the animals' welfare as it entails minimisation of pain, suffering, distress or lasting harm that may be experienced by the animals. Therefore, this review will focus on the R of Refinement in ferret research, with particular emphasis on refinement through adequate monitoring and reduction of pain and other forms of discomfort.

In this review, discomfort is used to describe suffering, distress, lasting harm and/or pain. Pain is an important aspect of discomfort and is described as the unpleasant experience that often follows nociception, i.e. the process whereby information regarding the presence of tissue damage is transmitted from primary afferent nociceptors to the spinal cord, brainstem, thalamus, and subcortical structures. In contrast to nociception, pain can only be experienced following higher brain

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processing, during which nociceptive information is conveyed by the thalamocortical network (for an overview see Loeser and Melzack, 1999). To correctly diagnose and treat pain, precise and adequate pain assessment is important. When assessing pain, various aspects should be taken into consideration, i.e. the time course (acute versus chronic), intensity (mild to severe), origin (visceral, somatic, neuropathic, inflammatory) and clinical implications of the painful stimulus (physiologic versus pathologic).

However, pain assessment is not always straightforward, as animals cannot communicate their level of pain to us and (in particular prey animals) hide their pain (Short, 1998). Additionally, the signs might be too subtle or we might not be adequately familiar with the normal physiology and behaviour of the animal to recognise deviations (Mayer, 2007). Thus, as a general rule it should be assumed that procedures or conditions that would cause pain in humans are likely to also cause pain in animals, i.e. use the analogy principle (Lichtenberger and Ko, 2007). This principle, however, cannot be used as the sole guideline, as animals might also experience unexpected pain (i.e. pain not caused by a procedure or medical condition) and species differences have been found (e.g. animals recover quicker and seem to tolerate some disease conditions better than humans)(Morton and Griffiths, 1985). Therefore, it is necessary to be able to recognise the indicators of pain in a specific animal species.

Efforts to recognise pain and other forms of discomfort in laboratory animals are primarily focused on (patho)physiological and behavioural parameters. In this review, an overview of parameters for recognition of discomfort in laboratory animals and the possible application and limitations of these parameters for use in laboratory ferrets are given.

General changes

Descriptions of parameters that can be used for the evaluation of discomfort in (laboratory) animals in general can give an indication where to focus our efforts when evaluating discomfort in laboratory ferrets.

Physiological parameters

One way to assess discomfort is by measuring (patho)physiological parameters that change in response to discomfort. Nociception leads to activation of the sympathetic nervous system, which in turn leads to catecholamine release (epinephrine, norepinephrine), causing an increase in temperature, heart rate, respiratory rate, blood pressure and peripheral vasodilatation. Additionally, the Hypothalamic-Pituitary-Adrenal-axis (HPA-axis) will be activated: the hypothalamus produces antidiuretic hormone (ADH) and corticotrophin-releasing hormone (CRH), which stimulate the release of adrenocorticotropic hormone (ACTH) from the pituitary gland, resulting in the production of glucocorticoids (e.g. cortisol) in the adrenal cortex, causing an increased availability of glucose (which over time can cause weight loss) and suppression of the immune system. Other alterations that may be seen in animals experiencing discomfort include changes in mucous membrane colour due to hypoxemia and fluid and electrolyte imbalances (Gregory, 2008; Morton and Griffiths, 1985; Short, 1998; Stasiak et al., 2003).

Behavioural parameters

Discomfort can also be assessed through the measuring of behavioural parameters. Examples of behavioural parameters that change when an animal is experiencing discomfort are: (1) changes in posture/gait (e.g. protection of affected area, unsteady gait/lameness); (2) changes in grooming behaviour (e.g. lack of grooming, excessive biting/licking of a painful body part, piloerection); (3) changes in behaviour/reaction to stimuli (e.g. twitching/shivering, sluggish or absent reflexes, muscle flaccidity/rigidity/weakness, hypersensitivity to touch/sound/temperature change, decreased activity/lethargy or restlessness, decline in exploration and play, isolation/withdrawal, changes in temperament/aggression); (4) increased vocalising and (5) changes in ingestion (e.g. decreased food and water intake and teeth grinding)(Gregory, 2008; Mayer, 2007; Morton and Griffiths, 1985; Short, 1998; Stasiak et al., 2003; Weary et al., 2006).

Facial expressions

Aside from physiological and gross behavioural changes, signs indicative of discomfort may also be more subtle (e.g. changes in facial expression). Due to activation of the sympathetic nervous system an animals" pupils dilate when it is experiencing discomfort (Morton and Griffiths, 1985). Additionally, ocular squinting, sunken eyes and flattened ears have been associated with discomfort (Morton and Griffiths, 1985; Stasiak et al., 2003). These observations of facial changes can be summarised in a facial grimace scale, which was first developed for mice (Langford et al., 2010a), and has since been adjusted to be utilised on rats (Sotocinal et al., 2011), rabbits (Keating et al., 2012), horses (Dalla Costa et al., 2014; Gleerup et al., 2015a), cats (Holden et al., 2014), cattle (Gleerup et al., 2015b), sheep (McLennan et al., 2016), piglets (Di Giminiani et al., 2016) and lambs (Guesgen et al., 2016). Overall, these grimace scales list changes in the eyes, cheek, nose, whiskers and ears as indicative of discomfort. These grimaces are hypothesised to serve in the soliciting for social support from conspecifics that can potentially help to diminish the experienced discomfort (Langford et al., 2010b). Additionally, a grimace might serve to minimise sensory input by closing the eyes, ears and nose (Susskind et al., 2008).

Changes in ferrets

Some of the physiological and behavioural parameters that are listed as useful for the evaluation of discomfort in (laboratory) animals in general have also been documented for ferrets.

Physiological parameters

It is expected that through activation of the sympathetic nervous system and the HPA-axis, a ferrets' temperature, cardiac rate, respiratory rate, blood pressure and cortisol levels increase when experiencing discomfort, while peripheral blood flow and bodyweight decrease (see Table 6.1 for reference values). When using these parameters for the evaluation of discomfort in ferrets, it has to be kept in mind that ferrets are sexually dimorphic: adult males are twice the size of females (Wolfensohn and Lloyd, 2008). Additionally, a seasonal 30-40% weight gain and loss in autumn and winter, respectively, is normal (Wolfensohn and Lloyd, 2008).

Behavioural parameters

Our knowledge of discomfort behaviours in ferrets stems primarily from clinical descriptions and personal experiences from owners, caregivers and veterinarians. Possible discomfort related behaviours in ferrets are: (1) pulled up abdomen (Figure 6.1a), hunched back (Figure 6.1b) – bear in mind that a slightly hunched back is normal in ferrets), stiff gait with head elevated and extended forward, a change in sleeping position preference; pulled up and curved tail (Figure 6.1c); (2) a lack of grooming (Figure 6.1d), piloerection of the tail (Figure 6.1e); (3) shivering/trembling in the presence of normal body temperature, inactivity/lethargy; (4) high pitched vocalisations when handled; (5) bruxism when presented with food, and decreased appetite/anorexia (Figure 6.1f, Table 6.2, Brown, 1997; Johnston, 2005; Lichtenberger and Ko, 2007; Mayer, 2007; Pollock, 2002; Sladky et al., 2000).

Table 6.1 Physiological indicators of discomfort (1=Jeans 1994, 2=Schoemaker 2002, 3=Wolfensohn and Lloyd 2008, 4= van Zeeland and Schoemaker n.p., 5=Hein et al 2012)

Parameter	Reference value for ferrets
Temperature[1-3]	37.8-40 °C
Cardiac rate[2,3]	200-250 beats/min
Respiratory rate[1-3]	33-36 /min
Blood pressure (diastole/systole)[3,4]	51-87/95-155 mmHg
Peripheral blood flow[3,4]	Warm extremities, pink mucous membranes
Body weight (females/males)[3]	600-2000 grams
Cortisol[5]	6.6 nmol/L

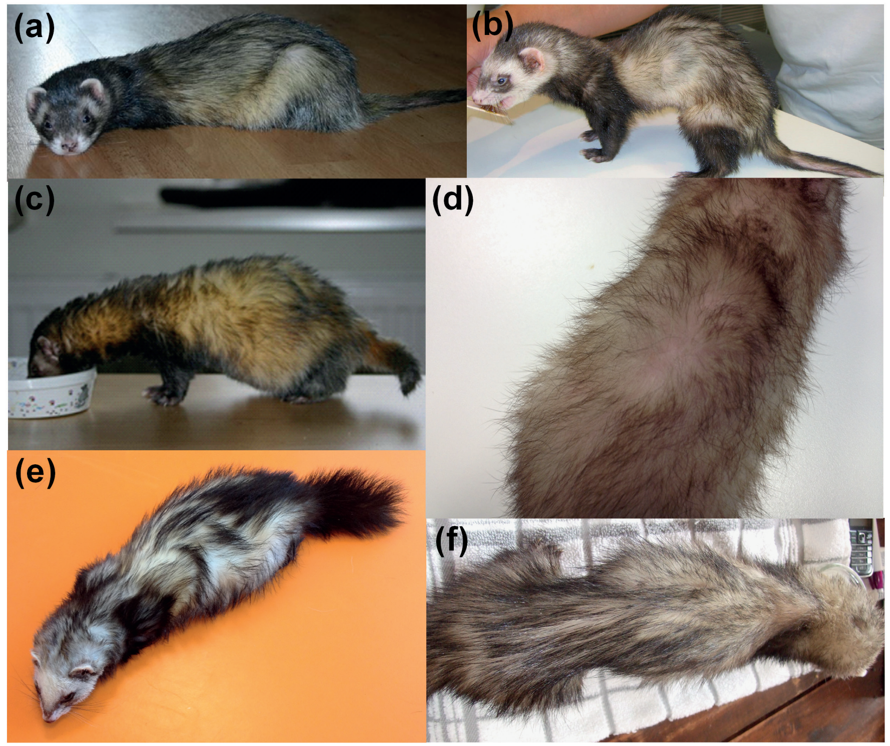

Figure 6.1 (a) ferret lying flat on the floor with the abdomen pulled up due to abdominal pain; (b) ferret with a hunched back due to abdominal pain; (c) ferret with a pulled up, curved tail due to nephritis; (d) ferret with a poor quality hair coat due to decreased grooming activity; (e) ferret with piloerection of the tail; (f) ferret with severe cachexia due to prolonged anorexia. Pictures are a courtesy of Birgit van der Laan, NL(a, c) and Hanneke Roest, De Frettenkliniek, Giessen, NL (b, d, e, f).

Table 6.2 Behavioural indicators of discomfort.

Parameter	Specification
Posture	Pulled up abdomen, excessive hunched back, protection of affected area
Gait	Stiff
Grooming	Poor quality hair coat due to lack of grooming
Twitching/shivering	In the presence of a normal body temperature
Activity	Increased sleeping (more/less curled up)/lethargy
Vocalisations	Scream, whine/moan, hiss, grunt
Piloerection	In particular piloerection of the tail (which is sometimes also pulled up and curved)
Bruxism	When presented with food
Appetite	Decreased food/water intake, leading to anorexia

Facial expressions

To the authors' knowledge, grimace scales have not yet been developed as an indicator for discomfort in ferrets. Many people even doubt whether ferrets would show a grimace as their ancestors are solitary animals. However, as hypothesised, the grimace could serve to limit sensory input and would therefore also serve a function in solitary animals. As the changes described in the grimace scales are generally the same over different species, it is expected that ferrets show comparable grimaces. In support of this expectation, a strained facial expression, squinting and dull, half-open, non-inquisitive eyes have been suggested as indicators of discomfort in ferrets (Mayer, 2007).

Limitations for use in ferrets

Many of the physiological and behavioural parameters that are documented for ferrets unfortunately come with limitations for the application in laboratory ferrets: they are unspecific for pain, they are inconsistent and often impractical in a laboratory setting.

Specificity

In general it can be stated that all parameters show a lack of specificity. Changes in physiological parameters (temperature, cardiac rate, respiratory rate, blood pressure, peripheral blood flow, bodyweight and cortisol) can also occur when the animal is active or in distress rather than experiencing discomfort. Changes in behavioural parameters (e.g. shivering or bristling of the tail fur) can be observed when waking up/excited or presented with an alarming stimulus (Johnston, 2005) Grimaces can also be observed during agonistic encounters (Defensor et al., 2012) and squinting of the eyes can also occur in response to bright lighting, when waking up or when anesthetised (Descovich et al., 2017). Additionally, a facial grimace can only be observed when an animal is experiencing pain with a particular severity and duration (between 10 minutes and 24 hours), so the absence of a grimace does not necessarily mean the absence of discomfort (Langford et al., 2010a; Miller and Leach, 2015). Therefore, all indicators of discomfort should always be viewed within the context, whereby the likelihood of the behaviour being related to discomfort increases if (potentially) discomfort-evoking stimuli are present and alternative explanatory contexts are absent. Additionally, none of the described parameters should be used on its own.

Consistency

A ferrets' behavioural expression of discomfort is not always consistent. For example, Johnston (2005) reported that ferrets experiencing discomfort will prefer to stay curled into a ball, whereas Brown (1997), Mayer (2007) and Pollock (2002) report the opposite. Another example is grooming: both an increase and a decrease are reported in literature. These conflicting observations might be explained when a different type and location of pain results in different behavioural responses. For example, the presence of abdominal pain could result in the ferret preferring to curl up in a ball to protect the painful area, while back pain might prevent the ferret from doing this. Also, when a specific joint or skin lesion causes a ferret pain, this area might be groomed excessively, while a ferret with a more general cause of pain, e.g. arthritis, might stop grooming itself as the movement causes pain. In addition, behaviour in response to acute and chronic pain may be different.

Practical limitations

There are some practical limitations to using changes in physiological parameters to evaluate discomfort in laboratory ferrets. For instance, it is possible that the restraint required to measure these parameters will itself influence the readings (Weary et al., 2006) and parameters such as blood pressure are difficult to measure in (awake) ferrets (Lichtenberger and Ko, 2007; van Zeeland and Schoemaker). Additionally, in some laboratory settings, it might be impractical or even impossible to measure physiological parameters. For example, when ferrets are used to model infectious diseases such as influenza, direct access to the animal might be limited due to the ferret being housed in an isolator. These housing systems also complicate the measurement of behavioural parameters as visual inspection is hindered and space is limited, thereby restricting the ferrets' normal activity and movements. Observation of behavioural changes is further limited by the high percentage of time spent sleeping: undisturbed ferrets can spend 70-80% of their day sleeping (Jha et al., 2006; Chapter 5), which might lead to oversight of a decrease in activity. Additionally, a decrease in activity might not be noticed due to the highly active reaction to stimulation that ferrets show (Jha et al., 2006). These factors render it unlikely that behavioural parameters such as inactivity will provide adequate information. Furthermore, recognition of behavioural indicators of discomfort may be complicated by the ferrets' high tolerance for discomfort: they will give little warning of illness due to their stoic nature (Plant and Lloyd, 2010).

Most of these practical limitations can be solved by implantation of a telemetry device. These devices provide the option for more continuous and remote monitoring of physiological and behavioural parameters, but implantation of a telemetry device is invasive and comes with the risk of considerable discomfort. Additionally, the interpretation of the data necessitates a good general knowledge of normal species-specific and individual behaviour.

In contrast to the physiological and behavioural parameters, evaluation of facial expressions is practically applicable in a laboratory setting, as the ferrets can be observed from a distance. Additionally, assessors can easily and reliably be trained to use a grimace scale (Leach et al., 2012). Also, a study in rats showed that only a short period of observation is needed to accurately discriminate animals that are experiencing discomfort from animals that are not (Leung et al., 2016).

Conclusion

The long list of limitations regarding the application of physiological and behavioural parameters for the evaluation of discomfort in laboratory ferrets emphasises the need for more specific and consistent parameters that are also practically applicable in a stoic, inactive animal in restricted housing conditions. A new addition to the toolbox for the evaluation of discomfort in laboratory ferrets might be facial expressions. Future efforts should therefore involve research into the possibility to develop a Ferret Grimace Scale. Until then, weight loss, changes in respiration and decreased activity, play and ingestion are the most promising parameters to evaluate pain and other forms of discomfort in laboratory ferrets, provided that they are combined and viewed within the context.

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Chapter 7

Pain recognition in ferrets: an owner survey

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Introduction
To assess the applicability of various physiological and behavioural parameters for the recognition of pain in laboratory ferrets, ferret owners were asked to describe what they observe when their ferret(s) is/are in pain, using an online survey.

Materials and methods
The survey was spread through social media, and sent to ferret vets, ferret societies and ferret owners throughout the world. The survey was part of a collaboration with Sarah Talbot (Charles Sturt University, Thurgoona, Australia). The survey consisted of four parts: one general part (11 questions), one part on personality (8 questions), one part on housing and enrichment (10 questions) and a final part on pain recognition (11 questions, see appendix 1). In the pain section, ferret owners were asked to indicate which changes in the following parameters – in their opinion – were indicative of discomfort in their ferret(s): breathing, body weight, posture, grooming, sleeping, demeanour, vocalisations, ingestion, facial expression, and other (which needed to be specified further, if applicable). One or multiple suggestions could be marked, following which the owners were asked to provide an exact description of the changes they noticed. The survey was online for three months (May-July) in 2015.

