DETECTION OF PAIN IN FERRETS (*MUSTELA PUTORIUS FURO*) VIA FACIAL EXPRESSIONS WITH THE USE OF A FERRET GRIMACE SCALE (FGS)

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ABSTRACT

Background: The recognition and alleviation of pain and distress is indispensable for safeguarding good welfare of laboratory animals. This is especially true for ferrets, as this species still endures a lot of unrelieved pain and discomfort in scientific research. One of the most used method of pain assessment is behavioral measures, which seem generally not applicable for pain recognition in ferrets and remains largely subjective. A novel means of pain assessment using facial expression changes has proven to be successful in different species. The objectives of the current study are to identify characteristic facial expressions of pain in ferrets (Facial Action Units) and with these develop a Ferret Grimace Scale (FGS) for pain recognition in this species.

Methods and Results*:** 19 female ferrets were photographed before and after abdominal surgery to supply no-pain/baseline and pain photographs. Baseline and pain photographs of each animal were compared to search for changes in facial expression, which resulted in the description of two Facial Action Units (FAU's): ''Orbital tightening'' and ''Whisker retraction''. The use of these Facial Action Units in a Ferret Grimace Scale for pain recognition was tested by means of scoring photographs by eight respondents. Results showed a significant increase of Ferret Grimace Scale-scores from baseline to five hours after surgery and a significant decline in Ferret Grimace Scale-scores 24 hours later. The Ferret Grimace Scale yielded an accuracy of 68.0% and an Inter-Rater reliability of 64.5%. ***Conclusion: The Ferret Grimace Scale can potentially serve as a method for pain recognition in the ferret. However, further research is necessary to i) identify more Action Units, ii) increase reliability and accuracy and to iii) evaluate and validate its use for different painful stimuli and analgesic schedules.

Keywords*: Ferret; Pain; Welfare; Pain assessment; Facial Expressions; Grimace Scale; Refinement; Laboratory Animal*

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PREFACE

This thesis is written and submitted in partial fulfillment for a Master's degree in Veterinary Medicine at Utrecht University. It contains the work done in a 17 week period from October 2014 to April 2015 at the Department of Animals in Science and Society at Utrecht University and Intravacc (Bilthoven, the Netherlands). This report deals with pain recognition in ferrets via facial expressions, which can hopefully contribute to further refinement of scientific studies that use this species in their experiments.

Several persons have contributed academically, practically and with support in the process of this thesis. I would therefore firstly like to thank my supervisor M.L. Reijgwart for her time, inspirational input and support during my research internship. Also special thanks to J.M. de Jonge. for letting me intervene in his influenza experiment, to dr. Y.R.A. van Zeeland, dr. N.J. Schoemaker and dr. M.C. Leach for helping me in my thought process during the development of the experimental design, to drs. J.C.M. Schouten for executing the valuable dissection of one animal for anatomical purposes and to dr. H.A. van Lith for helping me with the statistical analysis of the obtained data. Furthermore I would like to thank the Department of Animals in Science and Society from Utrecht University and Intravacc for the opportunity and their hospitality. I am also indebted to all participants for filling in my score-forms which helped me obtain my data and of course all my fellow students who gave constructive comments to this thesis when I needed it and made this internship a wonderful and fun experience. At last, a great thank you to all the animals involved in the experimental part of this thesis, which made the execution of this work fun and very instructive.

Melanie Stodel

1. INTRODUCTION

1.1 Laboratory animal welfare

The use of animals for biomedical scientific purposes provides essential information for mankind. Although in vitro studies are becoming increasingly important, in vivo studies, including those that use ferrets, are still indispensable (Olfert & Godson 2000). The use of laboratory animals for scientific purposes is often subject to ethical discussions where the future benefits and significance of the planned research are weighed against the possible discomfort and declined welfare of the animals (Bateson 1986; de Boo et al. 2005).

In order to assess the welfare of animals in laboratory conditions, the concept of the 'five freedoms' as formulated by the UK Farm Animal Welfare Council (FAWC) in 1993 has long been adhered. It states that an animal perceives its state as positive when it experiences i) Freedom from hunger and thirst, ii) Freedom from discomfort, iii) Freedom from pain, injury and disease, iv) Freedom to express normal behavior and v) Freedom from fear and distress (McCulloch 2012; Korte et al. 2007). The use of these freedoms as a basis for welfare assessments and its reliability has been argued recently (Baumans 2005) and the concept of welfare has been updated with biological aspects such as the need for allostasis or coping skills to maintain a positive welfare state (Korte et al. 2007) (Broom 1986; Broom 1991). Following the new concept of welfare, its description has been reformulated to ''*the freedom to display normal behavioral 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 & van der Staay 2012). Despite this clear description, the focus of concern in animal welfare and its importance still largely depend on differences in culture, religion, time, and one's personal opinion (Ohl & van der Staay 2012; Stafleu et al. 1996; Cohen et al. 2009; Yeates 2010). Welfare issues therefore cannot be exclusively resolved with the use of objective biological measures and science, but are also largely subject to the complexity of personal values and public perception within the moral values of society (Mason & Mendl 1993; Fraser 2008; Ohl & van der Staay 2012). As a result, stating values of normality and objective measurement of welfare remains difficult and questionable (Krohn et al. 2001).

Animal welfare in scientific studies is ensured and protected by the obligatory implementation of the 3R's by international and national legislation (Anon n.d.; Wolfensohn & Lloyd 2003; Boo et al. 2005; Overheid.nl n.d.). The 3R's as stated by Russel and Burch in 1995 encompass: i) Replacement, which involves the replacement of animals with inanimate research methods; ii) Reduction, which consists of the decrease of number of animals used and iii) Refinement, which has a focus on the minimization of pain and distress to the animal (Gauthier & Griffin 2005; Zurlo et al. 1996). Total replacement of animals in research is the ultimate goal for the protection of animal welfare. If replacement is not possible, the areas of reduction and refinement should be exploited (NKCA n.d.). But, even though the implementation of the 3R's meets moral, ethical, and law-restricted needs, its practical feasibility should not be overestimated. Replacement can be complicated due to the fact that animals are the main research subject, or the whole animal has to be studied. The implementation of reduction is often denied because it can possibly deficit statistical significance. Refinement however is seen as the lesser obstacle by scientists (Fenwick et al. 2011). In order to ensure further refinement in research, proper recognition and alleviation of pain in the used species is indispensable.(Flecknell et al. 2007).

1.2 Pain assessment

The physical and physiological components of pain, nociception and the emotional feeling (Anil et al. 2002), are embodied in the widely accepted definition of pain as defined by The International Association for the Study of Pain (IASP): '' *an unpleasant sensory and emotional experience associated with potential or acute tissue damage, or described in terms of such damage* '' (Carstens & Moberg 2000; Anon 2000; IASP 2012) with the note that '*The inability to communicate verbally does not negate the possibility that an individual is experiencing pain'* (Sneddon et al. 2014; IASP 2012). The consensus that all vertebrates and many invertebrates are capable of suffering and experiencing pain is widely accepted (Hawkins 2002; Sneddon et al. 2014).

Because there are no well-defined objective scientific assessments for the recognition of pain (Committee on Recognition and Alleviation of Distress in Laboratory Animals 2008), this often remains difficult. Measures of general body functioning, physiological responses and behavior (Weary et al. 2006) are currently in use for pain assessment in laboratory animals of which physiological and behavioral measures are mostly used (Molony & Kent 1997; Mayer 2007). Typical changes in behavioral patterns, posture, gait, appearance and response to handling are often seen in animals that endure pain and are one of the earliest signs for animal care staff (Anon 2000; Mayer 2007; Association of Veterinary Teachers and Research Workers 1989). Behavioral indices have been recognized as one of the most applicable measures of pain because changes can be seen immediately and interpreted rapidly without further intervention to the animal (Mellor et al. 2000) (Wright-Williams et al. 2007). Because of this great usability, behavioral effects of pain have been studied intensively for various species (Roughan & Flecknell 2003; Leach et al. 2009; Molony & Kent 1997; Flecknell & Roughan 2004; Sladky et al. 2000; Fitzpatrick et al. 2006). Although the existence of behavioral signs appears to be very helpful in the recognition of pain, the quantification often remains precarious and correlation with objective pain indicators is not always possible (Anil et al. 2002). Behavioral responses can be influenced by the animals early experience, age and its physiological state, which ultimately leads to intra-animal variability (Anon 2000). In addition, measures of pain are still prone to poor reliability because of the risk of inter- and intra- observer bias (Weary et al. 2006). The animals' rate of discomfort, pain or distress is often interpreted with underlying emotions and past experiences of observers which makes the assessment even more subjective (Anon 2000). And even though the presence of distress and even pain in animals might seem evident for observers, assessing and defining the degree can result in difficulties (Anil et al. 2002); how much of a particular behavior indicates suffering in the animal? (Duncan & Dawkins 1983). It is thought that a more thorough evaluation of pain related behavior can be attained by the use of trained, educated and skilled observers that are familiar with normal behavior of the used species (Anon 2000; Mayer 2007), but it has been shown that even well trained observers (irrespective of occupation, gender and experience) focus their attention first, longer and more frequent on the face of the animal than anywhere else on the body when attempting to assess pain. This change of focus may lead to inappropriate observation and assessment of behavior which may cause the subtle, infrequent, short during, novel or location specific behaviors remaining unobserved (Leach, Coulter, et al. 2011).

All in all it can be said that even though differences in behavior might seem an evident reaction to distress for most observers, strictly defining if this is directly related to pain remains questionable. Therefore, assertions that some specific behaviors can be used as real time measures of pain have to be made with cautiousness (Mogil et al. 2010).

1.3 Ferrets in scientific research

Ferrets (*Mustela putorius furo*) are largely used as an animal model for studies on the virulence, pathogenesis and transmission of influenza viruses (Belser et al. 2011; Ball 2004). A total of 405 ferrets were used for scientific purposes in the Netherlands in 2013, and were applied for the development, production, control or calibration of vaccines and drugs (213); educational purposes and scientific questions (66) (Nederlandse Voedsel- en Warenautoriteit 2013). These animals endured discomfort that ranged from little (36 animals) to little/moderate (44 animals), moderate (62 animals), moderate/severe (156 animals) and severe (107 animals) (Nederlandse Voedsel- en Warenautoriteit 2013). In addition, a total of 191 ferrets in 2013 did not receive pain relief because it was not compatible with the experimental goal, (Nederlandse Voedsel- en Warenautoriteit 2013) even though a lot of potential pain or discomfort experienced by ferrets can be prevented or reduced with anesthetics, analgesics or tranquilizers (Zurlo et al. 1996). This reluctance in the use of pain relief in ferret studies is often due to the possible inhibitory or excitatory effects of particular analgesics on the immune system of the animals, which could ultimately have an effect on the experimental outcome (Sacerdote 2006; Fenwick et al. 2011). However, proper application of the 3R's and especially refinement is mandatory and also improves the quality of studies as ''happy animals make good science'' (NKCA n.d.). In order to ensure further refinement of ferret studies, knowledge on pain recognition in this species is necessary.