Results
The part of the survey regarding questions on pain recognition was completed by 404 ferret owners from 16 different nationalities (AR, AU, BE, BR, CA, CZ, DE, FI, HR, JP, MT, NL, PL, SI, UK and US; Figure 7.1).

Physiological parameters
Ferret owners indicated that their ferrets' weight decreases (*N*=296/404, 95%) and their breathing becomes shallower (*N*=187/404, 68%), faster (*N*=178/404, 59%) and/or more difficult (*N*=163/404, 59%) when they are experiencing pain. (Table 7.1).

Behavioural parameters
All behavioural parameters found in the literature were also recognised by ferret owners. Most owners indicated that their ferret shows a change in demeanour (*N*=382/404, 95%). These owners specified to observe diminished activity (*N*=335/382, 88%) and play (*N*=323/382, 85%) in their ferrets. In line with these owner observations, the majority of owners specified to see an increase in sleeping behaviour (*N*=279/331, 84%). Additionally, a decrease in ingestion (*N*=302/341, 89%) and grooming (*N*=198/277, 71%) were reported. The specifications for changes in posture (*N*=367/404, 91%) and vocalisation (*N*=315/404, 78%) were more varied. (Table 7.2)

Facial expression
A majority of the owners (*N*=327/404, 81%) indicated to observe changes in the facial expression of their ferrets when they are experiencing discomfort. More specifically, the ferret owners indicated that in particular squinting the eyes (*N*=141/307, 46%), pulling back/down the ears (*N*=106/327, 32%) and drooping/pulled back whiskers (*N*=58/327, 31%) are indicative of discomfort. (Table 7.3)

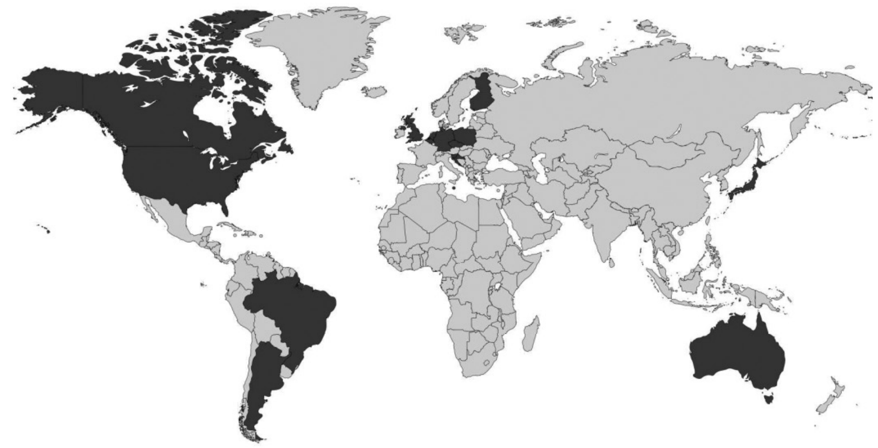

Figure 7.1 World map with the nationalities of the respondents highlighted (AR, AU, BE, BR, CA, CZ, DE, FI, HR, JP, MT, NL, PL, SI, UK and US).

Table 7.1 Number and percentage of respondents (*N*=404) that positively identified changes in physiological parameters as indicators of discomfort in their ferret(s).

Category	*N*	%	Specification	*N*	%
Weight	312	77%	Decrease	296	95%
			Increase	31	10%
Breathing	277	69%	Shallower	187	68%
			Faster	178	64%
			More difficult	163	59%
			Slower	81	29%
			Deeper	61	22%
			Other	28	10%

Table 7.2 Number and percentage of respondents (*N*=404) that positively identified changes in behavioural parameters as indicators of discomfort in their ferret(s).

Category	*N*	%	Specification	*N*	%
Posture	367	91%	Hunched	211	57%
			Shivering	177	48%
			Twitching	119	32%
			Tucked belly	113	31%
			Piloerection	110	30%
Grooming	277	69%	Less frequent	198	71%
			More frequent	80	29%
Sleeping	331	82%	More	279	84%
			More curled up	140	42%
			Change in timing	125	38%
			More on belly	72	22%
			Shorter	62	19%
			Less curled up	48	15%
			Less frequent	38	11%
			Less on belly	19	6%
Demeanour	382	95%	Less active	335	88%
			Less play	323	85%
			Less inquisitive	251	66%
			Slower walking pace	237	62%
			More biting	188	49%
			More teeth baring	67	18%
Vocalisation	315	78%	Scream	215	68%
			Whine/moan	154	49%
			Hiss	129	41%
			Grunt	81	26%
			Bark	62	20%
			Dook/Chuckle/Buck	53	17%
Ingestion	341	84%	Eating less	302	89%
			Drinking less	206	60%
			Eating less frequently	165	48%
			Eating slower	133	39%
			Drinking less frequently	121	35%
			Eating more selectively	114	33%
			Drinking slower	88	26%
			Drinking more	81	24%
			Drinking more frequently	65	19%

Table 7.3 Number and percentage of respondents (*N*=404) that positively identified changes in facial expression as indicators of discomfort in their ferret(s).

Category	*N*	%	Specification	*N*	%	Specification	*N*	%
Facial expression	327	81%	Eyes	307	93%	Squint	141	46%
						Less bright	74	24%
						Watery	43	14%
			Ears	106	32%	Pull back	25	23%
						Pull down	22	21%
			Whiskers	58	31%	Droop	15	25%
						Pull back	6	10%
			Nose	102	18%	Wet	19	18%
						Pale	15	15%
						Dry	14	14%
						Twitching	13	13%
			Mouth	11	3%	Clenched	5	45%
						Open	3	27%

Discussion

Ferret owners indicated to see weight loss and shallower/faster/more difficulty breathing, which should be practically applicable to monitor in laboratory ferrets. However, some owners also indicated to see a weight increase and slower/deeper breathing, confirming the inconsistency in physiological parameters that can be seen as a result of pain.

A majority of owners indicated that they saw a hunched posture, less frequent grooming, more sleeping, less activity, less play, less inquisitiveness, slower walking, more screaming and less eating and drinking as an indication of pain in their ferrets. However, there was a lack of consensus on the change in sleeping position preferences and grooming, which is also found in the literature (Chapter 6). Unfortunately, we were not able to ask for specific contexts in the owner survey. Therefore, the hypotheses regarding the lack of consensus on sleeping position preferences and grooming behaviour (Chapter 6) cannot be tested using the available data.

A majority of owners indicated to be able to recognise pain in their ferrets' facial expression, particularly in the eyes. The specific facial expressions that were mentioned as indicative of pain by the most owners were squinting eyes, pulled back ears, drooped whiskers, a wet nose and a clenched mouth. The descriptions for the eyes, ears and mouth are in accordance with the expressions that are described in e.g. the rat and mouse grimace scale. This is a first indication that these grimaces scales might also be adapted for use in ferrets.

Conclusion

The international survey revealed that ferret owners recognise many of the pain indicators that are also mentioned in the literature. The owners also have provided some indicators that are not previously published, which could be promising ventures for future research. A new addition to the toolbox for pain evaluation might be the development of a Ferret Grimace Scale.

Appendix 1: survey questions

What are the indications for you that your ferret(s) may be in pain? You may choose none, one or more than one response in each section.

1. Facial expression:
a. Changes in eyes
b. Changes in ears
c. Changes in whiskers
d. Changes in nose
e. Other (please specify)

Could you describe the changes in facial expression you selected and explain how they indicate pain to you?

2. Posture:
a. Shivering
b. Twitching
c. Erection of the hair
d. Tucked belly
e. Hunched
f. Other (please specify)

Could you describe the posture(s) you selected and explain how they indicate pain to you?

3. Demeanour:
a. More or less biting
b. More or less teeth baring
c. More or less active
d. Faster or slower walking pace
e. More or less inquisitive
f. More or less play
g. Other (please specify)

Could you describe the changes in demeanour you selected and explain how they indicate pain to you?

4. Sleeping:
a. More frequent periods of sleeping
b. Less frequent periods of sleeping
c. Longer periods of sleeping
d. Shorter periods of sleeping
e. Changes in timing of sleeping periods

Could you describe the changes in sleeping you selected and explain how they indicate pain to you?

5. Sleeping position:
a. More or less on belly
b. More or less on back
c. More or less curled up
d. Other (please specify)

Could you describe the changes in sleeping position you selected and explain how they indicate pain to you?
6. Grooming behaviour:
a. More frequent
b. Less frequent
c. Other (please specify)

Could you describe the changes in grooming behaviour you selected and explain how they indicate pain to you?

7. Eating and/or drinking behaviour:
a. Eating and/or drinking more (quantity)
b. Eating and/or drinking less (quantity)
c. Eating and/or drinking more frequently
d. Eating and/or drinking less frequently
e. Eating and/or drinking faster
f. Eating and/or drinking slower
g. Eating more selectively
h. Other (please specify)

Could you describe the changes in eating and/or drinking behaviour you selected and explain how they indicate pain to you?

8. Weight:
a. Increase
b. Decrease
c. Other (please specify)

Could you explain how the changes in weight you selected indicate pain to you?

9. Breathing:
a. More difficult
b. Change in frequency (faster / slower rate)
c. Change in depth (shallower / deeper)
d. Other (please specify)

Could you describe the changes in breathing you selected and explain how they indicate pain to you?

10. Vocalisations:
a. Scream (high pitched screech)
b. Hiss
c. Bark (loud chirp)
d. Dook / chuckle / buck (low or high pitched series of chortles or chucks strung together)
e. Whine / moan
f. Grunt
g. Other (please specify)

Could you provide a little more information about the vocalisations you have selected and explain how they indicate pain to you?

11. Do you recognise any other indications of pain that have not been mentioned? If so, please describe and explain these below.

Chapter 8

The composition and initial evaluation of a grimace scale in ferrets after surgical implantation of a telemetry probe

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Abstract

Reliable recognition of pain is difficult in ferrets as many currently available parameters are non-specific, inconsistent and/or impractical. Grimace scales have successfully been applied to assess pain in different animal species and might also be applicable to ferrets. To compose a Ferret Grimace Scale (FGS), we studied the facial musculature of ferrets and compared lateral photographs of 19 ferret faces at six time points before and after intraperitoneal telemetry probe implantation. We identified the Action Units (AUs) *orbital tightening*, *nose bulging*, *cheek bulging*, *ear changes* and *whisker retraction* as potential indicators of pain in ferrets. To evaluate whether these AUs could reliably be used to identify photographs taken before and after surgery, the photographs were scored 0, 1 or 2 (not, moderately or obviously present) by 11 observers that were blinded to the treatment and timing of the photographs. All AU-scores assigned to the photographs taken five hours after surgery were significantly higher compared to their time-matched baseline scores. Further analysis using the weights that were obtained using a Linear Discriminant Analysis revealed that scoring *orbital tightening* alone was sufficient to make this distinction with high sensitivity, specificity and accuracy. Including weighted scores for *nose bulging*, *cheek bulging* and ear change did not change this. As these AUs had more missing values than *orbital tightening*, their descriptions should be re-evaluated. Including *whisker retraction*, which had a negative weight, resulted in lower accuracy and should therefore in its current form be left out of the FGS. Overall, the results of this study suggest that the FGS and the AU *orbital tightening* in particular could be useful in a multifactorial pain assessment protocol for ferrets. However, before applying the FGS in practice, it should be further validated by incorporating more time points before and after applying (different) painful stimuli, and different levels of analgesia.

Introduction

Ferrets (*Mustela putorius furo*) are routinely used as an animal model of human disease (e.g. influenza), during which these animals often undergo a disease process associated with a high risk of (unrelieved) pain (note: in this article, pain will be used as a definition including pain, discomfort, distress and suffering, as these definitions overlap and are difficult to measure in animals (Anil et al., 2002). To optimise animal welfare during these studies, refinement strategies should be implemented, such as timely and accurate recognition and treatment of pain. Unfortunately, scientific data on the assessment of pain in ferrets is scarce, complicating the recognition of pain in these animals (van Oostrom et al., 2011).

The latest approach in the assessment of pain in animals are the species-specific grimace scales that appear to utilise our tendency to focus on the face of an animal (Leach et al., 2011). Also, grimace scales can easily and rapidly be taught to animal caretakers (Leach et al., 2012; Miller and Leach, 2015) and appear to be less time consuming than other pain assessment methods, e.g. activity monitoring (Leach et al., 2012). Moreover, live-scoring in a laboratory setting has been suggested to be practically applicable (Leung et al., 2016).

Grimace scales are based on the Facial Action Coding System (FACS) for humans, which describes changes to the surface appearance of the face using action units (AUs) such as brow lowering, tightening and closing of the eye lids, nose wrinkling and upper lip raising (LeResche, 1982; Prkachin, 1992). The FACS has been used successfully to assess pain in humans that are unable to communicate with their clinicians (e.g. people with cognitive impairment and neonates, Grunau and Craig, 1987; Jordan et al., 2011). The first grimace scale that was developed and validated for laboratory animals was the mouse grimace scale (Langford et al., 2010). This was followed by the development of grimace scales for rats (Sotocinal et al., 2011), rabbits (Keating et al., 2012), horses (Dalla Costa et al., 2014; Gleerup et al., 2015a), cats (Holden et al., 2014), cattle (Gleerup et al., 2015b), sheep (McLennan et al., 2016), piglets (Di Giminiani et al., 2016), and lambs (Guesgen et al., 2016). To the authors' knowledge, a grimace scale for ferrets has not yet been developed. The current grimace scales share changes in a number of the similar AUs that correspond to changes in the shape and/or position of the eyelids, cheek, nose, whiskers and ears, which also correspond with those observed to change in humans when in pain (Chambers and Mogil, 2015). This similarity suggests evolutionary conservation of key expressions of pain (Williams, Amanda C de C, 2002), which led to the hypothesis that ferrets would exhibit similar visible facial changes when experiencing pain, provided they possess the facial musculature to do so.

Understanding which muscles could be involved in facial expressions associated with pain could aid in identification of AUs and interspecies grimace scale comparisons. As no anatomical illustrations of the facial musculature of ferrets are readily available and ferrets are anecdotally said to lack the muscles to express the AUs seen in other species, an anatomical study was performed to document the different facial muscles in ferrets. Subsequently a Ferret Grimace Scale (FGS) with five AUs was composed, based on the information provided by the anatomical study and by using the grimace scales of other species. The clarity of the AU-descriptions as well as the sensitivity, specificity, and accuracy was assessed. Photographs were taken of ferrets' faces before and after undergoing a moderate to severe pain stimulus (i.e. surgical implantation of an intraperitoneal telemetry probe without subsequent analgesics) to see if double-blind observers could differentiate pre- and post-surgery photographs.

Animals, Materials & Methods

Ethical note

This study was carried out in accordance with the European legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU, European Commission, 2010) and ethically approved by the Institutional Animal Care and Use Committee (IACUC) of Intravacc (DEC201200260). The authors were kindly allowed to observe and photograph the ferrets that were undergoing a surgical procedure as part of this unrelated influenza study.

Facial musculature

To study the anatomy of the facial muscles, three humanely euthanised ferrets (from DEC201400137) were used to document the facial musculature. Dissection was started with a surgical cut through the skin layer in caudo-rostral direction. For proper investigation of the superficial musculature, the skin layer was removed in direction of the eye and muzzle at one lateral side. This was followed by the removal of the superficial muscles to further investigate the deep musculature. Methods of dissection were the same for both sides of the face. The facial muscles were compared to detailed anatomical drawings of the facial musculature of the cat, dog and rodents (Williams 2002) and a professional veterinary anatomic illustrator (R. van Deijk) composed a detailed anatomical drawing of the ferrets' facial muscles.

Animals and husbandry

To compose and evaluate the FGS, we used 19 domestic intact female ferrets of different coat colour and length (12 regular sables, 3 angora-like sables, 4 regular black-eyed whites). The ferrets were 16-32 weeks old, weighed 783±86 grams (range: 660-940 grams), and were obtained from Schimmel BV, The Netherlands. The ferrets were group-housed indoors in open phenolic face plywood ground cages (94.5x 166 x 64.6 cm) on wood shavings (IRS LIGNOCELL® Hygienic animal bedding). Ferrets were maintained on a 8:16 hour light:dark cycle (lights on from 8.00AM to 16.00PM) at 18-25 °C. Auditory stimulation in the form of a radio was present 24 hours/day. Food (Hope farms® Ferret balance pellets) and water were given *ad libitum* in stoneware bowls. Cage enrichment consisted of a ferret ball (25 cm diameter with 6 holes of 7.5 cm each) and a large, 24L flexible sleeping bucket. The animals were acclimated to their housing conditions for 27 days prior to the surgery. The ferrets were weighed weekly and their health was monitored daily.