Ferrets are known to have a high tolerance for pain and discomfort and in general give little warning of illness because they are more or less stoic creatures (Poole n.d.). This coping strategy could be worthwhile, because hiding possible expressions of pain can give an important advantage on survival in an antagonistic encounter (Williams 2002). Signs of illness and pain in ferrets mentioned in the literature all comprise of nonspecific behavioral descriptions which are largely subjective, sometimes contradictory and described with very little consistency (Mayer 2007; Johnston 2005; Sladky et al. 2000; Brown 1997; Pollock 2007; Lichtenberger & Ko 2007; van Oostrom et al. 2011; Chattipakorn et al. 2002). This inadequate description of pain behavior might also be subject to the low activity levels of this species. Undisturbed ferrets can sleep up to 70% of the time over a 24h period (Jha et al. 2006) and although stimulation can cause long bouts of activity up to 60 minutes (Jha et al. 2006), waking the animals for behavioral measures can result in inadequate or biased outcomes.

The discrepancy in the literature and the fact that behavioral indices generally seem not applicable for stoic creatures such as ferrets ultimately leads to the knowledge gap on proper pain recognition in this species, resulting in inadequate assessments. This underlines the great necessity for a new reliable and easy to use pain assessment tool to ensure further refinement in experimental methods for this species.

1.4 Facial expressions for pain recognition

The evidence about the universal facial expression of some emotions has first been described in ''The expression of Emotions in Man and Animals'' by Charles Darwin (Darwin 1872). Over the years, the connection between emotional experiences and facial expression in humans has remained a study of interest (Williams 2002), which ultimately resulted in the development and publishing of a Facial Action Coding System (FACS) by Ekman and Friesen (1987). This research tool for the measurement of facial movement, based on anatomical features and the visible distinguishable movements and activity in the human face, uses the description and intensity of 44 individual unique Action Units (AU's). The FACS has been used for the recognition and quantification of pain in humans (Ekman & Rosenberg 1997) because it brings the advantage of an easily observable and convenient information source, without the need for special equipment (LeResche & Dworkin 1984). As a result, characteristic and consistent patterns of facial expressions associated with intense acute and chronic pain of different types have been identified, containing AU's for brow lowering, tightening and closing of the eye lids, nose wrinkling and upper lip raising (LeResche 1982; LeResche & Dworkin 1988; Prkachin 1992). Measures on the facial expression of pain and adjusted pain coding systems have been intensively studied for (non-cognizant) elderly (Herr et al. 1998; Brignell 2003; Hadjistavropoulos et al. 2002; Kunz et al. 2007; Hsu et al. 2007), children (Bieri et al. 1990; Hunter et al. 2000; Tomlinson et al. 2010) and neonates (Schiavenato et al. 2008) with promising and valid results when used as a pain assessment tool.

The expression of emotions has been considered essential to the welfare of group-living animals as facial expression and vocalizations are involved in social interactions and communication of feeling states (e.g.: pleasure, joy or affection; anger; pain and astonishment or terror) (Chevalier-Skolnikoff 2006) even though hiding expressions of sickness and pain can be of significant importance for the survival of an individual animal within the group structure. The capability for animals to express emotions and therefore also pain through facial expressions also arises from the proposition of the continuity of species as stated by Darwin (Darwin & Ekman 1998). Subsequently, adapted from the original FACS for humans, FACS for identifying and coding facial movements in different species such as chimpanzees (Vick et al. 2007), macaques (Parr et al. 2010), gibbons (Waller et al. 2012) dogs (Waller et al. 2013), orangutans (C.C. Caeiro et al. 2013) and cats (Cátia C. Caeiro et al. 2013) have been developed. Furthermore, standardized facial coding systems that use intensity scores for changing facial features (Action Units) called Grimace Scales have been developed and validated lately for mice (MGS) (Langford et al. 2010). rats (RGS) (Sotocinal et al. 2011) rabbits (RbtGS) (Keating et al. 2012) and horses (HGS) (Dalla Costa et al. 2014). For cats, significant facial changes associated with pain were found (Holden et al. 2014). Grimace scale studies have used different painful stimuli which emitted spontaneous acute or chronic (post-operative) pain, yet the methods of identification the AU's of a pain face seemed roughly the same for each species. AU's in all species were mainly associated with the eye, ear, muzzle/whiskers and nose area (*Table 1).*

Animal	Eye	Nose	Ears	Cheeck	Whisker	Mouth
Mouse[1]	Orbital tightening	Bulge	Change	Bulge	Change (back /forward)	
Rat[2]	Orbital tightening	Flattening	Change		Change (forward)	
Rabbit[3]	Orbital tightening	Pointed		Flattening	Change	
Horse[4]	Orbital tightening, tension above eye	Strained nostrils, flattened profile	Stiff backwards	Strained chewing muscles		Strained
Cat[5]	Orbital tightening		Change			Change

Table 1. Action Units discovered in different species. OT=orbital tightening; 1= Langford *et al.* (2010); 2=Sotocinal *et al*. (2011); 3=Keating *et al.* (2012); 4=Dalla Costa *et al.* (2014); 5=Holden *et al.* (2014).

The relevance of the use of Grimace Scales for pain recognition in animals has already been demonstrated as the MGS has successfully been used to evaluate the effectivity of and responsiveness to (post-operative) analgesics (Matsumiya et al. 2012; Miller & Leach 2014; Faller et al. 2015) and the amount of pain associated with ear notching (Miller & Leach 2014) and vasectomy (Leach et al. 2012).

1.5 Facial expression in ferrets

There is a strong belief that more solitary living animals do not show as much facial expression for social communication. However, more recently a FACS for the domestic cat (*Felis catus*) has been developed (Cátia C. Caeiro et al. 2013) and several FAU's for pain expression in this species have been discovered (Holden et al. 2014), The social organization of the ferret has shown similarities with that of feral cats (Biró et al. 2004; Yamane et al. 1994): individual home ranges within each sex have found to be usually separate with little overlap of boundaries, day time resting is mostly solitary (Moors & Lavers 1981; Norbury et al. 1998) and overlap in core areas and home range size seem to be influenced by food supply (Moller & Alterio 1999). Even though feral ferrets tend to live solitary lives, *Mustela putorius/Mustela furo* hybrids have shown social play behavior accompanied with facial expressions of play ('play face') which could indicate that ferrets show facial expression in means of social communication (Poole 1978). Assuming that they do, developing a Grimace Scale for ferrets for pain recognition in this species could accompany great advantages.

In comparison to the widely used behavioral measures, pain scoring with Grimace Scales have proven to be less time consuming (Dalla Costa et al. 2014) and usable in the assessment of a range of different painful conditions from mild to severe and chronic to acute pain in different species (Langford et al. 2010; Keating et al. 2012; Dalla Costa et al. 2014; Sotocinal et al. 2011). In addition, the tendency to focus on the face when looking for behavioral indices of pain (Leach, Klaus, et al. 2011; Leach, Coulter, et al. 2011) can be utilized and observers can be easily and rapidly trained to look for species specific facial differences (Miller & Leach 2014; Leach et al. 2012; Sotocinal et al. 2011; Keating et al. 2012; Dalla Costa et al. 2014). Altogether, changes in facial expression can be a very promising and easy to use objective pain assessment tool when demonstrated that they are exclusive and can specifically distinguish pain from other types of distress in the ferret.

1.6 Aim, hypothesis and predictions

Following the need for an objective pain recognition system in laboratory ferrets and the good results with the use of Grimace Scales for pain recognition in other species, the aim of the present study is to identify FAU's in the ferret that indicate pain and with these develop a FGS which after further validation studies can be used for future pain assessment in this species.

This aim is accompanied by the main hypothesis that ferrets show pain by means of facial expression. Regarding this hypothesis, the first sub-hypothesis states that FAU's of pain can be found in the ferret. With the prediction that they can be found mainly in the same area's as shown by other Grimace Scales in different species (*Table 1*); eyes, nose, ears, cheek, whiskers, mouth/muzzle.

For the detection of FAU's and the development of a FGS, the animals needed to be photographed in a condition without pain (baseline) and after a painful stimulus to search for facial changes. For the current study, reduction of animal use was taken into account and ferrets from an influenza (H2N2) study that underwent abdominal surgery without further post-operative analgesics were used. It was assumed that this surgery, because of the manipulation of viscera, its moderate duration and the lack in use of analgesics, would lead to sufficient postoperative pain in the ferrets to identify pain-faces (Johnson-Delaney & Mayer 2006; Sotocinal et al. 2011; Langford et al. 2010).

Consequently, this leads to the formulation of the second sub-hypothesis that ferrets after the painful stimulus can be differentiated from their baseline condition with the use of the described FAU's in a FGS.

Photographs of animals in the painful condition were captured on two time points after surgery and on the same time points 24 hours later. Taking into account the duration of the anesthesiological effects and the estimated recovery time, (Arnemo & Søli 1992; Fournier-Chambrillon et al. 2003; FDA 2009; Sadove et al. 1971) the first time-point of photography was planned two hours after surgery. Because studies on laparotomy induced pain in rats showed that signs of pain were most prominent approximately two hours after surgery and sufficiently lasted for four to six hours (Roughan & Flecknell 2004; Roughan & Flecknell 2001) it was assumed that signs of pain would be mostly visible between two and six hours after surgery. Taking this into account, the second time-point of photography was chosen five hours after surgery.

Regarding the second sub-hypothesis on the use of the FGS, it was predicted that this second time-point would lead to the highest individual FAU-scores and highest FGS-score. Furthermore it was predicted that FGS-scores would decline in time towards baseline position as they did in the RGS after laparotomy (Sotocinal et al. 2011).

Because Grimace Scales in general are known to differentiate pain from no pain with high reliability and accuracy in different species and painful conditions (Langford et al. 2010; Dalla Costa et al. 2014; Sotocinal et al. 2011; Keating et al. 2012), it was predicted that the FGS would have the same characteristics.

2. ANATOMY

Because of the analogies in AU's and facial changes accompanied with pain in other species, it was assumed that the facial changes and so FAU's in ferrets would be mainly visible in the areas of the eyes, nose, ears, cheeks, whiskers and mouth/muzzle. To gain anatomical evidence for the existence of these possible facial changes, a ferret was dissected by a pathologist of the University of Utrecht to identify facial muscles in this species, because no anatomical drawings of the facial musculature in ferrets were available.

In preparation for this dissection, AU's found in Grimace Scale studies were compared from which the results are shown in *Table 1*. Muscles needed for the movement of eye, ear, muzzle, whiskers and nose in the cat were determined with the use of CatFACS (Cátia C. Caeiro et al. 2013). Dissection was started with a surgical cut through the skin layer in caudo-rostral direction. For proper investigation of the surficial musculature, the skin layer was removed superficially in direction of the eye and muzzle at one lateral side. In addition to that, superficial muscles were removed for further investigation of the deep musculature. Methods of dissection were the same for both sides of the face. Because no anatomical drawings on the facial musculature of ferrets were available, facial muscles of the dissected animal were investigated and compared to detailed anatomical drawings of the facial musculature of the cat, of which an example is visible in *Appendix 2* (Text Atlas of Cat anatomy by James E. Crouch). The identified analogies in the ferret with their function are shown in *Table 2.*

Table 2. Identified muscle directions with the proposed analogies. Numbers in this table correspond with the numbers in Figures 1,2 and 3.

Muscle direction / shape	Possible analogies	Function
1. Lateral eye corner towards ear	*M. frontoscutularis (*)* *M. frontoauricularis* *M. frontalis*	(*)Draws ear cranial
2. Lateral side of the head towards neck. Thin muscle, Triangular shape	*M. platysma*	Stretch skin over pectoralis and deltoid muscles
3. Caudal part of the neck area in dorsal direction	*M. sphincter colli superficialis*	Tighten skin on neck below
4. Around mouth	*M. orbicularis oris*	Close lips
5. Corner of the mouth towards dorsal ear area	*M. zygomaticus major*	Draw corner of mouth dorso-caudal and external ear ventro-cranial
6. Ear towards the jugular vein area in the neck	Unidentified	Ear movement?
7. Head area	*M. temporalis*	Close jaw
8. Above M. temporalis : medial towards ear	3 to 4 unidentified thin muscles	Ear movement?
9. From the lateral side of the ear in caudo-ventral direction over salivary gland	*M. depressor conchae*	Draw external ear ventral
10.	*M. submentalis*	Draw external ear ventral
11. From snout and whiskers towards eye	*M. caninus (**)* *M. proprius*	*(**)* Retract whiskers + raise upper lip
12.	*M. epicranius frontalis*	Move integument of dorsal side of head
13. Between all whiskers	Unidentified	Whisker movement?
14. Around the eye	*M. orbicularis oculi*	Close eyes
15. Lateral sides of the head and jaw	*M. masseter*	Elevate mandible

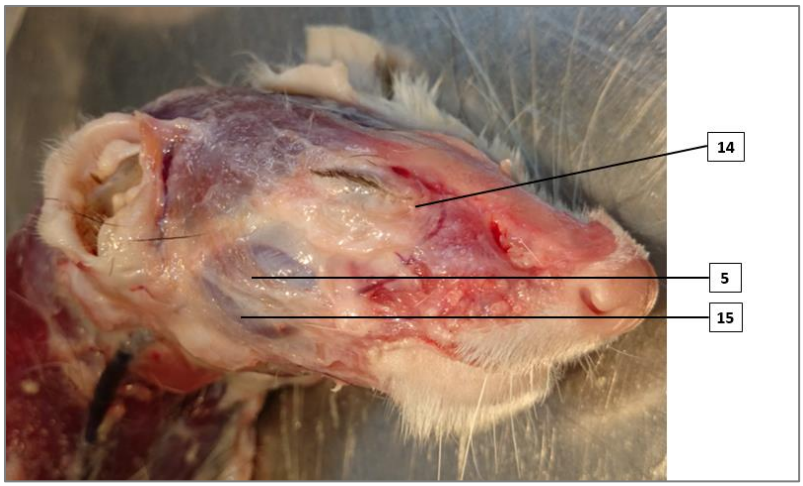


Figure 1. Right lateral side of the head after removal of superficial skin layer and muscles. Numbers correspond with *Table 2.*

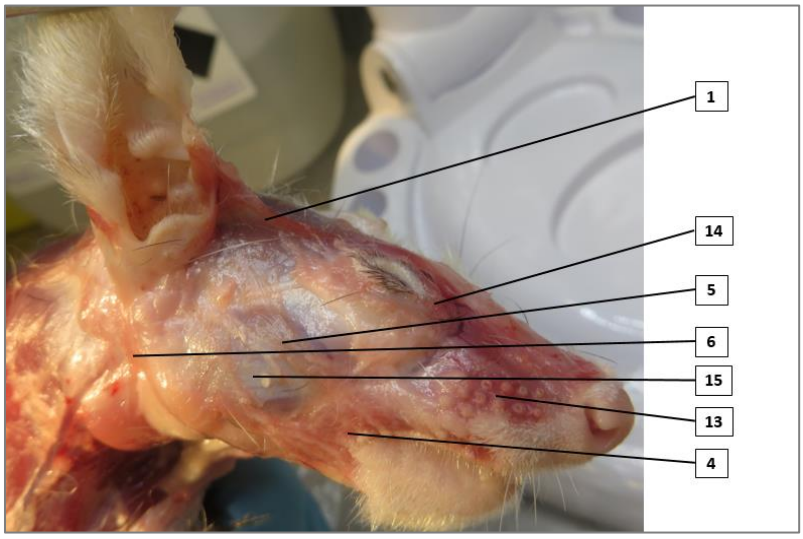


Figure 2. Right lateral side of the head after removal of superficial skin layer and muscles. Numbers correspond with *Table 2.*

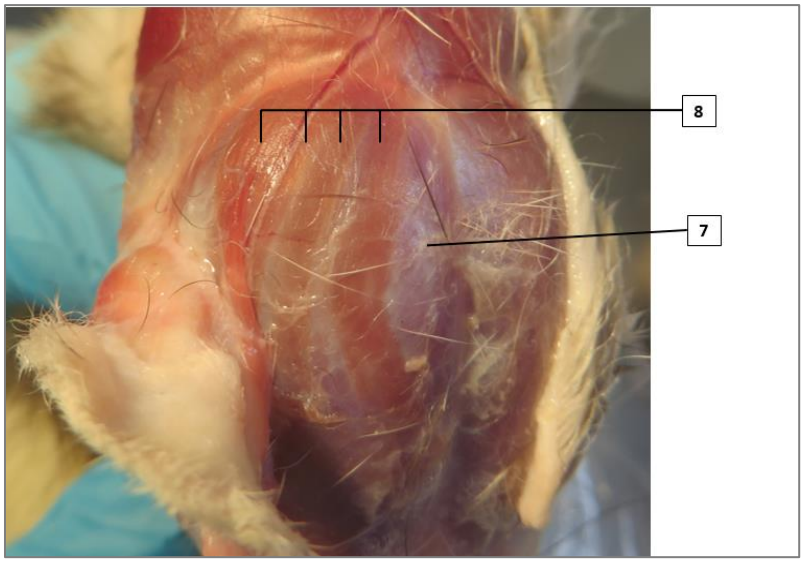


Figure 3. Dorsal view of the head after removal of superficial skin layer. Numbers correspond with *Table 2.*

3. MATERIALS AND METHODS

3.1 Ethical statement and general approach

Fulfilling the aim of reduction, no animals were specially obtained for the development of the FGS. The animals used in this study were originally purchased and used for another experiment on H2N2 influenza in ferrets. The abdominal implantation of two telemetry probes in preparation for this experiment was used as the painful stimulus in the present study. No additional procedures that could cause a higher state of distress on the animals were performed for the present study. The outline of the present study was approved by the original investigator of the influenza experiment to ensure that this experiment and its outcomes were not altered due to our proceedings.

3.2 Animals and Husbandry

A total of 19 16 to 32 weeks old domestic female ferrets of different coat, color, and weight were obtained from Schimmel BV and used in the present study. Because of the course of the influenza study, all animals were serologically tested to be seronegative for antibodies against influenza and Aleutian viruses and were provided with an ID-chip, which was placed subcutaneously in the neck area. At arrival, the animal's weight, appearance, and overall health were checked and noted. In addition, 15cm² of hair was shaved in the neck area between the shoulder blades for another experiment, blood was collected via the jugular vein, and nose and throat swabs were taken under anesthesia (0.2 ml ketamine) for the influenza experiment. All animals were randomly selected on weight, housed and managed under the same conditions after arrival: six animals per cage (open wooden cages on concrete floor measuring 94.5x 166 x 64.6 cm) on wood shavings (IRS LIGNOCELL ® Hygienic animal bedding) of an average depth of 3-5 cm in a Specified Pathogen Free (SPF) housing environment. Ferrets were maintained on a 8.00AM-16.00PM light cycle in a temperature and humidity controlled environment. Food (Hope farms ® Ferret balance pellets) and water were given ad libitum in stoneware bowls except for water restrictions after surgery. Cage enrichment consisted of a ferret ball (25 cm diameter with 6 holes of 7.5 cm each) and a large flexible sleeping bucket (24 liter). According to the experimental protocol of the influenza study, the animals were housed in the previously described conditions for 27 days to ensure habituation and acclimatization prior to the experiment. The animals were not deprived of food before surgery because of good experiences with this protocol.

3.3 Surgery and recovery

Surgery for the intraperitoneal implantation of the telemetry probes was performed 26 days after arrival starting at 9.00 AM. The two tube shaped probes (DST micro-T, STAR ODDI, 8.3 mm diameter x 25.5 mm length; DST micro-HRT, STAR ODDI, 8.3 mm diameter x 25.4 mm length) were inserted superficially to avoid too much manipulation of the gastrointestinal system. The animals were health checked and weighed prior to surgery to ensure reliable anesthetics. No pre- or peri-operational analgesics were given. For anesthesia, an intra-muscular injection in the caudal thigh area of 0.2 ml (9:1) Ketamine 10% - Dexdomitor (Terumo, 1ml syringe with needle, 25Gx 5/8) was given 5-10 minutes prior to surgery. The surgery consisted largely of the following steps: i) shaving and disinfection (with 70% alcohol), ii) incision with a scalpel (no. 24) through skin, abdominal muscles and abdominal viscera, iii) placement of the probes, iv) single knot sutures of the muscle layer and secondly the skin layer (Vicryl, Polyglactin 910, 3-0 FS-2 18.7mm, Johnson-Johnson Intl, Ethicon) and was seen as a routine surgery. Surgery was completed in less than 30 minutes per cage/group of six animals. After surgery, animals were placed in the SPF housings in their original groups with water deprivation for the first hours to prevent drowning. A single intra-muscular injection of 0.02 ml Antisedan (Atipamezole REG NL 7744 with Terumo, 1ml syringe with needle, 25Gx 5/8) was given to reverse anesthetics after surgery. Full recovery was estimated using a sedation scoring system (adapted from Lascelles et al. 1994). The animal was considered 'recovered' and ready for photographing when reaching a sedation score of '0' (fully alert and able to stand and walk) on the scale. No antibiotics, analgesics or other pain-relieving medication were administered post-surgery. Health of the animals was followed up for at least three days post-surgery by examining behavior in the group, appetite, and the possible unwanted exposure of intestinal elements through the sutures. All procedures were prepared and performed by bio-technicians employed by Intravacc at Bilthoven.