Surgery and recovery

Prior to anaesthesia a physical examination was performed on the ferrets to ascertain that they were in good health. Anaesthesia was induced by administering 0.2 ml of a 9:1 mixture of ketamine (Narketan, 100 mg/ml, range: 16-26 mg/kg) – dexmedetomidine (Dexdomitor®, 0.5 mg/ml, range 8-12 µg/kg) in the left caudal thigh musculature. The ferrets were placed in a dorsal recumbence and the ventral aspect was shaved and disinfected with 70% alcohol. A midline incision, with a maximum length of two centimetres, was made in between the most cranial nipples, through the skin, linea alba and peritoneum to enter the abdomen. The time of incision was registered as T_0. Two telemetry probes (DST micro-T and -HRT, STAR ODDI, 8.3 mm diameter, 25.5 long) were inserted into the abdomen. The linea alba and skin were separately closed with interrupted sutures (Vicryl® 3-0, Ethicon). All surgeries were performed by experienced technicians who were able to perform the surgery procedure on a single animal within 15 minutes, thereby enabling all surgeries to be completed between 9:00 and 11:30 AM of the same day. Because of the short duration of the surgery, no additional heat support was needed to allow the ferrets to maintain their body temperature. Following surgery, a single intra-muscular injection of 0.02 ml Antisedan® (Atipamezole, 5 mg/ml) REG NL 7744, average: 121 µg/kg, range: 92-145 µg/kg) was given in the right caudal thigh area to reverse the anaesthesia. The ferrets were then returned to their group-housing for recovery, where the water bowl was temporarily removed for safety reasons. The ferrets were continuously monitored until they were fully awake, at this time the water bowl was returned. Slowly recovering animals were placed in a separate cage with extra towels and a warm-water bottle wrapped in a towel for thermoregulation. As part of the influenza study protocol and with approval of the IACUC of Intravacc no post-surgical antibiotics or analgesics and other pain-relieving medication were administered.

Photographs

In order to take photographs, the ferrets were individually taken out of their home cage and carried to an adjacent room. The ferret was then placed on a table in front of a PVC tube (75 mm diameter, 30 cm length), through which the ferret had to walk. For five days prior to the surgery the ferrets were individually habituated to this procedure, once per day for a period of 5 minutes.

Based on the anaesthesia recovery time (Arnemo and Søli, 1992; Fournier-Chambrillon et al., 2003) and grimace scale studies in other animal species (e.g. rats Sotocinal et al., 2011), photographs were taken when the ferrets were fully recovered from the anaesthesia (i.e. 2 hours after surgery, T2) and when they were expected to show the most obvious grimace (as was seen in rats Sotocinal et al., 2011) (i.e. 5 hours after surgery, T5). Baseline photographs were taken at time-matched moments on the day prior to surgery (i.e. 22 and 19 hours prior to surgery, T_{B2} and T_{B5}). Additionally, time-matched photographs were taken on the day after surgery (i.e. 26 and 29 hours post-surgery, T_{P2} and T_{P5}) (Figure 8.1).

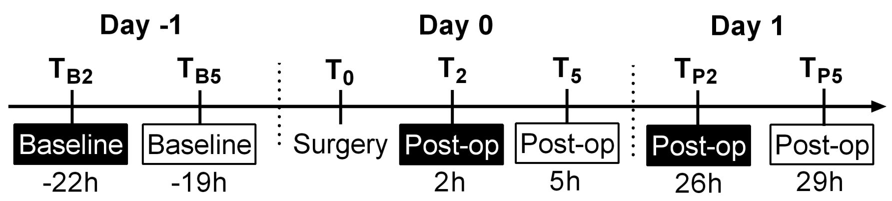

Figure 8.1 Timeline of the study. The photographs were taken at the time points in boxes. The black and white boxes are time-matched (T_{B2} was used as a baseline for T_2 and T_{P2}, while T_{B5} was used as a baseline for T_5 and T_P).

The photographs were taken with a camera (Canon EOS 7D Mark II Body + Canon EF 50 mm 1.8 f II objective) that was placed lateral to the table at 58 cm distance from the end of the tube on a tripod. When the ferrets' head was just outside of the tube, lateral photographs of the ferrets' head were taken in burst mode (10 frames/second, 1/250, *F*=5.0, ISO 10.000). This was repeated for the other side of the face. In total, each ferret walked through the tube 2-6 times per time point, depending on the suitability of the photographs taken. Selection of the photographs for further analysis was based on full visibility of the eyes, ears and whiskers. In addition, the position of the head needed to show the least angled (both horizontal and vertical). The selection of the photographs was performed blinded for time point and before identifying AUs to prevent selection bias. Selected photographs were cropped to contain only the head of the ferret and edited to contain a neutral background using Adobe Photoshop CC®.

Composition of the Ferret Grimace Scale

Time-point blind comparisons were made between individual baseline photographs from each animal and the matching post-surgery photographs (T_{B5} vs T_5) (within-subject comparison). Changes in facial expression within each ferret were recorded. Changes that were observed in five or more ferrets (>25%) were listed as action units (AUs). All AUs were listed in a Ferret Grimace Scale (FGS), designed to be scored on a three-point scale that is generally used for grimace scales (e.g. Keating et al., 2012; Langford et al., 2010; Sotocinal et al., 2011). A score of "0" indicated that the change in the AU was not present, a score of "1" indicated that the change in the AU was moderately present and a score of "2" indicated that the change in the AU was obviously present. Additionally, a short training module was composed for each AU, which showed three example photographs of each score (i.e. 0, 1 and 2) with lines indicating the changes visible for the AU.

Evaluation of the Ferret Grimace Scale

To evaluate whether each of the five selected AUs could be used to differentiate ferrets before and after surgery, a survey was designed in Survey Monkey® and distributed to eleven observers. These observers included three ferret veterinarians, five ferret owners, one ferret researcher and two members of a ferret society/rescue that were blinded to the study objectives and time points. Each observer was asked to complete a 7-part survey, with each survey part sent to the observer one week after completion of the previous part.

Five of these survey parts, (i.e. part 2-6) dealt with the individual AUs. In each of these, the observers were asked to rate each photograph (*N*=114: 19 ferrets, 6 time points, i.e. 1 photograph per ferret per time point) on the three-point scale (0=not present, 1=moderately present, 2=obviously present or ?=I do not know), after having completed through a short training module for that specific AU. The order in which the AUs and the photographs were scored was randomised between observers (the order of the AUs was randomised using a balanced Latin square; the order of the photographs was randomised automatically by Survey Monkey®; Table 8.1).

Two survey parts (i.e. parts 1 and 7) comprised questions regarding the general assessment of pain. In these survey parts, the observers were asked to rate each photograph on the overall pain that the observer estimated the ferret to be in, using a subjective scoring method from an overall discomfort assessment scale used during animal experiments (0=no pain, 1=mild pain, 2=moderate pain, 3=severe pain or ?=I do not know). The scores from the first part (overall pain score) are the "pre-training" scores, as the observers are not (yet) familiar with the AUs and have to rely on their own experience to assign a score. The scores from the last part (also overall pain score) are the "post-training" scores, as the observers completed the survey part and the accompanying training for each AU (*orbital tightening, nose bulging, cheek bulging, ear changes* and *whisker retraction*)(Table 8.1).

Table 8.1 Time schedule of the 7-part survey with the scores that were assigned to the photographs of the ferrets. AU1-5= action unit 1-5 of the Ferret Grimace Scale (*orbital tightening, nose bulging, cheek bulging, ear changes and whisker retraction*), presented in random order. Pre-training = intuitive overall pain assessment, post-training = overall pain assessment after being trained on the Ferret Grimace Scale.

Part 1	Part 2-6					Part 7
Pre-training	**Ferret Grimace Scale**					**Post-training**
Overall pain	**AU1**	**AU2**	**AU3**	**AU4**	**AU5**	**Overall pain**
0 = no pain 1 = minor pain 2 = moderate pain 3 = severe pain	0 = not present 1 = moderately present 2 = obviously present					0 = no pain 1 = minor pain 2 = moderate pain 3 = severe pain

Statistical analysis

All analyses were performed using R (R core team, R Foundation for Statistical Computing). Packages are listed between brackets for each analysis. Differences were considered statistically significant if $P<0.05$. The p-values were corrected for multiple comparisons using the False Discovery Rate (FDR, Benjamini et al., 2001).

First, to get an indication whether the descriptions of the AUs were clear and whether training aided in assigning an overall score to the photographs, the percentage of missing data (scored with "I don't know") per AU per time point ($=\frac{(\Sigma \text{ missing } \textit{data observer}_{1\text{-}11} \text{ on } T_x}{209\ (11 \textit{ observers x } 19 \textit{ ferrets})}$ x100) and per ferret ($=\frac{(\Sigma \text{ missing } \textit{data ferret}_x \text{ on } T_{\text{-}22\text{-}29}\ \textit{for AU}_{1\text{-}5}}{30\ (6 \textit{ timepoints x } 5 \textit{ AUs})}$ x100) were calculated. In addition, to further determine whether the descriptions of the AUs were clear and whether training aided in the agreement on the overall pain assessment, the inter-observer agreement was calculated for each AU and for the overall pain scores and the intra-observer agreement was calculated for the overall pain scores using an Intraclass Correlation Coefficient (ICC, "irr" package) (McGraw and Wong, 1996). A score of <0.40 is regarded as poor agreement, 0.40-0.59 as fair agreement, 0.6-0.74 as good agreement and 0.75-1.00 as excellent agreement (Cicchetti, 1994).

Second, to assess whether the AUs could be used to differentiate photographs of ferrets' faces before and after surgery, a Cumulative Link Mixed Model (CLMM, "ordinal" package) (Agresti, 2010) was performed to assess how the time-point, the observer and the ferret influenced the score for each AU. This method is analogous to a linear mixed model, but specific to ordinal response values. The CLMM was performed with time point as a fixed effect and observer and ferret as random effects. The baseline scores (T_{B2} and T_{B5}) were compared with each other and with the time-matched post-surgery (T_{B2} was compared to T_2 and T_{P2}, T_{B5} was compared to T_5 and T_{P5}).

Third, the importance of each AU in discriminating the pain condition of a ferret was measured using a Linear Discriminant Analysis (LDA, "MASS" package) (Venables and Ripley, 2002). To quantitatively assess this importance, LDA assigns a weight to each AU, with the most important AUs having higher weights with respect to the others. If the weights are all equal, the linear weighted combination of the five AU scores is the mean of the scores. However, the weights are often not equal and the linear weighted combination of the five AU scores were used to maximise the discrimination between the photographs taken five hours after surgery (T_5) and the corresponding baseline photographs (T_{B5}). The AUs that were assigned a negative or near-to-zero weight were removed from the analysis and another LDA was performed. Using the optimal weights of the AUs obtained by performing the LDA, the sensitivity, specificity and accuracy with which the AUs could be used to discriminate photographs taken five hours after surgery (T_5) from the corresponding baseline photographs (T_{B5}) were calculated. Here, it is assumed that all AUs are not present at T_{B5} (i.e. score 0 would be "correct") and obviously present at T_5 (i.e. score 2 would be "correct") in each photograph. The sensitivity (i.e. true positive rate) is the proportion of photographs taken at T_5 (i.e. five hours after surgery) that were assigned a high score for the AUs properly weighed by the LDA weights $\frac{T_5 \textit{ scored high according to LDA}}{19\ (\textit{all } T_5 \textit{ pictures})}$. The specificity (i.e. true negative rate) is the proportion of photographs taken at T_{B5} (i.e. 19 hours before surgery) that were assigned a low score for the AUs properly weighed by the LDA weights $\frac{T_{B5} \textit{ scored low according to LDA}}{19\ (\textit{all } T_{B5} \textit{ pictures})}$. The accuracy (i.e. the ratio of the sum of true positives and true negatives, to the total number of correct or incorrect classifications) is the proportion of photographs taken at T_{B5} and T_5 that were assigned a low and high score, respectively, properly weighed by the LDA weights $\frac{T_5 \textit{ scored high according to LDA} + T_{B5} \textit{ scored low according to LDA}}{38\ (\textit{all } T_5 + \textit{all } T_{B5} \textit{ pictures})}$.

Results

Facial musculature

The detailed anatomical drawing of the ferrets' facial muscles is shown in Figure 8.2. The name, origin, insertion and action of these muscles are listed in Table 8.2.

Table 8.2 Name, origin, insertion and action of the facial muscles of the ferret (Goldfinger, 2004).

Name	Origin	Insertion	Action
M. Levator nasolabialis	Just off the nose bridge midline, start at eye-level	Side of nose/front of upper lip	Lifts upper lip and wrinkles skin of the nose
M. Levator anguli oculi medialis	Upper surface of skull, above eye	Top of eye region, merging into the orbicularis oculi	Pulls skin above the eye upward, rearward and slightly inward
M. Orbicularis oculi	Surrounding the eyes	Inner end attaches to the skull	Closes the eyelid, primarily by depressing the upper eyelid
M. Retractor anguli oculi lateralis	Side of head to rear of the eye	Outer (rear) corner of eye region	Pulls the corner of the eye caudally
M. Fronto-scutularis	Lateral eye corner	Scutiform cartilage	Draws the ear cranial
M. Temporalis	Upper rear part of skull	Top of upward projection of lower jaw	Closes the mouth by lifting and pulling back the lower jaw
M. Orbicularis oris	Corner of mouth	Into lips as it surrounds mouth	Closes the mouth by tightening the lips
M. sphincter colli profundus	Near corner of mouth	Into orbicularis oculi, extending upward to lower eyes	Pulls the lower eyelid down, and/or lifts the upper lip
M. Zygomaticus	Base of ear	Corner of mouth, merging with fibers of orbicularis oris	Draws the corner of the mouth dorso-caudal and external ear ventro-cranial
M. Masseter	Lower edge of zygomatic arch	Side of lower jaw, into lower and rear edges of lower jaw	Closes mouth by lifting lower jaw
M. Deep sphincter colli: intermediate part	Base of ear	Lateral to masseter muscle, fuses with fascicles of same muscle on opposite side	Turns and pulls ear down
M. Platysma	Midline on back of the upper neck	Passes over side of lower jaw into corner of mouth, fusing with orbicularis oris	Pulls corner of the mouth backwards

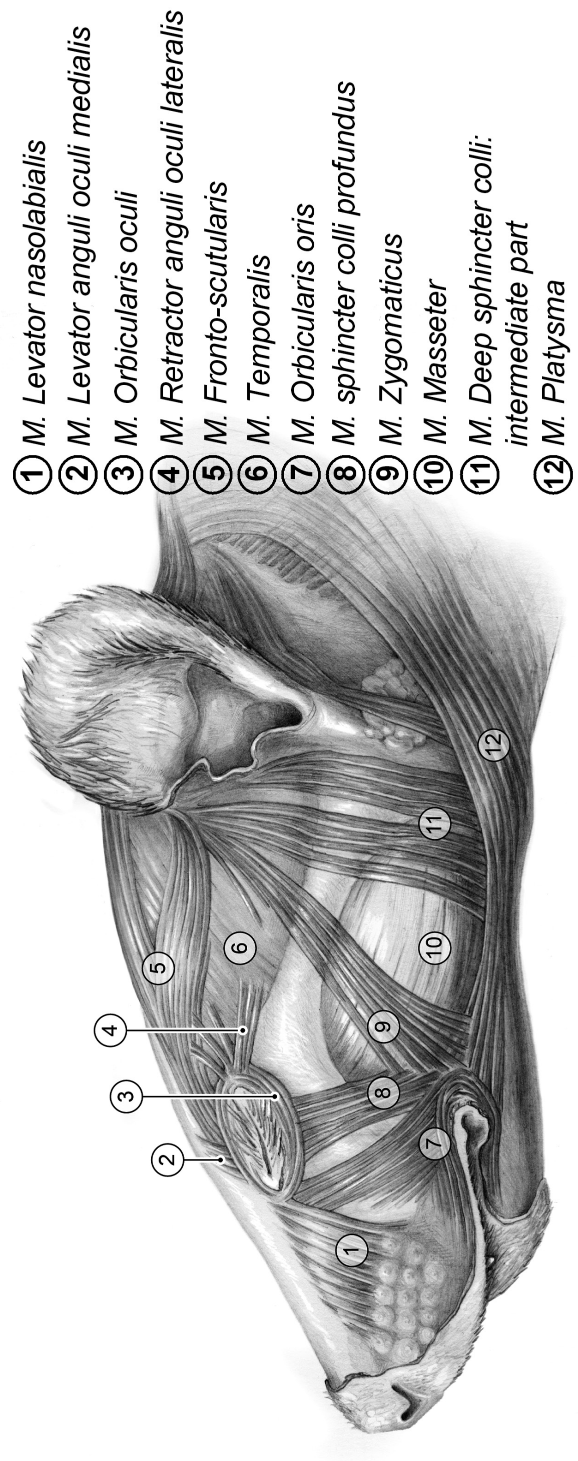

Figure 8.2 Ferret Facial anatomy. Skin and superficial muscles were removed during dissection, drawing made by R. van Deijk.

The Ferret Grimace Scale

Five action units (AUs) were defined from the within-subject comparisons: *orbital tightening*, *nose bulging*, *cheek bulging*, *ear changes* and *whisker retraction*. *Orbital tightening* consists of closing of the eyelids, which can cause a wrinkle to become visible around the eye. *Nose bulging* consists of pulling the nose down, causing the nose to round off, the nostrils to point down instead of straight forward and bulging of the bridge of the nose. *Cheek bulging* occurs due to constriction of the cheek muscles, making the contour of the cheeks become visible. Additionally, the cheek may be pulled up at the side of the ear. *Ear changes* consist of pulling back the ears against the body, possibly forming a pointed shape and folding over. *Whisker retraction* consists of pulling back the whiskers against the cheek, clumping together of the whiskers and caudal convergence of the whisker follicles (Figure 8.3).