3.4 Photographs

3.4.1 ALIGNMENT AND EQUIPMENT

The experimental design was chosen after a number of pilot studies with ferrets from the University of Utrecht and the ferrets used in the present study and after consulting two experts (*Figure 4*). PVC tubes (75 and 100 mm diameter, 30 cm length) were sawn lengthwise, held together with tie wraps and fitted on a coated wooden board with stabilizers and tie wraps attached to both sides to ensure steadiness of the tube. Additionally, a coaster (10x10 cm) was mounted on a hinge in front of the tube, with a wooden handle. All ferrets were tested on both tubes in pilot studies to determine the tube which was most suitable for each animal (*Figure 4*). The described setting was placed on a table (90x59 cm) of 74 cm height for the experiment. All photographs were taken with a Canon EOS 7D Mark II Body combined with a Canon EF 50 mm 1.8 f II objective. A graphic card (Lexar CF pro 1066x UDMA7 16GB 160MB/s) and tripod (Vanguard® Alta + 224CP) were also purchased and used in this experiment. Camera settings (1/250, F=5.0, ISO 10.000) and placement (58 cm distance from the subject and both horizontally and vertically leveled) were standardized. Manual settings and focus were chosen and photographs were shot in burst mode (10 frames /second). Photographs were taken under the same SPF conditions as where the animals were housed. Ferrets had to be taken out of their home cages and brought to the separate photographing room to ensure that their light regime was not disturbed. No additional flash or light source was used besides the TL-light in the room to avoid shadows and squinting of the eyes in the animals.

3.4.2 EXPERIMENT

Baseline (''no-pain'') recordings were taken one day prior to surgery (day-1) at approximately two and five hours after planned surgery time. So called 'Pain-recordings'' were taken two and five hours directly after surgery and on the same times 24 hours later following the timetable as shown in *Table 3*. The equipment, as mentioned above, was set up as shown in *Figure 5* and an additional sheet with the ID number of the animal (length 6 cm) was placed alongside the tube. The ferret was lead though the tube by an assistant with the coaster in front of the tube and turning in the tube was prohibited by the same person. When the ferret reached the end of the tube, the coaster was let down and the moment that the animal was located within the set focus point of the camera a burst of photographs was taken. If necessary, ferrets were gently restrained at the level of the scapula to ensure a clear and sharp photograph. This procedure was followed with each ferret in frontal and lateral (profile) view (for lateral view, the experimental setup was turned 45 degrees, camera position remained the same) until suitable photographs of each ferret and view were taken (*Figure 5 and 6*).

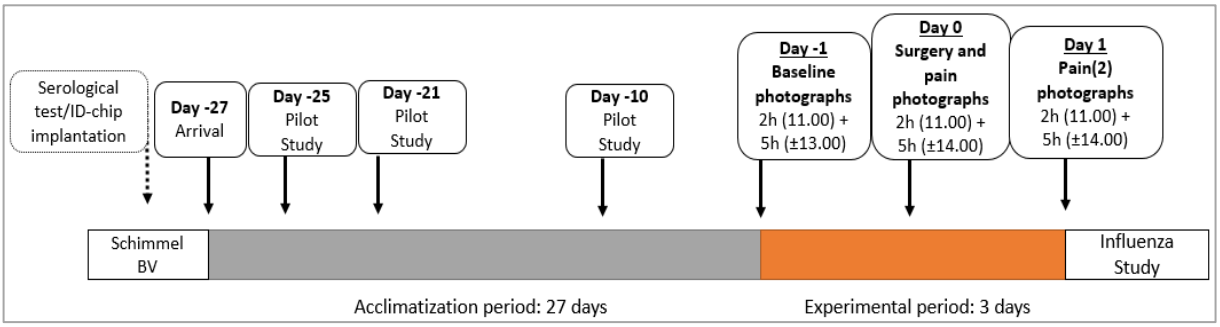
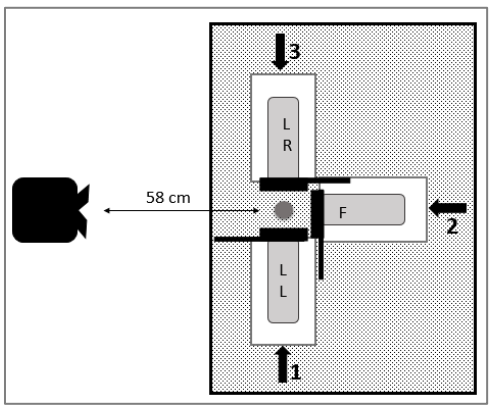


Figure 4. Experimental setup. Pilot studies comprise testing the tubes and photography.

Table 3. Time schedule photography FGS. Originally 24 ferrets and grouped per six, which resulted in a total of four groups. Ultimately 19 were used for FGS development.

Time	Groups photographed
11.00	Group 1
11.35	Group 2
12.10	Group 3
12.45	Group 4
14.00	Group 1
14.35	Group 2
15.10	Group 3
15.45	Group 4
16.00	End



Figures 5 (Left) and 6 (Right). Experimental set up. LL and LR represent the setup for lateral photographs, F represents the setup for frontal photographs. The dot represents the focus point on which the ferrets head should be for photographing.

3.5 Grimace Scale development

For development of the FGS, methods adapted from Langford et al.,(2010); Sotocinal et al., (2011); Keating et al., (2012); and Dalla Costa et al. (2014) were used.

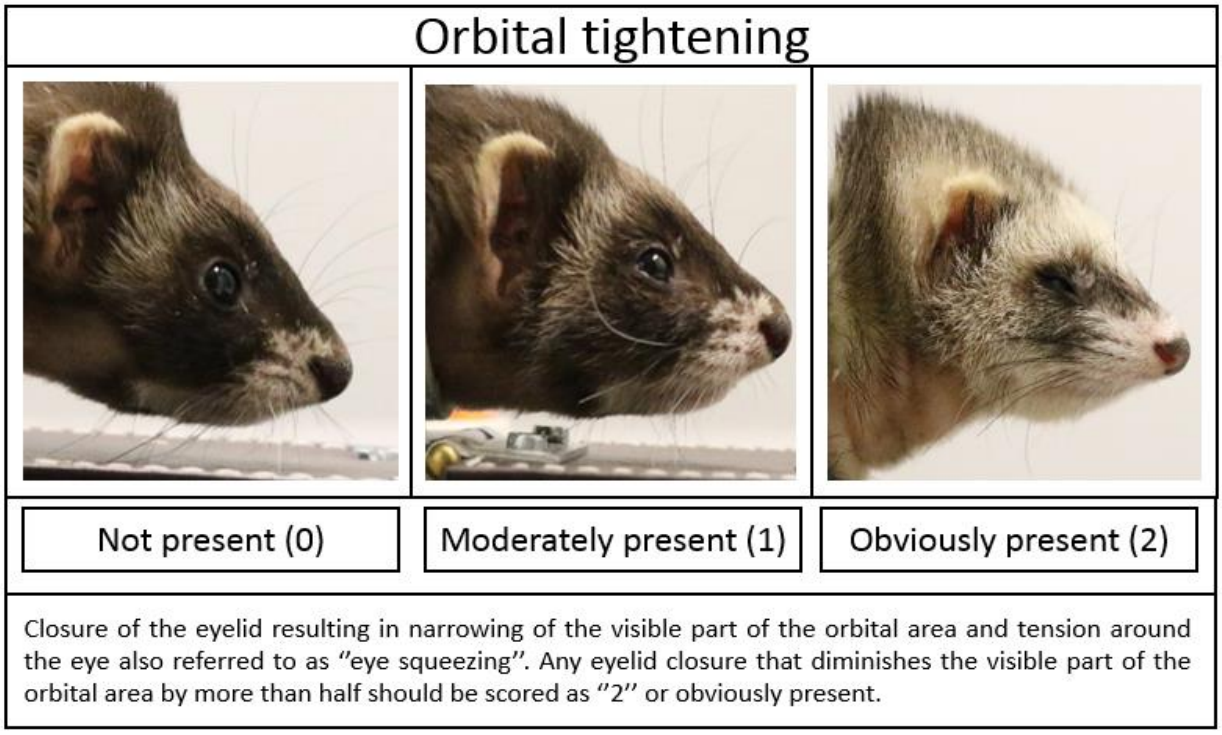
Photographs were structurally stored in maps and labeled by animal number, day and time. (B= baseline, P=Pain, P(2)= Pain 24 hours later, all additionally with time points). The most suitable photographs for manual scoring were selected for each animal, time point and view to reduce the number. For suitable frontal photographs eyes, ears and nose had to be visible with as less upward or downward turning of the head. Suitable lateral photographs were those where eyes ears and whiskers were fully visible and where the head was in the least angled position. This selection procedure resulted in at least one frontal, one left lateral and one right lateral photo per time point per day per animal. Selected photographs were then cropped with a cropping tool from Cyberlink PhotoDirector 4 software in such way that only the head including whiskers were fully visible and surroundings and body could not serve for possible bias.

Non-treatment blind comparisons were made between Individual baseline (no-pain) photographs from each animal and the matching pain photographs of that time point with the use of FastStone Image Viewer 5.3. This resulted in a comparison of B2h -P2h / B4h -P5h / B2h-P(2)2h and B4h-P(2)5h. Changes in facial expression within each ferret were listed and facial changes of all animals were compared to search for similarities. Furthermore, a session blind comparison between baseline and pain photos was made to identify changes in facial expression associated with the painful procedure. Randomized and unlabeled duos of high resolution baseline and pain photo pairs were selected and shown in comparison next to each other (randomly distributed left or right) on a computer screen with the use of the FastStone Image Viewer 5.3 comparison tool. For each duo, the session blind scorer mentioned the differences between the left and right photograph. A non-blind observer listed the mentioned differences per photograph in the matching baseline and pain section of an Excel sheet. Frontal images turned out to be difficult to score and were therefore excluded from further investigation

Based on these comparisons two FAU's were defined as follows:

1. Orbital tightening: closure of the eyelid resulting in narrowing of the visible orbital area and tension around the eye, also referred to as ''eye squeezing''
2. Whisker retraction: retraction of the whiskers where they are pulled back towards the cheeks instead of their normal position

Each FAU was then divided in three possible intensity scores in with a score of ''0'' indicating a high confidence of the Action Unit being absent, a score of ''1'' indicating either the dubiety of the Action Unit being present or high confidence of the action unit being moderately present and a score of ''2'' indicating high confidence of the Action Unit being obviously present. The combination of these AU's in the final Ferret Grimace Scale resulted in a maximal total pain score of 4 for an animal. Descriptions of each of the two FAU's and their scoring possibilities for intensity scores were made (*Figure 7*).