Missing scores

The number [percentage] of photographs that were scored “I do not know” (missing scores) by the observers was different between the AUs (LMM: F_{55}=3.108, P=0.022). More specifically, *orbital tightening* (N=8[4%]) had significantly less missing scores than *ear changes* (N=19[9%], P=0.001) and *cheek bulging* (N=15[7%], P=0.027). The number [percentage] of photographs that were scored “I don’t know” for overall pain by the observers was significantly lower after completing the training module on each of the AUs than before this training (LMM: F_{22}=10.333, P=0.004, before: N=41[20%], after: N=10[5%]). The number [percentage] of photographs with missing scores for the AUs also varied per ferret (LMM: F_{18}=5.846, P<0.001). More specifically, two of the three long-haired (angora-like ferrets) had significantly more missing scores for the AUs than the other ferrets (N=10[13%] versus N=5[6%], P<0.001 for both ferrets). (Table 8.3).

Intra- and interrater agreement

The inter-observer agreement for all AUs and for overall pain score were excellent: *orbital tightening*: ICC=0.97 ($F_{79,790}$=29.505, P<0.001), *nose bulging*: ICC=0.85 ($F_{67,670}$=6.514, P<0.001), *cheek bulging*: ICC=0.86 ($F_{63,630}$=7.038, P<0.001), *ear changes*: ICC=0.88 ($F_{55,550}$=8.443, P<0.001), *whisker retraction*: ICC=0.88 ($F_{54,540}$=8.321, P<0.001), overall pain pre-training: ICC=0.89 ($F_{5,50}$=10.882, P<0.001), overall pain post-training: ICC=0.89 ($F_{73,730}$=8.503, P<0.001). The intra-observer agreement between overall pain score pre- and post-training was good: ICC=0.67 (F_{968}=3.017, P<0.001).

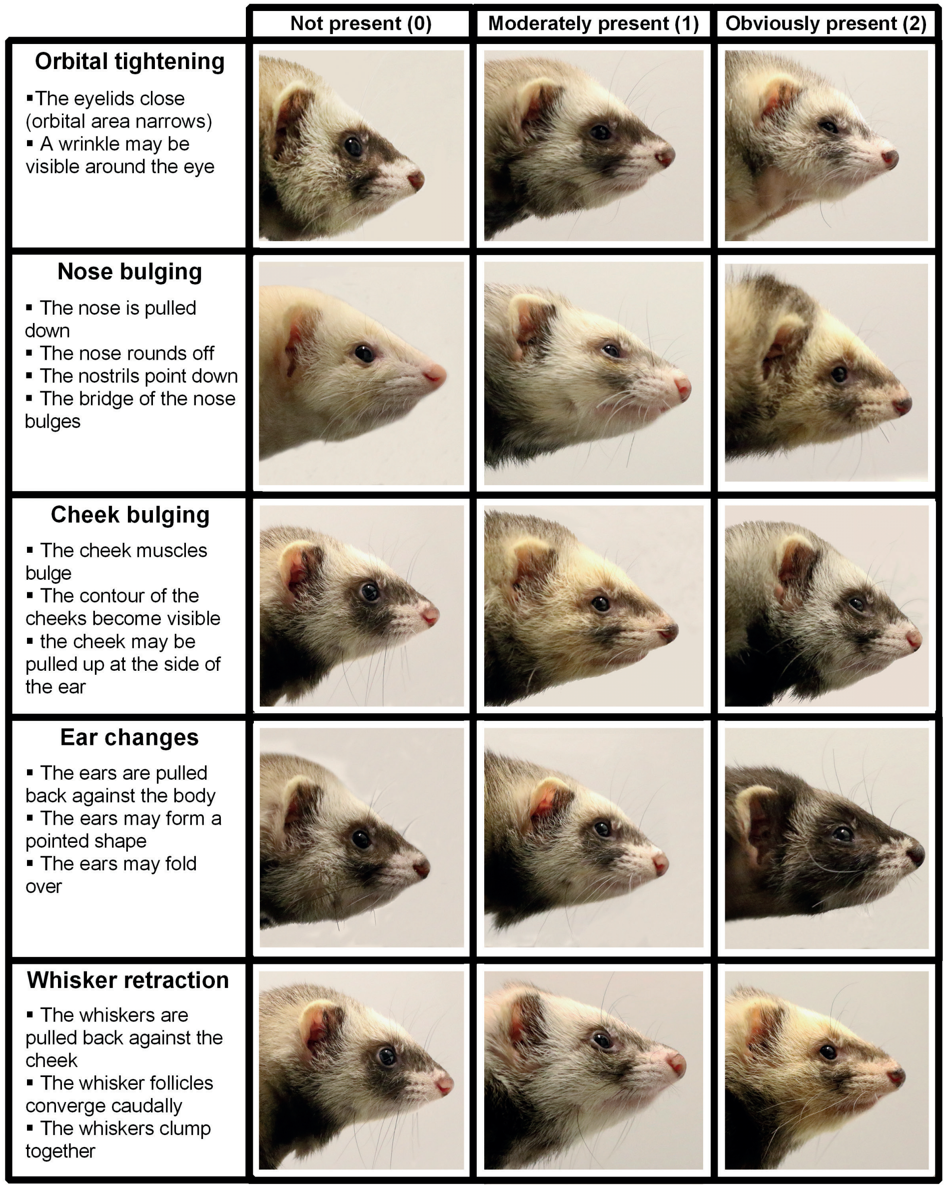

Figure 8.3 The ferret grimace scale. Photographs visualising the normal appearance and changes (0=not present, 1=moderately present, 2=obviously present) of the five Action Units that are used in the Ferret Grimace Scale.

Table 8.3 Number and percentage of pictures (N=209) that were scored "I not to know" for *orbital tightening*, *nose bulging*, *cheek bulging*, *ear changes*, *whisker retraction* and overall pain pre- and post-training by 11 observers of 19 ferret faces at different time points (T_{B2}, T_{B5}, T2, T5, T_{P2} and T_{P5}).

	Photographs with missing scores (of 209)													
	Orbital tightening		Nose bulging		Cheek bulging		Ear changes		Whisker retraction		Pre-training		Post-training	
Time	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
T_{B2}	6	3%	12	6%	14	7%	10	5%	11	5%	42	20%	11	5%
T_{B5}	10	5%	8	4%	19	9%	23	11%	16	8%	46	22%	4	2%
T_2	7	3%	9	4%	14	7%	20	10%	14	7%	45	22%	10	5%
T_5	5	2%	17	8%	15	7%	17	8%	12	6%	27	13%	8	4%
T_{P2}	7	3%	18	9%	12	6%	18	9%	16	8%	41	20%	12	6%
T_{P5}	12	6%	14	7%	15	7%	23	11%	15	7%	46	22%	14	7%

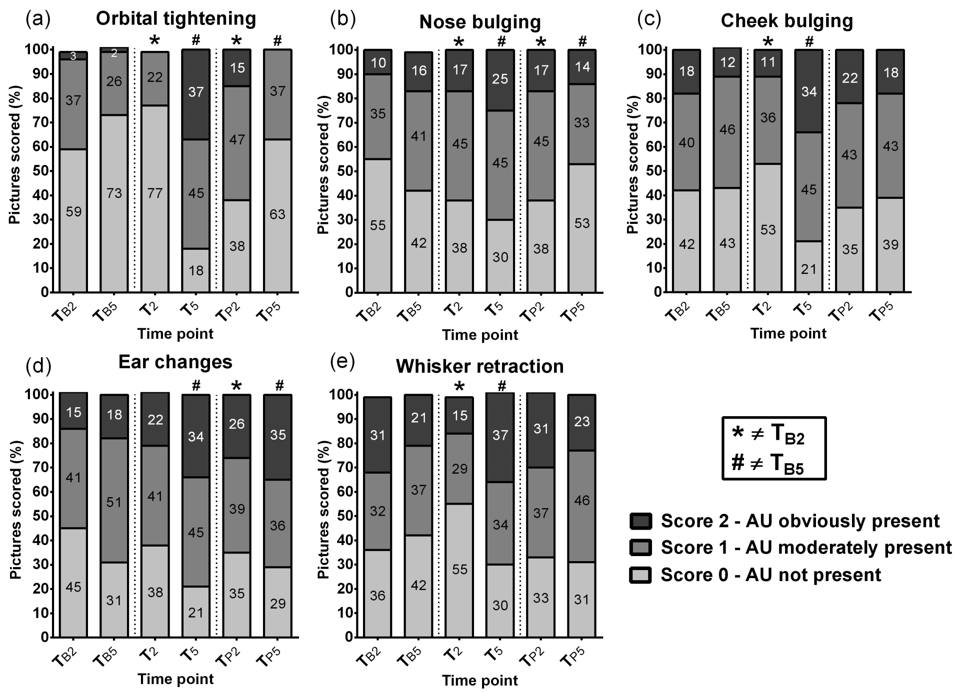

Figure 8.4 Average percentage of ferrets (N=19) that were assigned score 0, 1, and 2 for each of the AUs in the FGS by 11 observers at different time points. a) *orbital tightening*, b) *nose bulging*, c) *cheek bulging*, d) *ear changes* and e) *whisker retraction* on six time points relative to the time of surgery (T0), *= score is significantly different from score on T_{B2}, #= score is significantly different from score on T_{B5} with P<0.05 (11 observers, 19 ferrets).

AU-scores over time

There were differences in baseline scores for *orbital tightening* (CLMM: LR=210.9036, P<0.0001), *nose bulging* (CLMM: LR=220.8676, P<0.0001), *ear changes* (CLMM: LR=180.4370, P<0.0001) and *whisker retraction* (CLMM: LR=217.2884, P<0.0001). More specifically, the scores at baseline for *orbital tightening* and *whisker retraction* were significantly lower at T_{B5} than at T_{B2} (P=0.0024 and P=0.0354, respectively). The scores for *nose bulging* and *ear changes* were significantly higher at T_{B5} than at T_{B2} (P=0.0024 and P=0.0162, respectively).

When evaluating the AU *orbital tightening*, time was found to exert a significant effect on the score assigned by the observers (CLMM: LR=210.9036, P<0.0001). More specifically, the post-surgery scores at T_2 were significantly lower than at T_{B2} (P<0.001). Additionally, the post-surgery scores at T_5, T_{P2} and T_{P5} were significantly higher than their respective baseline scores (T_{B5}: P<0.0001, T_{B2}: P<0.0001 and T_{B5}: P=0.0428 respectively)(Figure 8.4a).

Similarly, differences were present in score over time for *nose bulging* (CLMM: LR=220.8676, P<0.0001), *cheek bulging* (CLMM: LR=232.0036, P<0.0001), *ear changes* (CLMM: LR=180.4370, P<0.0001), and *whisker retraction* (CLMM: LR=217.2884, P<0.0001). More specifically, for *nose bulging* the post-surgery scores at T_2, T_5 and T_{P2} were significantly higher than their respective baseline scores (T_{B2}: P=0.0003, T_{B5}: P=0.0048, T_{B2}: P=0.0002 respectively), whereas the post-surgery scores at T_{P5} were significantly lower than at baseline (T_{B5}: P=0.0393)(Figure 8.4b). For *ear changes*, post-surgery scores at T_5, T_{P2} and T_{P5} were significantly higher than their respective baseline scores (T_{B5}: P=0.0004, T_{B2}: P=0.0039, T_{B5}: P=0.0107 respectively)(Figure 8.4d). For *cheek bulging* and *whisker retraction*, only the post-surgery scores at T2 and T_5 differed significantly from their respective baselines, with the post-surgery scores at T_2 being significantly lower than at baseline (T_{B2}: P=0.0029 and P<0.0001 for *cheek bulging* and *whisker retraction*, respectively) and the post-surgery scores at T_5 being significantly higher than at baseline (T_{B5}: P<0.0001 and P=0.0003 for *cheek bulging* and *whisker retraction*, respectively) (Figure 8.4c and 8.4e).

Weight, sensitivity, specificity and accuracy

From the LDA, it was clear that *whisker retraction* had a negative weight, close to zero (Figure 8.5a). Therefore, *whisker retraction* was removed from the model and the weights of the other AUs were re-estimated by performing another LDA. In this model, like the first model, *cheek bulging, nose bulging and ear changes* had weights close to zero (Figure 8.5b).

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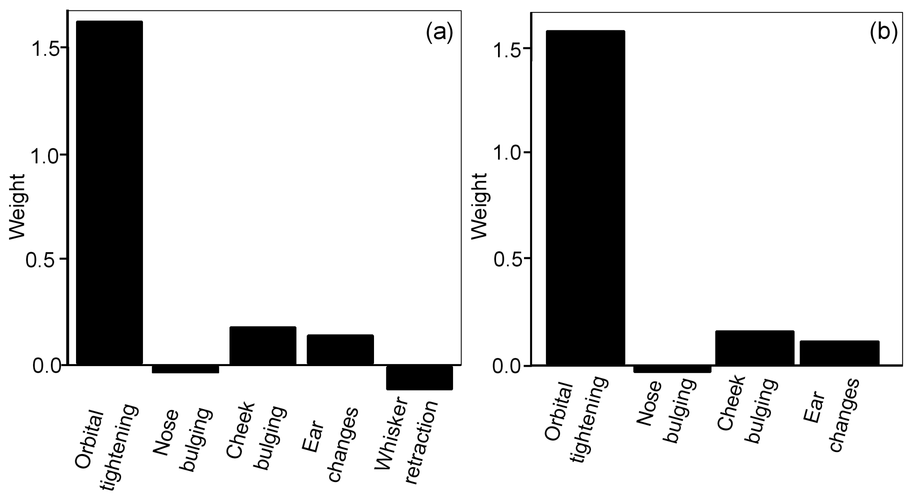

Figure 8.5 Optimal weights of each AU. Obtained by performing the LDA on the observer scores for *orbital tightening, nose bulging, cheek bulging, ear changes and whisker retraction*. a) using all AUs, b) without *whisker retraction*.

Using the optimal weights of the AUs obtained by performing the LDA, the sensitivity, specificity and accuracy of the observer scores were calculated 1) using all five AUs, 2) using the FGS without *whisker retraction* and 3) using only *orbital tightening*. In all these three options, the score 1 for *orbital tightening* AU was the cut-off value for which a photograph was classified as taken at T_{B5} (score of *orbital tightening* below 1, i.e. score of *orbital tightening* equal to 0) or at T_5 (score of *orbital tightening* equal to 1 or 2). The sensitivity (85%) and specificity (74%) were equal for all three options. The highest accuracy (80%) was achieved using the FGS without *whisker retraction* and using only *orbital tightening*.

Discussion

With this study, we aimed to compose a Ferret Grimace Scale (FGS) using action units (AUs) that were based on the facial musculature of ferrets. Additionally, we wanted to evaluate whether the descriptions of the AUs were clear and aided observers in assigning an overall pain score to the photographs that were taken before and after telemetry probe implantation surgery. Furthermore, we aimed to determine whether the AUs could be used to distinguish ferrets before and after this surgery. Finally, we aimed to make an informed decision on which AUs could be included in a final FGS in their current form and which AUs should be re-evaluated by assessing the weight, sensitivity, specificity and accuracy of the AUs comprising the scale.

Facial musculature and the FGS

The ferrets' facial musculature is very similar to that of mice and rats (Komárek, 2004; Komárek, 2000), containing all the necessary muscles to show different facial expressions. Therefore, it is not surprising that the potential AUs that were identified for the ferrets (*orbital tightening*, *nose bulging*, *cheek bulging*, *ear changes* and *whisker retraction*) are very similar to those in the other grimace scales, e.g. the mouse, rat and rabbit grimace scale (Keating et al., 2012; Langford et al., 2010; Sotocinal et al., 2011). Comparing the different grimace scales, the similarities are striking, raising the question whether it is necessary to develop a grimace scale for every animal species or whether we can reduce the amount of animals that are used in research by making a generalised vertebrate grimace scale. However, there are also species differences in the AUs of established Grimace Scales. For example, where rats and rabbits flatten their nose/cheek when in pain, mice (and ferrets) bulge their nose/cheek (Keating et al., 2012; Langford et al., 2010; Sotocinal et al., 2011). The only AU that seems to appear in the same form in all species is *orbital tightening*.

Missing values and agreement

Based on the number of missing values and the inter-observer reliability, the observers seemed to find the action unit (AU) *orbital tightening* the most clearly described and/or the most recognisable. The observers seemed to find the AUs *ear changes* and *cheek bulging* the least clear, as there were more missing values for these AUs. Therefore, it would be worthwhile to re-evaluate the descriptions of at least *ear changes* and *cheek bulging*, but preferably also *nose bulging* and *whisker retraction*.

The ability of observers to assign a score for the AUs could have potentially been influenced by the type of coat the ferret had, which seemed to be more difficult in the longer-haired ferrets. This has not previously been reported, but is not surprising, as the long hair might have obscured muscle tension and/or ear position. This should therefore be taken into account when applying the FGS and when selecting ferrets as animal models (e.g. selecting only short-haired ferrets).