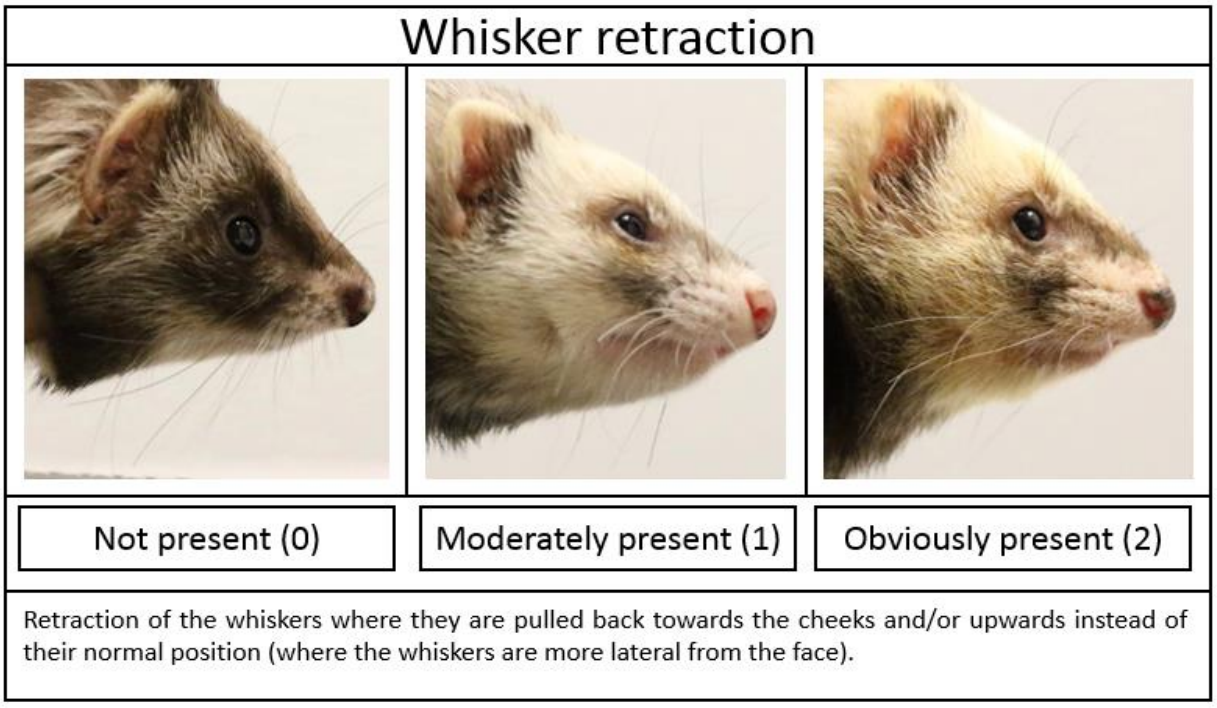


Figure 7. Ferret Grimace Scale. The FGS with images and descriptions of the FAU's 'Orbital tightening' (FAU1) and 'Whisker retraction; (FAU2). Within this FGS each FAU has to be scored to whether it is not present (score of 0), moderately present (score of 1) or obviously present (score of 2).

3.5.1 USABILTY OF THE FERRET GRIMACE SCALE

Because of experienced difficulties with scoring using the frontal photographs, only lateral images were selected for Grimace Scale scoring. In addition to that, because effects of the anesthesia were still present in the animals (yawning, inactivity) and too much manipulation was needed for clear photographs, the time point two hours post-surgery (P2h) was excluded for further FGS-scoring. A total of 95 photographs were randomly selected using an online randomizer tool ''random sequence generator'' (random.org). It was ensured that every animal was represented once at every time point either from the left or the right lateral view. For FGS-scoring, a PowerPoint presentation containing the 95 photographs (1 per slide) was used combined with an Excel score-form for the scoring of individual FAU's per photograph. The presentation comprised a preceding text which explained the content of the survey and the scoring methods using the prototype photographs with descriptions of each of the two FAU's and their scoring possibilities for intensity scores. All images were randomly distributed on the PowerPoint slides using a ''random sequence generator'' (random.org). The total of 95 images were scored by a total of eight treatment and session blind participants experienced with ferrets (3 veterinarians/scientists, 3 bio-technicians and 2 ferret owners). Each photograph was scored on a 3-point scale with scoring possibilities 0, 1 and 2 for each individual FAU. If the participants were unable to score an FAU at all they were asked to enter an 'x' for ''no score for FAU''. The maximum FGS score for each photograph was 4 (i.e. a score of 2 for each of the 2 action units). In addition to that, the participants were asked to give a dichotomous overall pain judgment on each photograph choosing from ''pain'' (score 1) or ''no pain'' (score 0).

3.6 Statistical analysis

All statistical analysis were performed using IBM SPSS Statistics 22®. Normality was tested for differences within subject between the compared time points with a Kolmogorov-Smirnov Exact significance (2-tailed) test (p>0,05). Sphericity and normality for the factor 'time' within each subject was calculated using a Kolmogorov-Smirnov test. Differences in Action Unit -scores on the different time points were analyzed using a repeated measures ANOVA. For Post-hoc analysis per individual FAU, paired sample T-tests were used to analyze the data with the time points as the 'within subjects factor' as shown in *Figure 8*. Cumulative combined scores of all respondents of both FAU's (FGS-scores), were also compared using paired sample T-tests with the same assumptions as mentioned earlier. Based on the number of different pairwise comparisons (6) the criterion was adjusted with the use of a Dunn-Šidák correction for significance level to $\alpha = 0{,}008513$. The reliability of the FGS was quantified by comparing average Action Unit scores across all eight participants, using the Intra-class Correlation Coefficient (ICC, Linear mixed model). Accuracy was determined by the comparison of the global pain vs. no pain dichotomous judgments with the actual pain state of the animal. For a valid comparison, the time point showing maximal FGS scores was used to supply 'pain' photos. This resulted in the use of 19 pain (time point 'P5h') and 19 no-pain photographs (time point 'B4h') for determination of accuracy. All values are described as average with standard error.

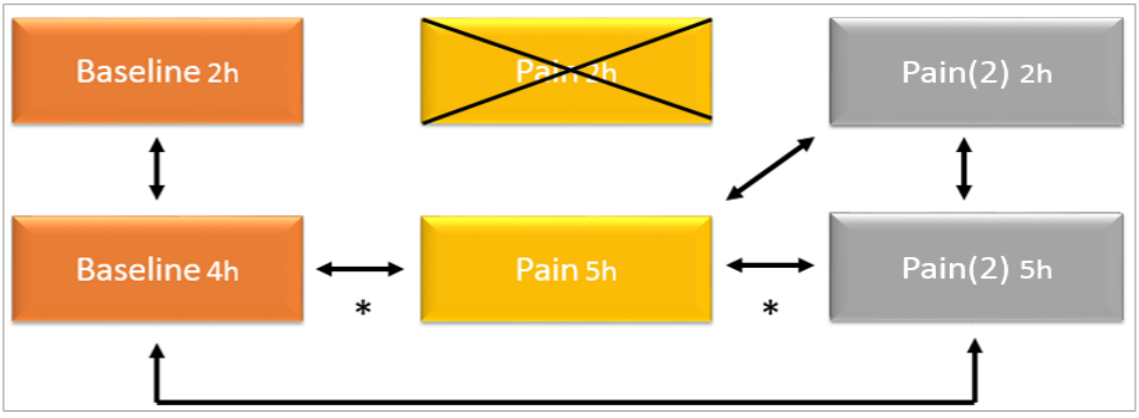


Figure 8. Statistical Post Hoc analysis of time points. Arrows indicate a comparison. Baselines were compared to see if time of day had an influence on Grimace Scale scores. B 4h-P5h, P5h-P(2)2h and P5h-P(2)5h were compared to look for

differences in Grimace Scale-scores due to the painful stimulus. P5h-P(2)2h, P(2)2h-P(2)5h and B4h-P(2)5h were compared to see if Grimace Scale-scores would decline in time.

4. RESULTS

4.1 Ferret Grimace Scale

The extent and time course of pain was assessed and quantified using the FGS (*Figure 5*). Because of the violation of Mauchley's test of sphericity for FAU1 (p<0,05,) with the estimates of sphericity (epsilon) >.75 for Greenhouse-Geisser (FAU epsilon = 0,542) the Huynh-Feldt modification was used (P<0,001) (Field 2012). For FAU2 and the FGS (FAU1+FAU2) the assumption of sphericity was met (respectively p=0,746 and p=0,465). Because of the use of only 'one' group (each ferret has its own baseline/control), there was no need to assume equality of variances. For the present study, it was chosen to use an Univariate general linear model / Repeated measures ANOVA (Algina&Keselman, 1997) which showed a significant effect of 'timepoint' in the 'Tests of Within Subject Effects' for FAU1 (df= 2,476, p=0,000152), FAU2 (df=4, p=0,000129) and FGS- scores (df=4, p=0,000006). All executed comparisons between time points for FAU1, FAU2 and FGS-scores were normally distributed (p>0,05) which led to the use of Post-Hoc multiple parametric paired sampled (two tailed) T-tests to search for differences between mean FGS-scores for time points.

4.1.1 SCORING FEEDBACK

Participants noted that some lateral pictures were not completely lateral and that the photo quality was not at its best for some photographs. Furthermore it was mentioned that coat-color distracted from scoring and that sometimes the background was troublesome. One scorer argued that some ferrets seemed more or less in fear instead of pain. Three scorers found whisker retraction (FAU2) far more difficult to score than eye closure (FAU1). Additional observed changes mentioned by scorers were: ear retraction, shape of the nose and pilo-erected fur on the head. Two scorers did not fill in the dichotomous overall pain score (0 or 1).

4.1.2 COMPARISONS BETWEEN TIME POINTS

4.1.2.1 Baseline

There were no significant differences between baselines for both FAU's (FAU1; t=-0.552, df=18, exact p=0.588, n=19)(FAU2; t=0.411, df=18, exact p=0.686, n=19) and FGS-scores (FGS; t=0.128, df=18, exact p=0.900).

4.1.2.2 Individual Action Units

FAU1 grimace scores significantly increased from B4h (0.2697±0.2468) to P5h (0.8891 ± 0.2928) (df=18; exact p=0.000891) and significantly decreased from P5h to P(2)5h (0.1918 ± 0.2115) (df=18; exact p=0.000922) (*Figure 9*).

FAU2 grimace scores showed the same patterns as FAU1 grimace scores, they significantly increased from B4h (0.6316 ± 0.4447) to P5h (1.2171 ±0.5089) (df=18; exact p=0.000393) and significantly decreased from P5h to P(2)5h (0.7058 ± 0.5234) (df=18; exact p=0,001060) (*Figure 9*).

Average difference scores (Pain (P5h) - No-pain (B4h)) were 0.619 For FAU1 and 0.585 For FAU2.