The observers scored the overall pain with excellent and good consistency (inter- and intra-observer, respectively), indicating that the observers agreed on which score to assign to a photograph, as well as that an observers' first (pre-training) assessment was equal to the second (post-training assessment). The intra-observer reliability might have lower agreement due to a training effect, i.e. as the observers have been trained to look more closely at the ferrets' faces in the survey-parts concerning the AUs. The observers did appear more confident to assign an overall pain score to the photographs after seeing the examples of and having scored the photographs on the AUs, as there

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were less missing values for the overall pain scores post-training than pre-training. This indicates that systematically looking at the ferrets' faces using the AUs can aid in formulating an overall pain score.

Scores over time

The baseline FGS scores at T_{B2} were significantly different from the baseline scores at T_{B5} for the AUs *orbital tightening*, *whisker retraction*, *ear changes* and *nose bulging*, which suggests that the time of day influences the facial expression of the ferrets. These differences in AU-scores might possibly be explained by the polycyclic sleep-wake cycle of ferrets: ferrets sleep in bouts of approximately three hours (Marks and Shaffery, 1996), which is exactly the window between the two baseline measurements. As the ferrets were used to being disturbed in the morning (around T_{B2}), but not in the afternoon (around T_{B5}) and they are known to adapt their sleep-wake cycle to human activity, the ferrets might have been awake around T_{B2} and just woke up around T_{B5}. This is supported by asleep, waking or sedated animals showing false positives with other grimace scales (i.e. mice: Langford et al., 2010; and rats: Sotocinal et al., 2011). Using more than two measurements on one day and using a smaller inter-measurement period could provide more information on the background of these differences in scores in ferrets.

Two hours after the surgery (T_2), the scores for *orbital tightening*, *nose bulging* and *cheek bulging* were significantly lower than baseline (T_{B2}), indicating that there might still have been a carry-over sedative or analgesic effect of ketamine (as the effects of dexmedetomidine were reversed using atipamezole). Studies in mink reported recovery within 60 or even 15 minutes after reversal with the chosen anaesthetic regime (Arnemo and Søli, 1992; Fournier-Chambrillon et al., 2003). However, possible carry-over effects of ketamine have not been addressed in these studies. Therefore, the changes observed in the ferrets' facial expressions two hours after surgery might be a reflection of recovery from the anaesthesia. This hypothesis is supported by the observation that some ferrets were repeatedly yawning during handling at this time point, which was not seen at other time points (but would be expected when yawning was expressed as a normal behaviour when waking up). It has been shown that isoflurane, and not buprenorphine, increased the mouse grimace score in male DBA/2 mice (Miller et al., 2015), which underlines the need to carefully choose the post-surgery time to ensure the animals are no longer experiencing the major sedative effects of anaesthesia or the anaesthetic regimes for grimace scale research. Additionally, it should be taken into account that the ferrets received equal amounts of anaesthetics, while their bodyweights differed. Some of the ferrets might therefore have experienced stronger/longer lasting sedation/analgesia than others.

The scores for all AUs and overall pain scores peaked five hours after surgery (T_5), which is in line with the results found in other grimace scale studies (e.g. the rat grimace scale: Sotocinal et al., 2011). This high FGS-score suggests that the ferrets were experiencing pain at that moment, supported by the observation that the ferrets showed repeating bouts of pronounced shivering when they were handled at this time point. The ferrets did not show shivering during handling at T_2 or at any other time point, which makes it unlikely that the shivering was caused by hypothermia, stress or as a result of recovery from the anaesthesia. However, due to the lack of control animals receiving analgesics, clear conclusions cannot be drawn on the pain-state of the ferrets. Follow-up studies using multiple analgesic schedules should be performed to confirm this as this will further confirm that the changes in the FGS observed here are pain-related. In these follow-up studies, it should be taken into account that there might be individual and sex differences in pain perception and therefore in pain expression.

The scores of the photographs taken the day after surgery (T_{P2} and T_{P5}) were significantly lower than the scores at T_5, but were still higher than baseline scores for *orbital tightening*, *nose bulging* and *ear changes*. This could possibly indicate that the chosen time frame for this study was too short to capture the point at which the ferrets' grimace (and pain-state) was back to baseline. It was unexpected that the ferrets would still show changes in the AUs 26 and/or 29 hours after surgery as the recovery time in rats was shorter, their grimace scores were back to baseline twelve hours after a laparotomy (Sotocinal et al., 2011). However, these differences between the species/studies might have occurred due to other pain-related causes, e.g. the size of the incision (relative to the animal) or the method with which the wounds were closed (sutures or tissue glue) or non pain-related causes, e.g. the anaesthesia that was used.

The decline in AU scores over time might indicate a slow recovery from pain, but again, this should be further confirmed using a control group that receives analgesics. In a follow-up study, it would be informative to measure more than two time points per day to identify the FGS-peak and to take FGS-scores later than 29 hours after surgery, to determine the point at which the AU scores are all back to baseline. Additionally, it would be prefereable to include other clinical signs that are considered indicative of pain (physiological and behavioural changes) to further validate this scale.

Weight, sensitivity, specificity and accuracy

Even though the scores for all AUs increased significantly from baseline to five hours after surgery (T_5), just the scores for *orbital tightening* were required to achieve a high sensitivity, specificity and accuracy, which is comparable to the findings of other studies (Dalla Costa et al., 2014; Keating et al., 2012; Langford et al., 2010; Sotocinal et al., 2011). Additionally, *orbital tightening* had the least missing values, indicating that it was the most clear/most visible AU for observers. It is not surprising that *orbital tightening* is the most important AU as this is also reported for other grimace scales (e.g. Di Giminiani et al., 2016) and humans tend to focus on the eyes of people/animals when assessing emotions such as pain (Dalton et al., 2005; Deyo et al., 2004).

Including the AUs *nose bulging*, *cheek bulging* and *ear changes* alongside *orbital tightening* in the FGS resulted in a similar accuracy of score assignment as *orbital tightening* alone. However, in comparison to *orbital tightening*, the weights assigned to these three AUs in the overall scoring were low, indicating that they contributed only slightly to the overall score. Moreover, many values were missing indicating that observers had difficulty in assigning a score for these three AUs. For the AU *whisker retraction*, a negative weight was even found and inclusion of this AU actually resulted in a lower accuracy, which indicates it might have confused the observers in the pain assessment. However, it should be taken into account that sensitivity, specificity and accuracy were calculated using the assumption (supported by LDA results) that all AUs were absent at T_{BS} and moderately or obviously present at T5 in each photograph, which might not have been the case as the FGS is not yet validated. This assumption might have led to an erroneously low or high accuracy. Moreover, since this, to the authors' knowledge, is the first study to assess the weight of individual AUs, it is currently not possible to compare our results with other grimace scales that incorporate *whisker retraction*, *nose bulging*, *cheek bulging* and *ear changes*, such as those of the mouse, rat and rabbit Keating et al., 2012; Langford et al., 2010; Sotocinal et al., 2011 . Nevertheless, our findings suggest that the description of the four AUs that resulted in low or negative scores should be re-evaluated to explore whether clarity and/or visibility can be increased. Additionally, the effects of live scoring, other photograph angles and settings should be explored as this might result in more reliable results for all AUs.

Conclusion

Overall, the results of this study suggest that the FGS and the AU *orbital tightening* in particular, could be useful for pain assessment of ferrets. The other AUs (*whisker retraction*, *nose bulging*, *cheek bulging* and *ear changes*) should be re-evaluated before they can be included in the FGS. Furthermore, prior to incorporating the FGS in a multifactorial pain assessment protocol, it should be further validated using different painful stimuli, analgesic regimens and measuring more time points.

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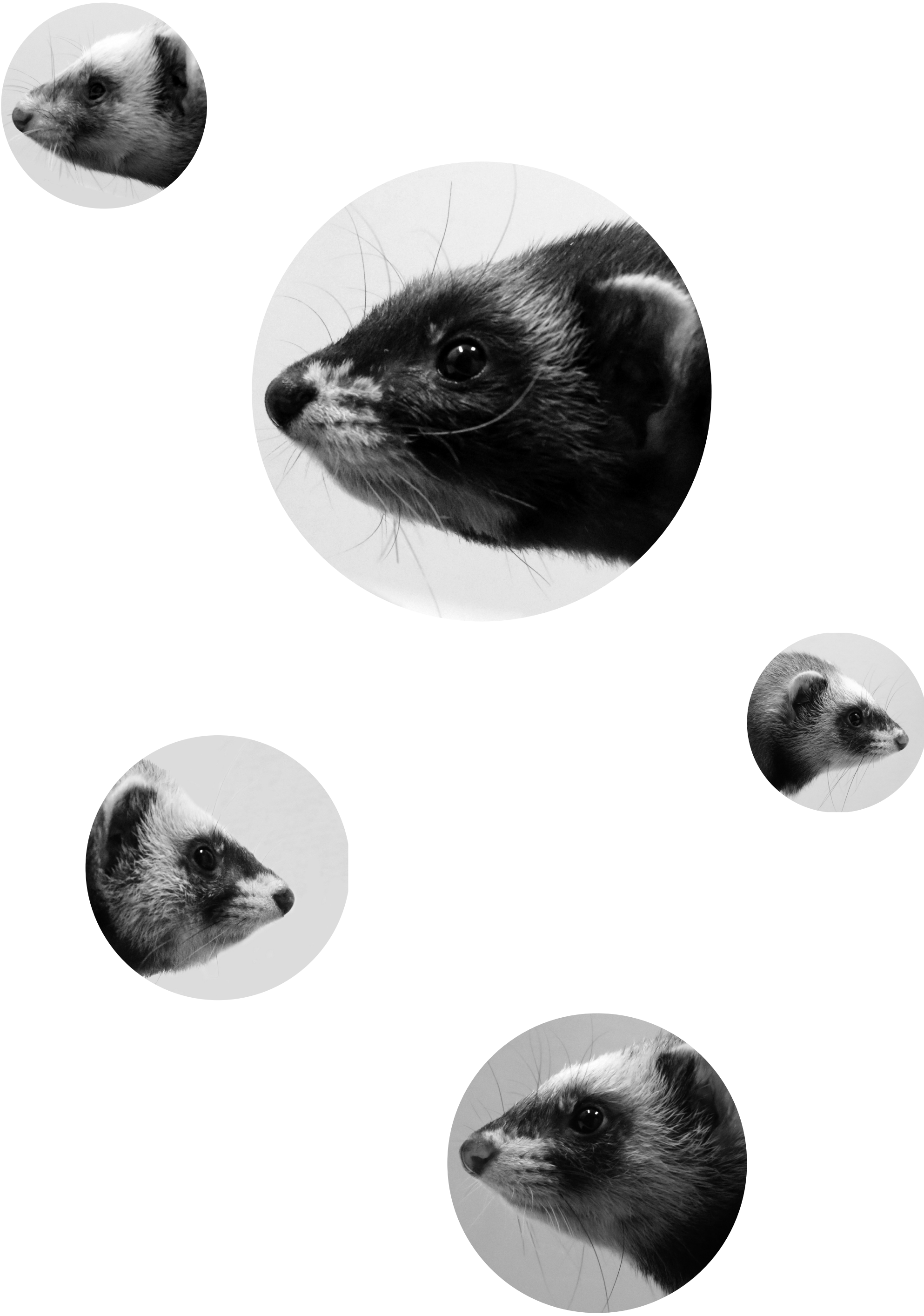

Chapter 9

Summarising discussion

Brief summary

Ferrets (*Mustela putorius furo*) are a valuable animal model in biomedical research, e.g. for studying human diseases such as influenza. Legislation requires studies using animals to adhere to the principle of the 3Rs, i.e. to replace the use of animals where possible; to reduce the number of animals used without compromising the scientific requirements; and to refine the use of animals. Refinement includes "any approach which avoids or minimises the actual or potential pain, distress and other adverse effects experienced at any time during the life of the animals involved, and which enhances their well-being" (Buchanan-Smith et al., 2005). However, when it comes to refinement of the care and use of laboratory ferrets, there is a knowledge gap, which is also reflected in the limited amount of ferret-specific information in the EU Directive (European Commission, 2010). The studies presented in this thesis are performed with the aim of giving insight into possible ways to refine the care and use of laboratory ferrets, focusing on the value and effect of environmental enrichment (Part I), and the recognition of pain and other forms of discomfort in ferrets (Part II).

Part I of this thesis focused on refinement of the care and use of laboratory ferrets through the provision of environmental enrichment. As consumer demand studies are commonly regarded as a valid method to assess an animals' motivation for different types of enrichment, this type of studies if used to substantiate which changes to an animals' housing should be made to improve their welfare. Therefore, in Chapter 2, the functionality of a 2-chamber closed economy consumer demand study using a push door for ferrets was assessed. For this set-up to be functional, it had to fulfil three prerequisites: 1) the motivation for food (i.e. an essential resource reflects the maximum push capacity of the ferrets; 2) pushing the weighted door is strenuous for the ferrets; and 3) the maximum weight the ferrets pushed for an empty chamber is significantly lower than the weight that they pushed for food. Prerequisite 1 was met, as the ferrets still attempted to push open the door for food, but were not able to do so at the highest weights. Prerequisite 2 was also met, as the number of visits to the chamber with food decreased with increasing weights on the door, whereas the duration of these visits increased. In other words, the ferrets rescheduled their behaviour to defend food consumption time with increasing weights (as seen in mink: Cooper and Mason, 2001; Sherwin and Nicol, 1997). Prerequisite 3 was not met as, despite the door being a strenuous task, the ferrets pushed 89% of the weight that they pushed for food for an empty chamber. To compare, this was only 67% in mink (Mason et al., 2001), indicating that ferrets might have had alternative motivations to push the weighted door, other than the resource provided in it. We hypothesised that this might be due to the ferrets having a high motivation for a second chamber (e.g. to defecate far away from the nest box) or to see/explore what is in the other chamber. To assess whether these aspects of the set-up were responsible for the high motivation for an empty chamber, three functional alternative set-ups were tested: a set-up with an extra freely available chamber; a set-up with a see-through divider instead of a solid divider; and a set-up with a freely accessible mesh tunnel around the perimeter of EC. Additionally, four non-functional set-ups (i.e. with different ways of free access to the empty chamber) were tested. The weight that the ferrets would push for an empty chamber was not lowered in either one of the functional set-ups. Allowing free access to the empty chamber did reduce the weight the ferrets were willing to push for the empty chamber, the effect being the largest when the ferrets were provided with a manipulable item (two-way cat flap or flexible bucket) in the home chamber. As we were unable to improve the functionality of the 2-chamber consumer demand set-up, an alternative set-up needed to be designed.

There were two possible alternatives to a 2-chamber consumer demand study set-up, the first being a 3-chamber set-up, with one home chamber, one empty chamber and one enrichment chamber. In a 3-chamber set-up, the motivation for different enrichments is tested consecutively, limiting the risk of substitutes influencing the ferrets' motivation. The other alternative was to test the ferrets in a multi-chamber set-up, with one home chamber, one empty chamber and multiple enrichment chambers. In this set-up, the motivation for different enrichments is tested concurrently, increasing the chances of the ferrets showing a low motivation for "luxury" items. As it was unknown which of these set-ups would be (most) functional for ferrets, we compared the results from a 3-chamber consumer demand study set-up to the results from a 7-chamber consumer demand study set-up in Chapter 3. Additionally, as results from the previous study (Chapter 2) indicated that the items present in the home chamber seemed to influence the ferrets' motivation to enter an empty chamber, the influence of the items that are present in the home chamber was assessed in an all-but-one set-up (compared to the 3-chamber set-up). In both the 7-chamber and the all-but-one set-up the ferrets showed a lower motivation for, less and shorter visits to, and shorter interaction times with the enrichment (chambers) than in the 3-chamber set-up. This appeared to be related to the ferrets making more economic choices in the 7-chamber set-up, as there was a differential effect on the less preferred enrichment and the empty chamber compared to the preferred enrichment chambers. For the all-but-one set-up, the explanation for the differences compared to the 3-chamber set-up may lie in the fact that the used set-up was not truly a closed economy, as the items in the home chamber and enrichment chamber were rotated. We therefore advise to use a multi-chamber consumer demand study set-up while keeping the number of items in the home cage to a minimum.

In Chapter 4, we used the 7-chamber consumer demand study set-up to test the ferrets' motivation for different enrichment categories, including their preference for specific items within each category. The motivation for sleeping enrichments (with a strong preference for the hammock) was as high as the motivation for food, i.e. they pushed to their maximum ability to gain access to sleeping enrichment. The ferrets also showed a higher motivation for sleeping enrichment, (drinking) water in a bowl, conspecifics, foraging enrichments and tunnels than for an empty chamber. However, there was a large individual variation for tunnels, i.e. some ferrets were very highly motivated to access these enrichments, while others were not. The only enrichments the ferrets were as motivated for as an empty chamber, were those in the category balls.

It is not only important to identify the ferrets' motivation for different enrichment items, but the effects of providing different types of enrichment should also be assessed. Therefore, in Chapter 5, the behavioural and physiological effects of providing preferred (i.e. two hammocks, a water bowl and a foraging ball) and non-preferred (i.e. two ferret balls, an extra food bowl and a golf ball) enrichment to laboratory ferrets for eight weeks were assessed. The ferrets that were housed in standard housing conditions (i.e. with bedding and a bucket) showed a greater increase in agonistic behaviour over these eight weeks than the ferrets that were housed with (non-preferred or preferred) enrichments. Moreover, the ferrets that were provided with preferred enrichment showed an increase in social play behaviour and a decrease in rearing behaviour after eight weeks, while the ferrets with non-preferred enrichment or in standard housing conditions did not. As agonistic behaviour is regarded as a negative welfare indicator and social play is regarded as a positive welfare indicator, studies using ferrets can probably be refined by providing these preferred enrichments (i.e. hammocks, a water bowl and a foraging ball).

Part II of this thesis focused on refining the care and use of laboratory ferrets by minimising the amount of pain that the ferrets experience. The literature review presented in Chapter 6 shows that most of the indicators of pain and other forms of discomfort in ferrets are impractical, nonspecific, inconsistent, subjective, and sometimes even contradictory. This lack of knowledge and unreliability of species specific pain scales underlines the great necessity for development and validation of a (new) reliable and easy to use pain assessment tool to ensure further refinement in experimental methods for ferrets.

To get an indication of possible new tools for the recognition of pain in laboratory ferrets, an international survey was spread amongst ferret owners, questioning which physiological and/or behavioural parameters they use to recognise (assumed) pain in their ferrets (Chapter 7). The results of this survey showed that ferret owners used general physiological and behavioural parameters, such as a decrease in weight, shallower/faster/more difficult breathing, diminished activity, less play, increased sleeping, decreased ingestion, decreased grooming, more vocalisation and changes in posture. Additionally, ferret owners indicated to use their ferrets' facial expressions to identify pain, whereby they particularly focused on squinting of the eyes, but they also indicated to see some changes in the ears, whiskers, nose and mouth. Using facial expressions to recognise pain in laboratory ferrets is a promising and practically applicable tool that had already been investigated in several other animals.

The possibility to compose a grimace scale for ferrets was explored in Chapter 8. A Ferret Grimace Scale consisting of five action units (*orbital tightening*, *whisker retraction*, *nose bulging*, *cheek bulging* and *ear changes*) was composed. Subsequent evaluation of this Ferret Grimace Scale showed that the facial expressions of ferrets and *orbital tightening* in particular, might be useful for pain assessment in (laboratory) ferrets. The other action units should be re-evaluated because they had negative or low weight and many missing values. Moreover, including these action units did not increase or even lowered the sensitivity, specificity and accuracy with which the Ferret Grimace Scale could be used by blinded observers to differentiate ferrets pre- and post-surgery. Also, the Ferret Grimace Scale should be further validated before it can be used for the assessment of pain in a multifactorial assessment protocol.

Implications and future of refinement of the care and use of laboratory ferrets

The results of the studies presented in this thesis, show that the care and use of laboratory ferrets can be refined 1) by providing them with hammocks, a water bowl and a foraging ball; and 2) by using the Ferret Grimace Scale to recognise pain. To reduce the possibility that conclusions are drawn based on a type I error, it is always necessary to replicate the results that were obtained. Also, the results should be viewed within their context, the limitations of the studies should be taken into consideration and the application of currently unexploited possibilities to refine studies that use ferrets (e.g. habituation and training) should be considered.

Part I – Value and effect of environmental enrichment for laboratory ferrets

The studies presented in part I of this thesis indicate that a multi-chamber consumer demand study set-up using a push door is the best method to identify beneficial enrichment for ferrets (i.e. hammocks, a water bowl and a foraging ball). Here we discuss the considerations regarding the consumer demand study set-up, the choice and categorisation of enrichments, the effect of enrichment on animal welfare, the limitations for generalisation of the results and possible reasons for reluctance to apply enrichment items to laboratory ferrets.

Consumer demand study set-up

We have performed several consumer demand studies to identify the enrichments that will most likely lead to a refinement of the care and use of laboratory ferrets. We initially started with a 2-chamber consumer demand study set-up, as a multi-chamber set-up comes with the limitation that choice in itself may be enriching; less preferred substitutes might be undervalued when tested concurrently with other enrichments; and the set-up comes with large space requirements, resulting in animals having to be tested consecutively, which may cause time effects that cannot be controlled for.

In the study described in Chapter 2, we established that a 2-chamber consumer demand study set-up was not functional for ferrets, even though this set-up has proven to generate valuable information regarding the motivation other animal species show to reach different kinds of enrichment (e.g. for parrots, van Zeeland, 2013). As discussed, the 2-chamber consumer demand study set-up was potentially unsuitable for ferrets due to their highly explorative (i.e. neophile) nature, which could have resulted in a high motivation to investigate novel items (Reijgwart et al., 2015). Possibly, a 2-chamber consumer demand study set-up will be functional for animal species that have the tendency to be more reluctant to explore novelty (i.e. are neophobic) and for that reason will not be motivated to push heavy weights for an empty chamber. Moreover, a multichamber consumer demand study set-up might even be overwhelming for neophobic animals and potentially lead to choice aversion (Hutchinson et al., 2005).

Another explanation for the difference in functionality of the 2-chamber set-up for different animal species might be that there are differences in how naturalistic a certain task is for that species. It has been reported in multiple species that different operant tasks may elicit a different motivation to interact with the task (e.g. Partridge, 1976; Young et al., 1994). For example, mink preferred and learned faster to pull a chain than pressing a lever, the first being assumed to better replicate natural foraging behaviour (Hansen et al., 2002). Similarly, pushing a door might present a less natural task for parrots than for ferrets. We used pushing a weighted door as a naturalistic task for the ferrets, as this is a very common task for consumer demand studies and has been used for several other animal species (e.g. starlings: Asher et al., 2009; fish: Galhardo et al., 2011; sheep: Jackson et al., 1999; mink: Mason et al., 2001; hens: Olsson and Keeling, 2002; rabbits: Seaman et al., 2008; dairy cattle: Von Keyserlingk et al., 2017). The use of the weighted door for ferrets was further supported by the observation that the ferrets did not need training to perform this task, which is suggested to reflect the naturalistic nature of the task (Hansen et al., 2002).

Generally, for the benefit of comparability of consumer demand studies in different species, it would be preferable to standardise behavioural tests. However, as discussed above, the conclusion that a multi-chamber consumer demand study set-up using a push door is the optimal set-up might not be applicable to all animal species. As testing an animal in a sub-optimal set-up for its species might result in biased results, not standardisation, but thorough knowledge of the natural behaviour and history of the species to be tested is required when designing a consumer demand study to ensure that each animal species is tested in a set-up that is optimal for measuring its behavioural priorities.

Choice and categorisation of enrichments

We have tested the ferrets' priorities and preferences for thirteen types of enrichment, divided over six enrichment categories (sleeping enrichment, foraging enrichment, tunnels, balls, social enrichment and water bowls) in the consumer demand studies presented in Chapters 3-4. These enrichment (categories) were chosen based on the natural history and behaviour of ferrets, as the most "natural" enrichments are suggested to have the most beneficial effects on the animals' welfare (Newberry, 1995). However, the more natural enrichments were not always preferred by the ferrets. For example, based on the literature on feral ferrets (Clapperton, 2001), we hypothesised that ferrets would prefer to sleep in a dark, enclosed area, comparable to their natural resting location (i.e. rabbit burrows), and would therefore prefer the bucket or the plastic cover for resting. Contrary to this hypothesis, the ferrets preferred to sleep in the elevated, exposed hammock and were highly motivated to do so, possibly due to the soft lining of the hammock, the rocking motion, warmth, space or the snugness of the hammock (Reijgwart et al., 2016). Moreover, provision of this hypothesised less natural enrichment (together with a water bowl and a foraging ball) resulted in positive behavioural changes (i.e. less aggression and more play) that were not observed in ferrets that were provided with the more naturalistic ferret ball and/or bucket (Chapter 5).

The ferrets showed a high motivation to gain access to sleeping enrichment, foraging enrichment, tunnels, social enrichment and water bowls. We chose to divide the enrichments into six enrichment categories to increase the chances of testing the favoured item to perform each categorised behaviour (i.e. sleeping, foraging, exploring, chasing, socialising, drinking, and eating). However, the categorisation of enrichments was most likely not absolute, as enrichments can have multiple purposes. For example, the ferrets did not only use the hammock for sleeping, but also to manipulate c.q. "play" with (i.e. pull at it). Therefore, enrichment options that were presented in separate chambers might have - in part – been substitutes and allowed for performance of similar behaviours. For example, both the category balls and the category foraging enrichment allowed for the expression of appetitive behaviour (i.e. chasing); and both the ferret ball and hammock allowed for sleeping or resting behaviour. The ferrets never rested in the ferret ball in the consumer demand study (Reijgwart et al., 2016), but preferred resting in the ferret ball over the sawdust and bucket when no hammock was present (Chapter 5). Therefore, the ferrets may have chosen to work for their most preferred option in the consumer demand study (i.e. hammock, foraging ball), despite the other enrichment option being of interest to the animal as well, resulting in undervaluation of the less preferred substitute (i.e. ferret ball). This highlights one of the important downsides of a multi-chamber consumer demand study set-up.

Within the most preferred enrichment categories, the ferrets preferred the hammock, water bowl and foraging ball (Reijgwart et al., 2016). Of course, only a limited number of enrichment categories and items could be tested, so we cannot rule out that there might possibly be other highly enriching items that were not included in the studies presented in this thesis. For example, we have not tested the ferrets' motivation and preferences for different types of bedding, but always provided them with wood fiber bedding, a bedding type that is routinely provided to laboratory ferrets in research facilities. However, as sawdust and wood shavings are suggested to cause upper respiratory tract problems, this might be detrimental in case of studies into the ferrets' respiratory system (e.g. influenza virus) and might therefore be better removed from the ferrets" environment. Nevertheless, the ferrets appeared to appreciate the bedding, as every time new bedding was added the ferrets would "weasel war dance" and dig in it while vocalising ("dooking"). This could indicate that provision of some kind of bedding is essential for the ferrets' wellbeing, especially when taking into account that digging is a natural behaviour for which ferrets will likely show a high motivation (Fisher, 2006). As such, simple removal of bedding is not advised if the behaviour cannot be fulfilled in another way. Instead, alternatives such as those used by private ferret owners (e.g. torn-up blankets, towels or sheets, rice or sand boxes (Lewington, 2007), unpublished ferret owner survey) may be considered. Future studies to assess the ferrets' motivation for different bedding types are therefore advised, to identify whether and for which types of bedding materials ferrets are highly motivated. This will allow for recommendations to be made on alternative bedding types that help to optimise the ferrets' welfare while simultaneously preventing potential adverse effects on lung function and study results.

The effect of enrichment on animal welfare

We hypothesised that the enrichments for which the ferrets were highly motivated in the consumer demand studies would also have the most value in refining the housing conditions of laboratory ferrets. However, before enrichment can be said to be beneficial, the effects on animal welfare should be considered (Würbel and Garner, 2007). Changes in an animals' behaviour and physiology are often used as an indicator of welfare. For example, agonistic behaviour is generally seen as a negative welfare indicator, whereas play behaviour is regarded as a positive welfare indicator (Blanchard et al., 2001; Held and Špinka, 2011). Therefore, in Chapter 5, the effect of the provision of different types of enrichment on the behaviour and physiology of laboratory ferrets was assessed. The results of this study showed that providing any type of enrichment to the ferrets resulted in less aggressive behaviour, compared to standard housing conditions. Moreover, the provision of preferred enrichment resulted in less rearing behaviour and more social play behaviour, compared to providing either non-preferred enrichment or housing the animals in standard housing conditions. In other words, preferred enrichment seemed to lower the performance of behaviours that are initiated by and result in stress (i.e. aggression), while simultaneously increasing the performance of rewarding behaviours that are indicative of relaxation (i.e. play behaviour). This finding might therefore be an indication that the ferrets housed with a hammock, foraging ball and water bowl experience a better welfare than the ferrets that are not.

It should be taken into account that the interpretation of all measured behavioural parameters is complicated, as we mostly do not know the (valence of the) emotion that is driving some of these behaviours. For example, as discussed in Chapter 5, tunnelling behaviour was categorised as exploration behaviour, but it might just as well represent play or scent marking behaviour. Also, the ferrets were highly motivated to be able to sleep next to two conspecifics, which might be driven by a desire for social contact or it might have a function as territorial behaviour. Nevertheless, even if we did exactly know how to categorise the behaviour, the emotional valence of the behaviour remains unknown. Therefore, before conclusions can be drawn regarding the effect of providing enrichment on animal welfare, the effects of enrichment on emotional perception (i.e. whether it actually increases the animals' feeling of wellbeing) should be studied (Duncan, 1993; Fraser and Duncan, 1998). For example, the valence of emotion can be estimated through measuring cognitive expressions of internal emotional states in a judgement bias test, which uses an animals' choices to infer whether it feels more pessimistic or optimistic (Mendl et al., 2009; Paul et al., 2005). This type of test was originally applied in human psychology and relies on the assumption that changes in affective state causes changes in judgement, attention and memory (Paul et al., 2005).

For example, people in a negative affective state (e.g. anxious or depressed) have been shown to make negative judgements about neutral events and stimuli (Eysenck et al., 1991; Gotlib and Krasnoperova, 1998; MacLeod and Byrne, 1996; Wright and Bower, 1992). The judgement bias test has been adapted for the use in animals and it has been successfully used to assess both negative and positive affective states as a result of changes in the animals' environment, e.g. in rats (Brydges et al., 2011; Harding et al., 2004), mice (Boleij et al., 2012), starlings (Bateson and Matheson, 2007; Brilot et al., 2010; Matheson et al., 2008), dogs (Burman et al., 2011; Casey et al., 2008), and pigs (Douglas et al., 2012; Murphy et al., 2013). Performing a judgement bias test in ferrets that were housed in standard conditions, with non-preferred enrichment and with preferred enrichment might therefore shed more light on the effect of these enrichments on the wellbeing of the ferrets.

Limitations for generalisation

The generalisability of the conclusion that a multi-chamber consumer demand study set-up using a push door is the best method to identify beneficial enrichment for ferrets (i.e. hammocks, a water bowl and a foraging ball) to laboratory ferrets in general is limited by three factors:

First, we have chosen to only use young, female ferrets in our studies, as this was the age and sex that is predominantly used at research facilities. Commonly, studies using ferrets last for only one or two months, whereas our behavioural studies lasted for several months to a year. Therefore, our ferrets were ovariectomised to prevent them from going into oestrus during these long studies, which would greatly influence the behaviour and possibly health of the ferrets. However, intact female ferrets, male ferrets and/or older ferrets might have different enrichment priorities and the enrichments might have differential effects on these animals. For example, (older) intact females and males might be more motivated to perform territorial behaviour and therefore have a lower motivation for contact with conspecifics and/or the effect of social housing might be different in these animals (Takahashi, 1990). Therefore, it might not be possible to generalise the results of the studies presented in this thesis to intact females and males. Replication of these studies using intact females and males is therefore recommended in order to be able to make general recommendations for laboratory ferrets.

Secondly, we have tested the ferrets solitary in all the consumer demand set-ups for both practical reasons and because – taking the natural history of the ferret into consideration (Moller and Alterio, 1999; Moors and Lavers, 1981) – we hypothesised that the ferrets would not be motivated for contact with conspecifics. However, the ferrets were highly motivated for access to two other ferrets. This motivation could have been driven by positive emotions, as the ferrets were mostly seen sleeping in proximity of the other ferrets (Reijgwart et al., 2016), but this could also be explained by the ferrets' wanting to keep an eye on the potential competitors in their territory (Chang et al., 2000; Takahashi, 1990). This again highlights the necessity to further study the emotional valence of behaviours. Nevertheless, it is stated in the EU Directive that ferrets should be housed with conspecifics and isolation should be regarded as causing severe discomfort to the animals (European Commission, 2007), which makes it most likely that ferrets will be housed socially in research settings. It has been shown for several (social) animal species that social testing generates different results than solitary testing (e.g. Mench and Stricklin, 1990; Pedersen et al., 2002), therefore it would be interesting to investigate whether the ferrets' priorities and preferences are different when they are tested in a social setting. Related to the social testing in the consumer demand study, the effects of enrichment on the behaviour and physiology of ferrets were now assessed using groups of six ferrets. Again, this was chosen as this was the common group size at the research facility. However, the effects of enrichment and the effects of social housing itself might be very different in smaller or larger groups. Possibly, the motivation for and the effect of enrichments that provide shelter or compartmentalisation of the enclosure might change greatly when the ferrets are tested in a social consumer demand study set-up or when the effects of enrichment are assessed using a different group size.

Thirdly, early life experiences, socialisation, habituation and differences in personality can influence the extent to which results from one study can be generalised to the entire (laboratory) ferret population. For example, the well-habituated ferrets that were used in our studies would gather in front of the cage when someone entered the room, whereas ferrets that had just recently arrived at the research facility and only received limited socialisation and habituation to people, would all hurry to hide in the bucket. Additionally, even though we used animals that originated from the same breeder and were equally socialised and habituated to the presence of and handling by people in the consumer demand studies, not all ferret were equally motivated for all enrichment categories. For example, there was a great variability in the motivation the ferrets' showed for access to the tunnels in the 7-chamber set-up (Reijgwart et al., 2016). When reviewing the video footage of this study, we observed that one of the animals that was highly motivated to go to the tunnels only went there at times that a person entered the animal room. She would hide in one of the tunnels during the cleaning tasks and would only emerge after a few minutes. Therefore, not only the extent to which the ferrets are socialised and habituated to humans might play an important role in the motivation for enrichment that e.g. enables hiding, also personality and individual preferences should be taken into account.