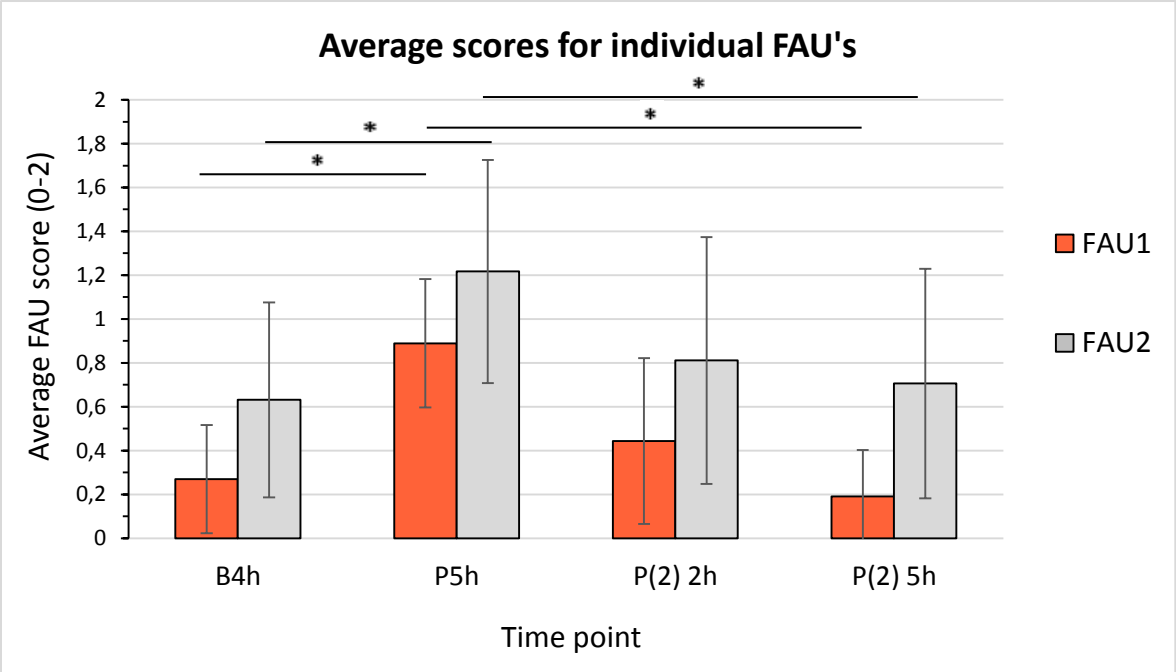


Figure 9. Average scores for each individual FAU on each time point. FAU scores can range from 0-2. Bars represent standard deviation. * = p<0.008513.

4.1.2.3 FGS scores (cumulative FAU-scores)

Significant differences in FGS-scores were found between the same time points as found for FAU1 and FAU2: B4h (0.9013 ± 0.6979) – P5h (2.1062 ± 1.0746)(df=18; exact p = 0.000227) and P5h – P(2)5h (0.8966 ± 0.7093) (df=18; exact p=0.000677) (*Figure 10*)

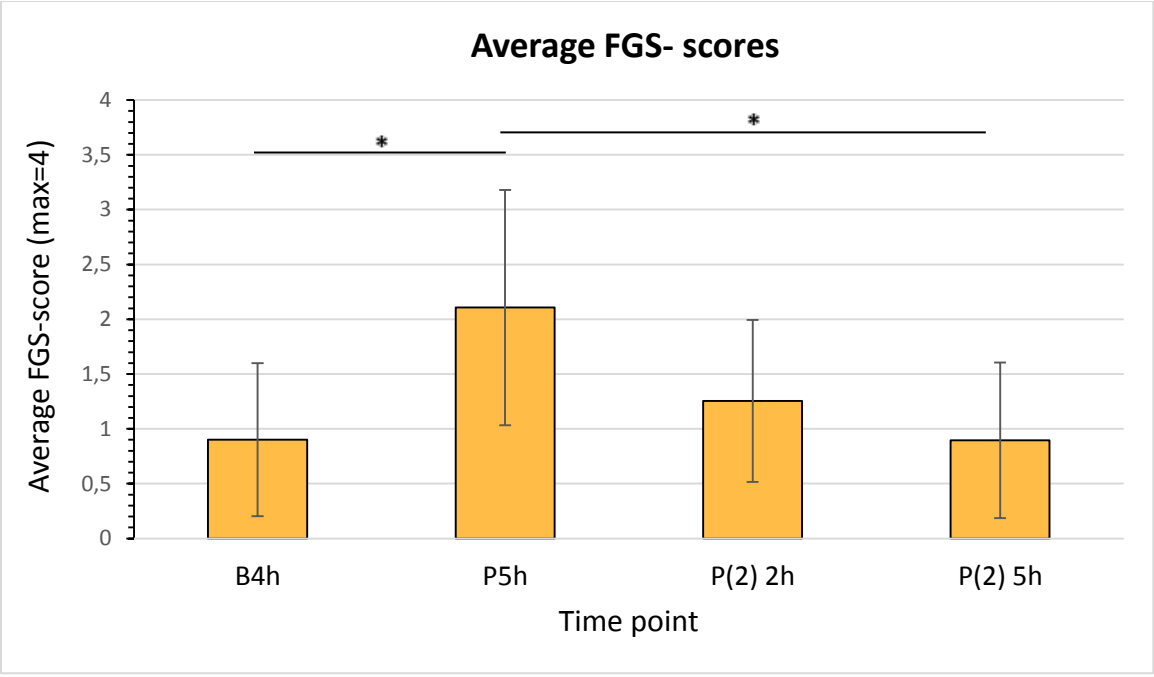


Figure 10. Average FGS scores (FAU1+FAU2) for each time point. FGS scores can range from 0-4. Bars represent the standard deviation. *= p<0.008513.

4.1.4 RELIABILITY AND ACCURACY

The FGS demonstrated a medium inter-rater reliability (IRR) with an overall Intra-class Correlation Coefficient (ICC) value of 0.645. Both separate FAU's also showed medium ICC values; 0.646 for eye closure (FAU1) and 0.545 for whisker retraction (FAU2). The accuracy of the global pain judgement *(Figure 11)* was 68.0% with misses ('pain' photographs scored as 'no-pain') being more prevalent (17.2%) than false alarms ('no-pain' photographs scored as 'pain') (14.8%). Individual scorers accuracy ranged from 59.4-72.9%.

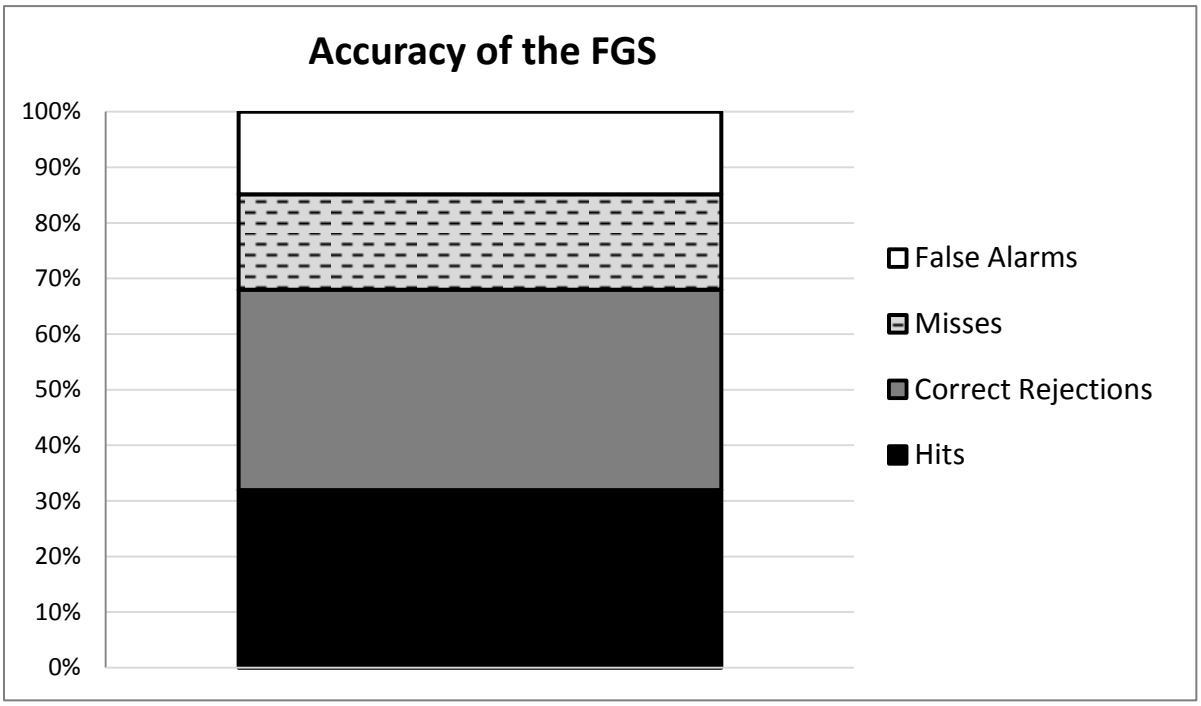


Figure 11. Accuracy of the FGS. The dichotomous scores of 19 no-pain (Baseline 4h) and 19 pain (Pain 5h) photographs were used to determine accuracy across 6 scorers (total of 38 photographs, half no-pain / half pain). *Hits*: pain photographs scored as pain; *Correct Rejections*: no-pain photographs scored no-pain; *Misses*: pain photographs scored as no-pain, *False Alarms*: no-pain photographs scored as pain.

5. DISCUSSION

As many ferrets are still largely used in scientific studies where often no sufficient analgesics are imbedded in the experimental outline, the need for recognition of pain in this species is high. However, the currently used methods for pain recognition in this species are highly subjective and ferrets tend to be reluctant in showing pain related behavior. In an effort to develop a new, easy to use and reliable objective pain assessment tool in ferrets, the present study identified FAU's in this species and with this developed a FGS.

5.1 Identification of Facial Action Units

Facial changes in the areas of the eyes and whiskers, previously described as FAU's ''eye closure'' (FAU1) and ''whisker retraction'' (FAU2), were identified and used in the FGS. These findings partly met our expectations. Even though the identification of the two FAU's in ferrets supports the first prediction that changes in facial expression in response to pain could be found in areas similar to other species, the quantity is relatively low and unexpected (*Table 2*). Both FAU's have also been identified as distinctive facial features of pain in other species. Eye closure was seen as a facial feature of pain in mice (Langford et al. 2010), rats (Sotocinal et al. 2011), rabbits (Keating et al. 2012), horses (Dalla Costa et al. 2014) and cats (Holden et al. 2014) and whisker change was found as a facial feature of pain in mice (Langford et al. 2010), rats (Sotocinal et al. 2011) and rabbits (Keating et al. 2012). The facial musculature of the ferret provided evidence for possible movement in the ear, lip, mouth, whisker and eye area even though the initial expectation on finding facial expression in ferrets was low because of their stoic nature. Additional observed changes of facial expression such as ear retraction, differences in nose-shape and pilo-erection on the cheeks were revealed trough scoring feedback. However, none of these were identified with the initial detection of AU's in the profile/lateral photographs by a session-blind investigator.