Practical application

We found positive effects on agonistic, play and exploration behaviour in ferrets that were provided with hammocks, a water bowl and a foraging ball (Chapter 5) and therefore advocate the application of these enrichments. However, research facilities might argue that providing these enrichments to ferrets is not always practically applicable or economically feasible in their setting.

One practical limitation for the provision of enrichment to ferrets might be that they are sometimes moved from Animal BioSafety Level (ABSL)-II to ABSL-III conditions (i.e. to an isolator). This limitation of the available space for enrichments and increased hygienic standards sometimes necessitates removing enrichment from the animals' environment, which has been suggested to have a negative effect on the welfare of animals (Bateson and Matheson, 2007; Latham and Mason, 2010). However, the effect of removing or changing enrichment to more practical items on the welfare of ferrets has not been investigated. Until this has been demonstrated, and taking into account that the animals spend the majority of their life in ABSL-II conditions or even less, and only a limited time in the more strict ABSL-III conditions, preferred enrichment should be provided to ferrets. If changing or removing the enrichments prove to have a negative effect on the welfare of the ferrets, examining which elements of the use of preferred enrichments are responsible for the high motivation for these items might point into the direction of alternative highly valued enrichments that are applicable in a research setting.

Another argument not to implement enrichment might be that there are concerns about the comparability with previous scientific results that were obtained using standard (i.e. impoverished) housed animals. However, I share the view that "it is unacceptable that the impoverishment that was imposed upon these animals by man's requirements for standardisation and optimalisation of economical and ergonomical factors, is now a reason to prohibit improvements of these conditions" (Van der Harst and Spruijt, 2007). Also, the rigorous standardisation and impoverishment of the environment of laboratory animals has been applied in an attempt to decrease within- and between-laboratory variability, thereby increasing replicability (Beynen et al., 2003a; Beynen et al., 2003b; Gartner, 1999), which has falsely been used as a proxy measure of external validity of experimental results, i.e. the "standardisation fallacy" has been applied (Baumans, 2005; Richter et al., 2009; Würbel, 2000). As externally valid experimental results should be both replicable and robust against minor variations in housing, provision of beneficial enrichment will actually result in scientific results of a higher quality by taking environmental effects into account and reducing the number of type I errors (Baumans, 2005; Richter et al., 2009; Würbel, 2002; Würbel, 2000).

Another argument against the rigorous standardisation and impoverishment of the environment of laboratory animals is that while variance in experimental results might be reduced to some degree by standardisation of e.g. housing and feeding conditions and ambient room conditions (Baker, 2011), maternal factors and early rearing conditions cannot be controlled (Holmes et al., 2005). Additionally, there are always environmental factors that cannot be standardised between laboratories (i.e. staff, animal rooms, noise levels Crabbe et al., 1999), so even if rigorous standardisation results in the intended increased within-laboratory replicability, it will unintendedly decrease between-laboratory replicability. This unproductive approach to minimise variation has also been referred to as "standardisation and the Red Queen" (Garner et al., 2006), in analogy to Alice running on the spot (Alice through the looking glass, Lewis Carroll).

Part II – Recognition and alleviation of pain in laboratory ferrets

The review presented in chapter 6 and the owner survey in chapter 7 show that recognising pain in animals is difficult (see also Sneddon et al., 2014). An additional complicating factor is the difficulty in defining whether the observed changes in the animal are indeed pain (that can be alleviated by the provision of analgesics) and not another form of discomfort (e.g. nausea). A relatively new, valuable measure for the assessment of pain is the use of facial expressions (Descovich et al., 2017). Since this was not developed yet for ferrets, part of this thesis focused on composing and evaluating a Ferret Grimace Scale (chapter 8). Here, we discuss the possibilities to further validate this grimace scale, the considerations for practical application of the Grimace Scale, and pain alleviation in laboratory ferrets.

Validation of the Ferret Grimace Scale

Part II of this thesis showed that facial expressions might aid in the recognition of (postoperative) pain in ferrets, leading to the development of a Ferret Grimace Scale that was composed of the action units *orbital tightening*, *ear changes*, *nose bulging*, *cheek bulging* and *whisker retraction* (Chapter 8). Evaluation of this grimace scale indicated that mainly the action unit *orbital tightening* could be used by blinded observers to differentiate ferrets pre- and post- surgery. However, we were not able to identify whether the grimace was sensitive and specific for pain, i.e. whether the ferrets that showed a grimace were actually experiencing pain and whether ferrets only show a grimace when experiencing pain and not also in other contexts. Therefore, this new tool for the recognition of pain in ferrets should be further validated before recommendations can be made regarding the provision of analgesia to ferrets that show a grimace.

To assess whether the Ferret Grimace Scale is sensitive to pain, a control group that receives analgesia should be used, as was done for other grimace scales (e.g. Langford et al., 2010). For the surgery that was used in this thesis, NSAIDs (e.g. meloxicam, carprofen, ketoprofen, flunixin meglumine) are good candidates to administer to the ferrets in the control group, as these have been reported to successfully alleviate post-operative pain in ferrets (Flecknell, 1998; Lichtenberger and Ko, 2007; Pollock, 2002). Of these NSAIDs, only the pharmacokinetics of meloxicam have been studied in ferrets, with male and female ferrets having peak plasma concentrations similar to those reported to provide adequate analgesia in other species one hour after a single subcutaneous injection of 0.2 mg/kg Meloxicam (Chinnadurai et al., 2014). However, the analgesic efficacy of Meloxicam was not specifically tested and there is a general lack of data on the clinical efficacy of the analgesic compounds available for use in ferrets (van Oostrom et al., 2011). Therefore, the identification of an effective analgesic substance and concentration should be a focus of future research. Once the Ferret Grimace Scale is validated with the use of one analgesic substance (e.g. meloxicam), the grimace scale can subsequently be used to provide data on the efficacy of other analgesic substances and doses (as was done in mice, Matsumiya et al., 2012).

Another approach to assess the relative sensitivity of the Ferret Grimace Scale is to correlate the grimace scores with a species-specific behavioural pain scale that includes behavioural and physiological indicators of pain as those mentioned in Chapter 6. For example, post-surgical pain assessment using the Mouse Grimace Scale was strongly correlated with manual scoring of pain behaviours (Leach et al., 2012). However, there are few validated pain-indicating behaviours in ferrets, as reviewed in Chapter 6. Novel techniques such as the measurement of ultrasonic vocalisations as an indicator of pain might be explored, which have been suggested to be a reliable indicator of chronic cancer pain and neuropathic pain in mice (Kurejova et al., 2010). Even though ultrasonic vocalisations have not been reported in ferrets, mink kits that are isolated from their mothers and nests perform complex ultrasonic vocalisations (Clausen et al., 2008), which might indicate that ferrets use this form of communication as well.

Application of the Ferret Grimace Scale

Aside from validation, application of the ferret grimace scale in practice requires other criteria to be met. First of all, grimace scales have been reported to be less useful to detect acute (<10 min) or chronic (>24h) pain (Langford et al., 2010). As we have only investigated the application of the Ferret Grimace Scale for the detection of post-operative pain, additional studies should focus on the efficacy with which it can be used to identify pain following various painful procedures, as.

Also, the practical applicability of the Ferret Grimace Scale might be improved by determining a general baseline grimace score (which is never 0) and an intervention threshold score (i.e. the grimace score above which the application of analgesia is indicated), as was done for rats and mice (Miller and Leach, 2015; Oliver et al., 2014). When these baseline and intervention scores are determined, it should be taken into account that there might be sex differences in both pain expression and pain perception (Langford et al., 2010; Miller and Leach, 2015; Wiesenfeld-Hallin, 2005).

Additionally, the amount of false negatives and false positives should be avoided as much as possible, as these reduce the practical applicability of the Ferret Grimace Scale. The amount of false negatives might be reduced by thorough socialisation and/or habituation to humans, as ferrets with limited socialisation and habituation might not show a grimace due to fear or anxiety while they actually are in pain. The observation that Mouse Grimace Scale scores were lower when live scoring was used compared to retrospective scoring from still images (that were made in the absence of human observers) might be illustrative of this effect (Miller and Leach, 2015). To prevent false positives, the environmental context in which the animals are assessed should be carefully considered, as the grimace scale has been shown not to be specific for pain. For example, facial expressions comparable to the pain-grimace are reported when the animal is assessed in a brightly lit room, when tired/sedated, and when being in an agonistic encounters (Defensor et al., 2012; Langford et al., 2010; Miller et al., 2015; Sotocinal et al., 2011).

Pain alleviation in laboratory ferrets

The Ferret Grimace Scale can aid researchers in recognising pain in ferrets and thereby allow them to minimise the pain that the ferrets experience, which will not only improve animal welfare, but also increase the validity of research, as pain is regarded as an adverse side effect in studies that are not explicitly intended to study pain. Significant unrelieved pain is a stressor that, if the animal cannot adapt to it, causes distress and negative physiologic consequences, not the least of which is immune dysfunction (Padgett and Glaser, 2003). Moreover, individual differences in the way the animals cope with this stressor (Koolhaas et al., 1999) might increase inter-individual variation in the results, resulting in more animals being needed to achieve sufficient statistical power. Nevertheless, the possible (negative) effects of analgesia on the study objectives and the fear of incomparability with previous studies (in which no analgesia was applied) have been used as an (invalid) argument to justify withholding analgesia. Again, it is my view that it is unacceptable that requirements for standardisation and optimisation of economic factors are now a reason to prohibit improvements of these conditions. Moreover, the lack of data on the clinical efficacy, pharmacokinetics, and pharmacodynamics of the analgesic compounds available for use in ferrets (van Oostrom et al., 2011) should not be a valid argument to withhold analgesia, but instead should galvanise our efforts to fill the gaps in our knowledge, with the aim of no longer withholding necessary pain medication for laboratory ferrets simply based on the potential for compromising the experimental outcome.

Unexploited possibilities for refinement

The studies presented in this thesis culminate in two recommendations to refine the care and use of laboratory ferrets: the provision of preferred enrichment and using a grimace scale to recognise pain. Additional to these measures, habituation and training of ferrets can provide a currently underexploited possibility for refinement.

The lack of proper socialisation and habituation of ferrets to human handling has given ferrets the reputation of being aggressive towards handlers, resulting in ferrets in research facilities being handled using a thick leather glove combined with a sudden, firm grip in order to prevent being bitten. However, when time is invested to habituate and positively reinforce the ferrets to the presence of and gentle handling by humans, they are actually very friendly animals that enjoy human contact. Also, my experience is that they seem to enjoy and solicit tickling, which has been shown to be a rewarding and fear-reducing form of human contact for rats (Burgdorf and Panksepp, 2001; Cloutier et al., 2012; Cloutier et al., 2013; Panksepp and Burgdorf, 2000). Gentle handling and playful interaction might therefore be quick and easy ways to provide the ferrets with a pleasurable experience. Moreover, it might even be used to mitigate the impact of aversive procedures such as injections (Cloutier et al., 2014). Human interaction can even be used as a positive reinforcer to train the ferrets to voluntary cooperate during e.g. weighing, injection or blood sampling (Davis and Pérusse, 1988) – for which of course other reinforcers can also be used - resulting in these procedures being less stressful to both the animals and technicians.

Limited socialisation and habituation can also cause routine activities and procedures, such as personnel entering the animal room and cage cleaning, to induce stress, which in turn may have major effects on the experimental outcome (Balcombe et al., 2004; Castelhano-Carlos and Baumans, 2009; Reinhardt and Reinhardt, 2006). Habituation and training should therefore also be implemented to improve the validity of experimental results (e.g. Rowan, 1990; van Driel and Talling, 2005; Verwer et al., 2009). One example of training that might be used to mitigate the stress that results from common procedures is the introduction of a safety signal, so the animals learn that nothing will happen as long as that signal is given.

Final remark

This thesis presents two promising methods to refine the care and use of laboratory ferrets that will hopefully be applied in every research facility. Nevertheless, this is just the tip of the iceberg and hopefully the suggestions presented in this discussion will inspire other researchers to further study possibilities for refinement in order to provide these wonderful animals with the best possible care.

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ADDENDUM

List of publications and abstracts

Scientific publications

- *M.L. Reijgwart, C.M. Vinke, C.F.M. Hendriksen, M. van der Meer, N.J. Schoemaker, Y.R.A. van Zeeland* (2015) Workaholic ferrets: Does a two-chamber consumer demand study give insight in the preferences of laboratory ferrets (*Mustela putorius furo*)? Applied Animal Behaviour Science 171:161-169. DOI: 10.1016/j.applanim.2015.08.032
- *M.L. Reijgwart, C.M. Vinke, C.F.M. Hendriksen, M. van der Meer, N.J. Schoemaker, Y.R.A. van Zeeland* (2016) Ferrets' (*Mustela putorius furo*) enrichment priorities and preferences as determined in a seven-chamber consumer demand study. Applied Animal Behaviour Science 180:114-121. DOI: 10.1016/j.applanim.2016.04.022

Accepted for publication

- *M.L. Reijgwart, C.M. Vinke, C.F.M. Hendriksen, M. van der Meer, N.J. Schoemaker, Y.R.A. van Zeeland* (2017) Are all motivation tests the same? The effect of two adaptations to a 3-chamber consumer demand study in ferrets. Accepted for publication in Animal Behaviour.
- *M.L. Reijgwart, N.J. Schoemaker, R. Pascuzzo, M.C. Leach, M. Stodel, L. de Nies, C.F.M. Hendriksen, M. van der Meer, C.M. Vinke, Y.R.A. van Zeeland* (2017) The composition and initial evaluation of a grimace scale in ferrets after surgical implantation of a telemetry probe. Accepted for publication in PlosOne.

Submitted manuscripts

- *M.L. Reijgwart, C.M. Vinke, C.F.M. Hendriksen, K. den Hoed, M. van der Meer, M. van der Meer, N.J. Schoemaker, Y.R.A. van Zeeland* **(2017)** The effect of provision of preferred and non-preferred enrichment on behavioural and physiological parameters in laboratory ferrets (*Mustela putorius furo*). Submitted to Applied Animal Behaviour Science .

Popular/scientific publications

- M.L. Reijgwart (2016) Verrijking voor fretten. Biotechniek nummer 55/4: 15-19

Abstracts in proceedings

- *M.L. Reijgwart, C.M. Vinke, C.F.M. Hendriksen, M. van der Meer, N.J. Schoemaker, Y.R.A. van Zeeland (2014)* Workaholic ferrets: pushing it to the limit. Proceedings ISAE Benelux 2014.
- *M.L. Reijgwart, C.M. Vinke, C.F.M. Hendriksen, M. van der Meer, N.J. Schoemaker, Y.R.A. van Zeeland (2015)* Ferrets' behavioural priorities and preferences as determined in a three- and seven-chamber consumer demand study. Proceedings ISAE Benelux 2015.
- *M.L. Reijgwart, C.M. Vinke, C.F.M. Hendriksen, M. van der Meer, N.J. Schoemaker, Y.R.A. van Zeeland* (2015) Ferrets' behavioural priorities and preferences as determined in a three- and seven-chamber consumer demand study. Proceedings NVG Meeting 2015.
- *M.L. Reijgwart, Y.R.A. van Zeeland, C.M. Vinke, M. van der Meer, N.J. Schoemaker, C.F.M. Hendriksen (2016)* Refinement of the care and use of laboratory ferrets (*Mustela putorius furo*). Proceedings FELASA 2016.
- *M.L. Reijgwart, C.M. Vinke, C.F.M. Hendriksen, M. van der Meer, N.J. Schoemaker, Y.R.A. van Zeeland (2016)* What ferrets want: studies into the enrichment priorities of ferrets (*Mustela putorius fu*ro). Proceedings ISAE 2016.
- *M.L. Reijgwart, C.M. Vinke, C.F.M. Hendriksen, M. van der Meer, N.J. Schoemaker, Y.R.A. van Zeeland, (2017)* Are all motivation tests the same? A comparison of three types of consumer demand studies in ferrets (*Mustela putorius furo*). Proceedings UFAW 2017.
- *M.L. Reijgwart, N.J. Schoemaker, R. Pascuzzo, M.C. Leach, C.F.M. Hendriksen, M. van der Meer, C.M. Vinke, Y.R.A. van Zeeland (2017).* Development and validation of a ferret grimace scale. proceedings UFAW 2017.

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Nederlandse samenvatting

Fretten (*Mustela putorius furo*) zijn een waardevol diermodel in biomedisch onderzoek, bijvoorbeeld voor het bestuderen van humane ziektes zoals influenza. De Nederlandse wetgeving vereist dat experimenten waarbij gebruik wordt gemaakt van dieren het principe van de 3V's wordt nageleefd. Dit betekent dat het gebruik van dieren daar waar mogelijk vervangen moet worden (vervanging); dat het aantal dieren dat gebruikt wordt verminderd moet worden (vermindering); en dat de pijn en het ongerief van de dieren geminimaliseerd moet worden waardoor het welzijn van de dieren verbeterd wordt (verfijning). Helaas is er weinig bekend over hoe de zorg voor en het gebruik van de fret verfijnd kan worden, wat ook zichtbaar is in de beperkte wet- en regelgeving betreffende deze dieren. De onderzoeken die zijn beschreven in dit proefschrift zijn uitgevoerd met het doel om inzicht te krijgen in mogelijke manieren om de zorg voor en het gebruik van fretten te verfijnen, waarbij de nadruk is gelegd op de waarde en het effect van kooiverrijking (deel I) en het herkennen van pijn en andere vormen van ongerief in fretten (deel II).

Verfijning door het aanbieden van effectieve kooiverrijking

Deel 1 van dit proefschrift richt zich op verfijning van de zorg voor en het gebruik van de fret als proefdier door het verschaffen van kooiverrijking. Om de motivatie van een dier voor verschillende types kooiverrijking (en daarmee de waarde van de verrijking) te bepalen worden zogeheten 'consumer demand' studies gebruikt. In een consumer demand studie moet het dier een bepaalde taak uitvoeren (een 'prijs' betalen) om toegang te krijgen tot verrijking, waarbij de taak iedere dag een beetje zwaarder wordt gemaakt. In dit proefschrift is gebruikt gemaakt van een deur die door het aanbrengen van gewichten moeilijker open gemaakt kon worden. Het gewicht waarbij de fret de taak te zwaar/de prijs te hoog vindt en geen toegang meer neemt tot de verrijking geeft de waarde van deze verrijking weer. In de eerste drie hoofdstukken van dit proefschrift is dit type onderzoek gebruikt om de waarde te bepalen die fretten aan verschillende typen verrijking hechten.

In **hoofdstuk 2** wordt een onderzoek beschreven waarbij de functionaliteit van een 2-kamer consumer demand studie opzet is getest. Een 2-kamer opzet bestaat uit een thuiskamer en een lege of verrijkingskamer. De 2-kamer opzet werd geacht functioneel te zijn wanneer: 1) de motivatie voor voer (een essentiële bron) de maximale duwkracht van de fretten weergaf; 2) het duwen van de deur zwaar was voor de fretten; en 3) het maximale gewicht dat de fretten duwden voor een lege ruimte significant lager was dan het gewicht dat zij duwden voor voer. De 2-kamer opzet voldeed aan voorwaarde 1, gezien de fretten de deur bij de hoogste gewichten

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nog open probeerden te duwen voor toegang tot voer, maar hier niet meer toe in staat waren. De opzet voldeed ook aan voorwaarde 2, gezien het aantal bezoeken aan de kamer met voer verminderde bij hogere gewichten, terwijl de duur van de bezoeken steeg. Met andere woorden: de fretten veranderden hun gedrag om bij de hogere gewichten hun voerconsumptie op peil te kunnen houden. De 2-kamer opzet voldeed echter niet aan voorwaarde 3, gezien de fretten 89% van het gewicht dat zij voor voer duwden, voor een lege kamer duwden. Dit was een indicatie dat de fretten mogelijk andere motieven hadden om de deur open te duwen, naast de bron die in de kamer verschaft werd. Onze hypothese was dat dit wellicht kwam omdat de fretten een hoge motivatie hadden om de beschikking te hebben over een tweede kamer (bijvoorbeeld om zich zo ver mogelijk van de nestbox te kunnen ontlasten) of om te kunnen zien/onderzoeken wat zich in de tweede kamer bevond. Om te onderzoeken welke elementen van de 2-kamer opzet mogelijk verantwoordelijk waren voor de hoge motivatie voor een lege kamer zijn drie alternatieve functionele 2-kamer opzetten getest: 1) een opzet met een extra vrij toegankelijke kamer; 2) een opzet met een doorzichtige scheiding tussen de twee kamers; en 3) een opzet met een vrij toegankelijke gazen tunnel langs de rand van de lege kamer. Naast deze drie functionele alternatieve opzetten zijn ook vier niet-functionele alternatieve opzetten getest, waarbij de fret op verschillende manieren vrij toegang had tot de lege kamer. Geen enkele van de alternatieve functionele opzetten verlaagde het gewicht dat de fretten bereid waren te duwen voor een lege kamer. Dit was wel het geval bij de niet-functionele alternatieve opzetten, waarbij het effect het grootste was in de opzetten waarin de fretten een object konden manipuleren in de thuiskooi (een kattenluikje dat naar twee kanten open kon of een plastic emmer). Omdat we de functionaliteit van de 2-kamer opzet niet konden verbeteren, zijn er alternatieve opzetten getest.

In **hoofdstuk 3** wordt een onderzoek beschreven waarbij drie verschillende alternatieve opzetten van de consumer demand studie zijn vergeleken. De eerste is een 3-kamer opzet met een thuiskamer, een lege kamer en een verrijkingskamer. In deze opzet wordt de motivatie voor verschillende verrijkingen na elkaar getest, waarmee het risico dat de motivatie van de fretten wordt beïnvloed door substituten wordt geminimaliseerd. De tweede is een 7-kamer opzet met een thuiskamer, een lege kamer en zes verrijkingskamers. In deze opzet wordt de motivatie voor verschillende verrijkingen tegelijkertijd getest, waardoor de kans dat de fretten een lage motivatie tonen voor ‘luxe’ verrijkingen gemaximaliseerd wordt. De derde is een alles-behalve-één 3-kamer opzet waarbij er meerdere verrijkingsobjecten in de thuiskamer aanwezig zijn. De resultaten die zijn verkregen in deze opzet zijn vergeleken met de resultaten uit de 3-kamer opzet, omdat het onderzoek in hoofdstuk 2 indiceerde dat de motivatie van de fretten beïnvloed kan worden door de items die aanwezig zijn in de thuiskamer. Zowel in de 7-kamer opzet als de alles-behalve-één opzet lieten de fretten een lagere motivatie zien voor de verschillende verrijkingen en bezochten ze deze minder en korter dan in de 3-kamer opzet. In de 7-kamer opzet leek deze lagere motivatie een resultaat te zijn van economische keuzes door de fretten, omdat er een verschillend effect waarneembaar was voor de minder gewilde verrijkingen en de lege kamer in vergelijking tot de voorkeursverrijkingen. Voor de alles-behalve-één opzet leek de lagere motivatie echter veroorzaakt te zijn doordat deze opzet niet een compleet gesloten economie had, omdat de verrijkingen in de thuiskamer rouleerden. Daarom volgt uit het onderzoek het advies om voor fretten een 7-kamer opzet te gebruiken waarbij het aantal verrijkingen in de thuiskamer tot een minimum worden beperkt.

In **hoofdstuk 4** wordt een onderzoek beschreven waarbij de 7-kamer studie opzet wordt gebruikt om de motivatie van fretten voor zes verschillende verrijkingscategorieën te testen. Hierbij is ook onderzocht wat de voorkeursverrijking binnen een categorie was. Uit het onderzoek bleek dat de motivatie voor de categorie ‘slaapverrijking’, waarbij de fretten een sterke voorkeur hadden voor de hangmat, net zo hoog was als de motivatie voor voer. In andere woorden, de fretten duwden tot hun maximale kunnen om toegang te krijgen tot een hangmat om in te slapen. De fretten toonden daarnaast een hogere motivatie voor toegang tot een waterbak, soortgenoten, voerverrijking en tunnels dan voor een lege kamer. Er was een hoge individuele variatie in de motivatie voor tunnels, waarbij sommige fretten heel hoog gemotiveerd waren voor toegang tot deze verrijking, terwijl anderen totaal niet gemotiveerd waren. De categorie ‘ballen’ was de enige waar de fretten geen hogere motivatie voor lieten zien dan voor de lege kamer.

In **hoofdstuk 5** wordt een onderzoek beschreven waarbij de gedragsmatige en fysiologische effecten van het huisvesten van fretten met verschillende soorten verrijking zijn onderzocht. Op basis van de resultaten uit hoofdstuk 4 werden twee hangmatten, een waterbak en een voerbal gecategoriseerd als voorkeursverrijking en twee frettenballen, een extra voerbak en een golfbal als niet-voorkeursverrijking. De fretten die gedurende acht weken in standaard omstandigheden (dus zonder verrijking) werden gehouden lieten een grotere toename in agonistisch gedrag zien dan de fretten die met (niet-)voorkeursverrijking werden gehuisvest. Daarnaast lieten de fretten die gedurende acht weken gehuisvest werden met voorkeursverrijking een toename in sociaal spel en een afname in ‘rearing’ zien, die niet gezien werd bij de fretten in standaard huisvesting of met niet-voorkeursverrijking. Omdat agonistisch gedrag wordt gezien als een negatieve welzijnsindicator en sociaal spelgedrag wordt gezien als een positieve welzijnsindicator, wijzen de resultaten van dit onderzoek er op dat studies waarbij fretten gebruikt worden waarschijnlijk verfijnd kunnen worden door ze te huisvesten met voorkeursverrijking (hangmatten, een waterbak en een voerbal).

Verfijning door het herkennen van pijn

Deel II van dit proefschrift richt zich op verfijning van de zorg voor en het gebruik van fretten als proefdier door het minimaliseren van de hoeveelheid pijn en ongerief die de fretten ervaren.

In **hoofdstuk 6** wordt een literatuuronderzoek beschreven dat aangeeft dat de meeste indicatoren van pijn en andere vormen van ongerief bij fretten niet praktisch toepasbaar, niet specifiek, inconsistent, subjectief en soms zelfs tegenstrijdig zijn. Dit gebrek aan kennis en onbetrouwbaarheid van soortspecifieke pijnschalen benadrukken de hoge nood voor het ontwikkelen en valideren van een (nieuwe) betrouwbare en makkelijk toepasbare pijnbeoordelingstool om verdere verfijning van experimentele methodes voor fretten te waarborgen.

In **hoofdstuk 7** worden de resultaten van een internationale enquête onder fretteneigenaren gepresenteerd, waarin gevraagd is welke fysiologische en/of gedragsparameters de eigenaren gebruiken om pijn bij hun fret(ten) te herkennen. De resultaten van deze enquête lieten zien dat fretteneigenaren algemene indicatoren zoals gewichtsafname, oppervlakkig/snel/moeilijk ademen, verminderde activiteit, verminderd spelgedrag, meer slaapgedrag, verminderde eetlust, verminderde zelfverzorging, meer vocalisatie en veranderingen in postuur gebruiken om pijn bij hun fret(ten) te herkennen. Naast deze algemene parameters gaven fretteneigenaren aan dat zij de gezichtsuitdrukking van hun fret(ten) gebruiken om pijn te herkennen. Hierbij gaven zij aan vooral het dichtknijpen van de ogen te zien, maar zij gaven aan ook veranderingen in de oren, snorharen, neus en mond te zien. Het gebruik van gezichtsuitdrukkingen om pijn te herkennen bij fretten die als proefdier gebruikt worden kan dus een waardevolle en praktisch toepasbare nieuwe tool zijn voor het herkennen van pijn bij deze dieren.

In **hoofdstuk 8** is dan ook onderzocht of een zogeheten 'grimace scale', zoals die al was ontwikkeld voor verschillende (proef)dieren, ook voor fretten opgesteld kon worden. Door de gezichten van fretten op verschillende tijdstippen voor en na intraperitoneale implantatie van een telemetrie probe te fotograferen en te vergelijken is een grimace scale voor fretten opgesteld. Deze grimace scale bestaat uit vijf 'action units': ogen dichtknijpen, snorharen terugtrekken, neus bollen, wang bollen en verandering in de oorstand. Evaluatie van de grimace scale liet zien dat de gezichtsuitdrukkingen van fretten en ogen dichtknijpen in het bijzonder, gebruikt kunnen worden om pijn bij fretten te herkennen. De vier andere action units moeten opnieuw geëvalueerd worden omdat deze weinig tot niets bijdroegen aan de beeldvorming en moeilijker te scoren bleken. Daarnaast zorgde het meenemen van deze action units voor een lagere sensitiviteit, specificiteit en accuraatheid waarmee de foto's die voor en na de operatie gemaakt waren konden worden geïdentificeerd. De resultaten van het onderzoek tonen aan dat het kijken naar de gezichtsuitdrukking bij fretten veelbelovend is, hoewel deze nog wel verder gevalideerd moet worden voor hij daadwerkelijk gebruikt kan worden in een multifactorieel protocol om pijn te herkennen.

Praktische adviezen

Samengevat tonen de resultaten van de onderzoeken die zijn gepresenteerd in dit proefschrift aan dat de zorg voor en het gebruik van fretten als proefdieren enerzijds kan worden verfijnd door het aanbieden van hangmatten, een waterbak en een voerbal; en anderzijds door de grimace scale voor fretten te gebruiken om pijn te herkennen bij deze dieren.

Curriculum Vitae

Marsha Reijgwart werd geboren op 7 februari 1987 te Boxmeer en groeide op in Beugen. Op het Elzendaalcollege in Boxmeer behaalde zij in 2005 het VWO diploma, waarna zij een propedeuse in dier- en veehouderij aan de HAS in 's Hertogenbosch heeft behaald. In 2006 begon zij aan de studie Biologie aan de Universiteit Utrecht. De bachelorfase heeft zij afgerond in 2010, waarna ze de master Environmental Biology – track behavioral ecology aan de Universiteit Utrecht heeft gevolgd. In haar masterfase heeft ze eerst 9 maanden stage gelopen bij de faculteit diergeneeskunde, onder begeleiding van Dr. Yvonne van Zeeland waar zij onderzoek deed naar de ethologie van grijze roodstaart papegaaien en een consumer demand studie ontwierp voor deze dieren. Daarna heeft zij 6 maanden onderzoek gedaan naar hokverrijking voor seniore asielhonden in Dierentehuis 's Hertogenbosch voor De hondenbescherming Den Haag. Tot slot heeft zij een masterscriptie geschreven over locomotiestereotypieën bij paarden onder begeleiding van Prof. Dr. Georgia Mason. Op 31 juli 2012 heeft zij het masterdiploma biologische wetenschappen met het programma Environmental Biology behaald. Van oktober 2010 tot december 2012 heeft zij ook de 2-jarige postgraduaat toegepast diergedrag aan de Katholieke Hogeschool Sint-Lieven gevolgd en succesvol afgerond. Vervolgens heeft zij haar eigen bedrijf opgericht, waarbinnen ze werkzaam is als kattengedragstherapeute. In maart 2013 is zij begonnen aan een promotieonderzoek, waarvoor zij als AIO werd aangesteld bij de faculteit Diergeneeskunde, afdeling Dier in Wetenschap en Maatschappij. Zij werd gedetacheerd bij Intravacc in Bilthoven, waar zij haar onderzoek heeft uitgevoerd. De resultaten van dit promotieonderzoek zijn beschreven in dit proefschrift. Marsha is momenteel werkzaam als beleidsmedewerker bij de Landelijke Inspectiedienst Dierenbescherming.

Dankwoord

Het is onwerkelijk: daar ligt hij dan, mijn proefschrift. In de afgelopen ontzettend leerzame jaren heb ik niet alleen veel over fretten geleerd, maar mezelf en mijn grenzen beter leren kennen. Dit was allemaal niet mogelijk geweest zonder de steun van collega's, vrienden en familie, waarvan ik een aantal personen hieronder extra in het zonnetje wil zetten.

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Tijdens mijn verdediging staat ook vriendin en paranimf **Lonneke** (letterlijk) achter me. Op de middelbare school hadden we een paar jaar nodig om te beseffen dat we elkaar best aardig vonden, maar dat heeft geresulteerd in een super sterke vriendschap! We zien elkaar te weinig, maar dat maakt onze vriendschap niet minder. Je bent een geweldig mens, eerlijk en staat altijd voor me klaar, bedankt daarvoor!

In een dankwoord kunnen ook mijn ouders, **Wilma** en **Ron**, niet ontbreken. Jullie zijn al mijn hele leven een grote steun (financieel en mentaal), mede waardoor ik nu zover ben gekomen. Bedankt voor de duwen in de rug (of de trein in) die ik nodig heb gehad, de stand-by telefoondienst in moeilijke tijden en de interesse in mijn werk!

Ik heb het geluk gehad mijn onderzoek te mogen doen terwijl ik omringd werd door geweldige collega's. Zonder mijn geweldig fijne werkplek bij DWM en met name de dames van de 'M'-kamer was het een stuk moeilijker geweest om de moed er in te houden tijdens de dalen die bij een PhD horen. **Maaike**, **Marcia**, **Marloes** en **Marijke:** jullie waren de beste kamergenootjes die iemand zich kan wensen! Stuk voor stuk knettergek (in a positive way!), met een goed gevoel voor humor en een hart van goud! Thanks for the laughs!

Janneke, wie had tijdens Ethology and Welfare gedacht dat onze wilde plannen (ongeveer) waar zouden worden: we zijn niet alleen allebei gedragstherapeut, maar waren ook collega's bij DWM! Ik heb 'het nest' nu verlaten, maar we gaan elkaar zeker nog zien!

Ook **Annemarie** heeft een speciale vermelding in dit dankwoord verdiend. Tijdens mijn discussie-writer's block gaf je mij precies de tip die ik nodig had om uit mijn peins-cirkel te breken en een logisch verhaal op te schrijven.

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