Although the continuity of species as described by Darwin suggests similarities, slight differences between the identification of FAU's in Grimace Scales and the low detection in the FGS can be expected and possibly explained. The outward pain responses after a painful stimulus can be highly dependent on the pain tolerance threshold of the used species (Short 1998), the used stimulus, stimulus intensity, the duration of the stimulus, and the applied area (Sotocinal et al. 2011; Dalla Costa et al. 2014) which have not been studied preliminary for ferrets. This could have caused some more subtle FAU's to remain unseen. Furthermore, less capturing of the optimal pain face and FAU's could have been caused by the fact that only two time points after surgery and two time points 24 hours later were chosen for acquiring pain photographs whilst no preliminary studies on the appropriate time interval of observations were done as seen in other grimace scales (e.g. HGS (Dalla Costa et al. 2014)). Premised that ferrets in fact do show more facial expression than initially observed, the use of solely lateral views could have contributed to the relatively low identification of facial changes. The inability to score FAU's in frontal photographs, which ultimately led to their exclusion, has also been reported in horses (Dalla Costa et al. 2014). Finally, the quality of observing could have been diminished due to the untrained assessor of the photographs in the search for AU's.

The current findings indicate that ferrets do show some facial expression in the areas of the whiskers and eyes but this relatively low identification FAU's might be i) species specific, ii) due to the unstandardized painful stimulus and time interval of photography iii) caused by the (low amount of) used views on the ferrets face or iv) because of the non-trained observer. Because the current FGS has a relatively low maximal score of four, differentiating with precision is more or less difficult; blinking of the animal and no whisker retraction combined in the same photograph would already give a FGS score of one out of four. In addition, when a scorer finds one of the FAU's ''unable to score'' the FGS-score of pain only depends on one single FAU. Next to this, it has to be taken into consideration that all visible facial changes can also independently be caused by other causes than pain (orbital tightening by blinking or sedation (Matsumiya et al. 2012), whisker change by sniffing). Therefore creating a FGS composing more than two FAU's is essential. In order to investigate the pain-face of ferrets more thoroughly, a pilot study with a high intensity painful stimulus combined with a dose-dependent analgesic reversal could be used (as seen in the MGS (Langford et al. 2010)). The use of high intensity painful stimuli might need to be ethically discussed, but it would help to truly recognize the facial activity of ferret in pain. Finally,

additional views on the ferrets face (e.g. dorsal view) and previous training of the observer could help in the search for FAU's of pain in this species.

5.2 Ferret Grimace Scale

5.2.1 COMPARISON BETWEEN TIME POINTS

The described Facial Action Units of pain in ferrets were compared separately and in their use in a Ferret Grimace Scale to determine whether these could differentiate ferrets in pain from ferrets in their baseline condition.

5.2.1.1 Grimace-scores (individual FAU's)

Both FAU's, ''eye closure'' (FAU1) and ''whisker change'' (FAU2), significantly increased from baseline to the time point five hours post-surgery (P5h) and were significantly decreased 24 hours later (P(2)5h). Despite no further statistical comparison was made, whisker retraction (FAU2) seemed to yield relatively higher Grimace Scores than eye closure (FAU1) on each time point. Furthermore, average difference scores of both FAU's (0.619 (FAU1); 0.585 (FAU2)) were roughly the same.

The significant increase from baseline, peak grimace score at time point P5h and decrease to baseline from P5h in both individual FAU's were expected. Despite the fact that comparing individual FAU's amongst time points gives insight in their individual ability and utility in differentiating ''pain'' from ''no pain'' and their influence on total Grimace Scale scores, most other Grimace-Scales did not study them separately. However, Sotocinal *et al.* (2011) did investigate the prominence of individual AU's at the peak of pain in rats and difference scores to look at their individual utility. Difference scores of ''orbital'' and ''whiskers'' in the RGS were comparable to those found in the present study (Sotocinal et al. 2011) and indicate that both have equal influence on scoring outcomes when used in a FGS.

The relatively higher scores for ''whisker change'' compared to ''eye closure'' draw attention despite no statistical analysis on this particular observation was done. Although great differences in scores between FAU's were not initially expected, the relatively higher average scores on whisker retraction (FAU2) can possibly be explained with some of the scoring feedback. Observers found whisker retraction rather difficult to score. Difficulty in scoring of particular AU's has also been observed in horses (Dalla Costa et al. 2014) and difficulties in scoring whisker changes were also found in the RbtGS but merely because many of the images were not of high enough quality for clear observation of this AU (Keating et al. 2012). Difficulties in scoring whisker retraction in the present study could have led to an ambiguous identification of ''not-present'' compared to ''present'' which could culminate to high scores (because a score of 0 is presumably less chosen than 1 or 2) in comparison to eye closure which is much more easy to distinguish.

All in all, the present findings indicate that both of the described FAU's are able to distinguish ferrets 5h post-surgery from their baseline position. They therefore can be cumulatively used into the FGS.

For future research, more clarifying descriptions or basic drawings as used by Holden et al (2014) could give observers more stability in scoring FAU's that are perceived as 'difficult.' Furthermore, the effects of image quality will be discussed in the ''reliability and accuracy'' section of this paper.

5.2.1.2 Ferret Grimace Scale-scores

The same significant increase from baseline to 5h post-surgery (P5h) and the decrease to baseline positon (B4h) were observable in the FGS as found with the individual FAU's. No statistical differences were found between both time points the day after surgery (P(2)2h and P(2)5h) and P(2)5h with baselines which indicates a trend towards baseline position over time.

The fact that FGS-scores peaked significantly at 5h post-surgery was as expected and appears to be comparable to the results found in the RGS of Sotocinal *et al.* (2011), where peak RGS-scores were seen at four hours post-laparatomy. However, significant differences in RGS-scores from baseline were seen in more than one time point, respectively at 1h, 4h and 6h post-surgery. Unfortunately in the FGS, the time point two hours after surgery (P2h) could not be taken into account because of the possible effects of anesthesia. Additional comparisons with time point P2h would have given more insight in the pain induced by the used painful stimulus and FGS scores over time. Furthermore, the fact that P(2)2h has not significantly decreased from Pain5h in the FGS may suggests that animals at this time point still showed some facial grimacing. However, because no comparisons to baseline were made regarding time point P(2)2h this statement cannot be supported. The decline of FGS-scores in time was as expected and not caused by time-based changes of facial expression in the animals because comparison of baselines showed no statistical differences. A decline in Grimace Scale scores over time (trend towards baseline) has also been observed in rats, but the time points were dependent on the used painful stimulus (Sotocinal et al. 2011). Furthermore, it has to be noted that the disappearance of facial grimacing and the lower detection of FAU's over time does not necessarily correspond to the diminishing of pain. The ability to adaptation of pain thresholds (Bodnar et al. 1978) and to suppress the pain response and thus facial expressions of pain has to be taken into consideration because of its functional advantages for the animal during stressful situations (Amit & Galina 1986).

Even though the aim of the use of grimace scales is to recognize pain in the used species, these results only indicate that a significant change in facial grimacing is visible. Whether these facial changes are directly related to post-operative pain or other types of distress (e.g. anesthesia, stress, surroundings) has to be investigated more thoroughly.

5.2.1.3 RELIABILTY AND ACCURACY

Inter-Rater-Reliability of the FGS was tested with the Intra-Class Correlation Coefficient which shows the consistency among observational ratings. The FGS yielded an overall Intra-class Correlation Coefficient of 0.645 which is lower than observed in other Grimace Scales (MGS: 0.90 (Langford et al. 2010), HGS: 0.92 (Dalla Costa et al. 2014) RGS: 0.90 (Langford et al. 2010) and RbtGS: 0.91 (Keating et al. 2012)) and therefore unexpected. Depending on the benchmarking system, an ICC of 0.645 can be classified as moderate to good (Kilem n.d.). The relatively lower consistency could arise because of the lack of universal training in the development of the present Grimace Scale, as coders from different disciplines and with different views on pain in animals participated. In accordance with other Grimace Scales applied to animals and humans, scorers gave no-pain (baseline) photographs low, but not zero FGS-scores. Due to possible reactions on the caregiver or surroundings, it might be possible for an animal to blink, or move whiskers in its normal behavior. This could lead to the visibility of these FAU's in low intensity on no-pain photographs which possibly lowers the reliability of this pain scale. To ensure further evaluation of the reliability in the use of the found FAU's in a FGS it is largely recommended to use more and trained scorers with similar knowledge and background in future research.

Evaluation of the FGS revealed an overall accuracy of 68% (*Figure 11*), which was lower than expected as the accuracies found in other Grimace Scales (MGS: 97% (Langford et al. 2010), RbtGS:84% (Keating et al. 2012) RGS: 82% (Sotocinal et al. 2011) and HGS: 73.3% (Dalla Costa et al. 2014)) were relatively higher. Firstly, this relatively low accuracy might be due to the assumption of pain: some of the ferrets might not have been in pain at the precise moment of photographing. We found that animals showed repeating bouts of muscle activity with handling for photography, which could possibly indicate pain. Photographs could have been made in between those waves of pain expression. Secondly, the images used in this study varied in quality, which might have resulted in a lower accuracy, as Langford *et al.* (2010) noted that a higher image quality improved the accuracy of the Mouse Grimace Scale from 72%

to 97%. In addition, scoring feedback showed that coat-color of the animals and the lack of standardized backgrounds made some photographs more difficult to score than others. This is supported by other studies which also discuss the negative impact of a lower image quality, dark background and coat color of the animals on scoring ability (Dalla Costa et al. 2014; Langford et al. 2010). Thirdly, another factor for the lower accuracy includes the method of pain face capturing. In the current study, photographs were used instead of the 15-30 minute HD filming sequence used in other Grimace Scales. This could have led to missing shots of pain-faces in ferrets that actually did endure pain. However, photographing the animals also has an advantage over filming, because it diminishes the bias caused by selecting usable photographs from film sequences. Furthermore, some ferrets had to walk through the tube multiple times, as they would look sideways after exiting the tube, which might have caused agitation and therefore effect the ferrets' facial expression. Finally, all scorers in the present study were untrained which probably also effected the accuracy; Langford *et al.* (2010) showed that a scorer with one year of experience did score with higher accuracy. Overall, the accuracy of the FGS after the present study can be seen as above chance.

6. Conclusion

The aim of the present study was to identify FAU's in ferrets that indicate pain and with these to develop a FGS. The present study identified two FAU's in ferrets, ''eye closure'' and whisker retraction'' with which, when used in a FGS, ferrets after the painful stimulus could be differentiated from their baseline position. Because no standardized pain evoking pilot studies or analgesic controls were used, it cannot be said with certainty if these facial changes are directly related to the post-operative pain or other types of distress. In addition, the detection of only two FAU's with relatively moderate accuracy and inter-rater reliability compared to other Grimace Scales is not yet sufficient for the development of a pain recognition tool. Therefore we cannot yet conclude with certainty that ferrets show pain by means of facial expressions. For the development of a FGS embedded within a multidimensional tool for use of pain recognition in laboratory or clinical settings, further investigations are necessary i) to identify more FAU's, ii) to increase reliability and accuracy, and iii) to evaluate its use with different painful stimuli and analgesic schedules.

REFERENCES

Amit, Z. & Galina, Z.H., 1986. Stress-induced analgesia: adaptive pain suppression. *Physiological Reviews*, 66(4), pp.1091–1120.

Anil, S.S., Anil, L. & Deen, J., 2002. Challenges of pain assessment in domestic animals. *JAVMA*, 220(3), pp.313–319.

Anon, 2000. ILAR Committee on Regular Issues in Animal Care and use. In *Definition of pain and distress and reporting requirements for laboratory animals*. Available at: http;//books.nap.edu/books/0309072913/html/index.html.

Anon, Legislation and implementation: EU legislative framework. Available at: http://ec.europa.eu/environment/chemicals/lab_animals/legislation/en.htm.

Arnemo, J.M. & Søli, N.E., 1992. Immobilization of mink (Mustela vison) with medetomidine-ketamine and remobilization with atipamezole. *Veterinary Research Communications*, 16(4), pp.281–292. Available at: http://link.springer.com/10.1007/BF01839327.

Association of Veterinary Teachers and Research Workers, 1989. *Guidelines for recognition and assessment of pain in animals*,

Ball, R.S., 2004. Issues to Consider for Preparing Ferrets as Research Subjects in the Laboratory. *ILAR Journal*, 47(4), pp.348–357.

Bateson, P., 1986. When to experiment on animals. *New Scientist*, (February), pp.30–32.

Baumans, V., 2005. Science-based assessment of animal welfare. *Rev. sci. tech. Off. int. Epiz.*, 24(2), pp.503–514.

Belser, J. a, Katz, J.M. & Tumpey, T.M., 2011. The ferret as a model organism to study influenza A virus infection. *Disease models & mechanisms*, 4(5), pp.575–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3180220&tool=pmcentrez&rendertype=abstract [Accessed July 10, 2014].

Bieri, D. et al., 1990. The Faces Pain Scale for the self-assessment of the severity of pain experienced by children : development , initial validation , and preliminary investigation for ratio scale properties. *Pain*, 41, pp.139–150.

Biró, Z., Szemethy, L. & Heltai, M., 2004. Home range sizes of wildcats (Felis silvestris) and feral domestic cats (Felis silvestris f . catus) in a hilly region of Hungary. *Mammalian Biology*, 69(5), pp.302–310.

Bodnar, R.J. et al., 1978. Stress-induced analgesia : Adaptation following chronic cold water swims. *Bulletin of Psychonomic Society*, 11(6), pp.337–340.

De Boo, M.J. et al., 2005. The interplay between replacement, reduction and refinement: considerations where the Three Rs interact. *Animal Welfare*, 14, pp.327–332.

Boo, M.J. De et al., 2005. The interplay between replacement, reduction and refinement: considerations where the Three Rs interact. *Animal Welfare*, 14, pp.327–332.

Brignell, B.A., 2003. Assessment of pain in non-cognizant elderly. *Pain Management/Dementia Care*, 14(1), pp.71–77.

Broom, D.M., 1991. Assessing welfare and suffering. *Behavioural processes*, 25, pp.117–123.

Broom, D.M., 1986. Indicators of poor welfare. *The British veterinary journal*, 142(6), pp.524–526. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3594185.

Brown, S. a., 1997. Clinical techniques in domestic ferrets. *Seminars in Avian and Exotic Pet Medicine*, 6(2), pp.75–85. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1055937X9780014X.

Caeiro, C.C. et al., 2013. OrangFACS: A muscle-based coding system for orangutan facial movements. *International Journal of Primatology*, 34(1), pp.115–129.

Caeiro, C.C., Waller, B.M. & Burrows, A.M., 2013. *CatFACS: The Cat Facial Coding System Manual*,

Carstens, E. & Moberg, G.P., 2000. Recognizing pain and distress in laboratory animals. *ILAR journal / National Research Council, Institute of Laboratory Animal Resources*, 41(2), pp.62–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11304586.

Chattipakorn, S.C. et al., 2002. Trigeminal c-Fos expression and behavioral responses to pulpal inflammation in ferrets. *Pain*, 99(1-2), pp.61–69. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0304395902000544.

Chevalier-Skolnikoff, S., 2006. Facial Expression of Emotion in Nonhuman-primates. In P. Ekman, ed. *Darwin and Facial Expression: A Century of Research in Review*.

Cohen, N.E., Stassen, E.N. & Brom, F.W. a, 2009. Fundamental moral attitudes to animals and their role in judgment: An empirical model to describe fundamental moral attitudes to animals and their role in judgment on the culling of healthy animals during an animal disease epidemic. *Journal of Agricultural and Environmental Ethics*, 22, pp.341–359.

Committee on Recognition and Alleviation of Distress in Laboratory Animals, 2008. *Recognition and Alleviation of Distress in Laboratory Animals*, Washington, D.C.: The national academies press.

Dalla Costa, E. et al., 2014. Development of the Horse Grimace Scale (HGS) as a pain assessment tool in horses undergoing routine castration. *PloS one*, 9(3), p.e92281. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3960217&tool=pmcentrez&rendertype=abstract [Accessed July 9, 2014].

Darwin, C.R., 1872. *The expression of emotions in man and animals* 1st ed., London: John Murray. Available at: http://darwin-online.org.uk/content/frameset?pageseq=1&itemID=F1142&viewtype=text.

Darwin, C.R. & Ekman, P., 1998. *The Expression of the Emotions in Man and Animals* 3rd ed., New York: Oxford University Press.

Duncan, I.J.H. & Dawkins, M.S., 1983. The Problem of Assessing Well-Being'' and Suffering'' in Farm Animals. In D. Smidt, ed. *Indicates Relevant to Farm Animal Welfare*. Springer Netherlands, pp. 13–24.

Ekman, P. & Rosenberg, E.L., 1997. *What the Face Reveals: Basic and Applied Studies of Spontaneous Expression Using the Facial Action Coding System (FACS)*, Oxford University Press.

Faller, K.M.E. et al., 2015. Refinement of analgesia following thoracotomy and experimental myocardial infarction using the Mouse Grimace Scale. *Experimental Physiology*, 100, pp.164–172. Available at: http://doi.wiley.com/10.1113/expphysiol.2014.083139.

FDA, 2009. NADA 141-033 Antisedan - original approval. Available at: http://www.fda.gov/AnimalVeterinary/Products/ApprovedAnimalDrugProducts/FOIADrugSummaries/ucm116093.htm.

Fenwick, N., Danielson, P. & Griffin, G., 2011. Survey of Canadian Animal-Based Researchers ' Views on the Three Rs : Replacement , Reduction and Refinement. *PloS one*, 6(8), pp.1–14.

Field, A., 2012. No Title. Available at: www.statisticshell.com/docs/repeatedmeasures.pdf.

Fitzpatrick, J., Scott, M. & Nolan, a., 2006. Assessment of pain and welfare in sheep. *Small Ruminant Research*, 62(1-2), pp.55–61. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0921448805002993 [Accessed October 4, 2014].

Flecknell, P., Gledhill, J. & Richardson, C., 2007. Assessing Animal Health and Welfare and Recognising Pain and Distress. *ALTEX*, 24(Special Issue), pp.82–83.

Flecknell, P.A. & Roughan, J.V., 2004. Assessing pain in animals - putting research into practice. *Animal Welfare*, 13, pp.71–75.

Fournier-Chambrillon, C. et al., 2003. Immobilization of free-ranging European mink (Mustela lutreola) an polecat (Mustela putorius) with medetomidine-ketamine and reversal by atipamezole. *Journal of wildlife diseases*, 39(2), pp.393–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12910767.

Fraser, D., 2008. Understanding animal welfare. *Acta Veterinaria Scandinavica*, 50(Suppl 1), p.S1. Available at: http://www.actavetscand.com/content/50/S1/S1 [Accessed September 22, 2014].

Gauthier, C. & Griffin, G., 2005. Using animals in research , testing and teaching Landmarks in animal-based research and key moral. *Rev. sci. tech. Off. int. Epiz.*, 24(2), pp.735–745.

Hadjistavropoulos, T. et al., 2002. Using facial expressions to assess musculoskeletal pain in older persons. *European journal of pain (London, England)*, 6, pp.179–187.

Hawkins, P., 2002. Recognizing and assessing pain, suffering and distress in laboratory animals: a survey of current practice in the UK with recommendations. *Laboratory animals*, 36(4), pp.378–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12396281 [Accessed September 24, 2014].

Herr, K.A. et al., 1998. Evaluation of the Faces Pain Scale for Use with the Elderly. *Clin J Pain*, 14(1), pp.29–38.

Holden, E. et al., 2014. Evaluation of facial expression in acute pain in cats. *The Journal of small animal practice*, (1872), pp.1–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25354833 [Accessed November 8, 2014].

Hsu, K.-T. et al., 2007. The application of facial expressions to the assessment of orofacial pain in cognitively impaired older adults. *Journal of the American Dental Association (1939)*, 138(7), pp.963–969; quiz 1021–1022. Available at: http://dx.doi.org/10.14219/jada.archive.2007.0293.

Hunter, M. et al., 2000. An Evaluation of the Faces Pain Scale with Young Children. *Journal of Pain and Symptom Management*, 20(2), pp.122–129.

IASP, 2012. IASP Taxonomy. Available at: http://www.iasp-pain.org/Taxonomy?navItemNumber=576#Pain.

Jha, S.K., Coleman, T. & Frank, M.G., 2006. Sleep and sleep regulation in the ferret (Mustela putorius furo). *Behavioural Brain Research*, 172, pp.106–113.

Johnson-Delaney, C.A. & Mayer, J., 2006. *The exotic mammal technician wet lab*, Association of Exotic Mamal Veterinarians.

Johnston, M.S., 2005. Clinical Approaches to Analgesia in Ferrets and Rabbits. *Seminars in Avian and Exotic Pet Medicine*, 14(4), pp.229–235. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1055937X0500071X [Accessed July 10, 2014].

Keating, S.C.J. et al., 2012. Evaluation of EMLA cream for preventing pain during tattooing of rabbits: changes in physiological, behavioural and facial expression responses. *PloS one*, 7(9), p.e44437. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3436883&tool=pmcentrez&rendertype=abstract [Accessed July 9, 2014].

Kilem, L.G., Chapter 6: Benchmarking Inter-Rater Reliability Coefficients. In *Handbook of Inter-Rater Reliability*.

Korte, S.M., Olivier, B. & Koolhaas, J.M., 2007. A new animal welfare concept based on allostasis. *Physiology & behavior*, 92(3), pp.422–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17174361 [Accessed August 22, 2014].

Krohn, T.C., Hejgaard, K. & Hansen, A.K., 2001. Methods for General Assessment of the Welfare of Laboratory Rats. *Acta Agriculturae Scandinavica, Section A — Animal Science*, 30, pp.118–123.

Kunz, M. et al., 2007. The facial expression of pain in patients with dementia. *Pain*, 133(1), pp.221–228. Available at: http://dx.doi.org/10.1016/j.pain.2007.09.007.

Langford, D.J. et al., 2010. Coding of facial expressions of pain in the laboratory mouse. *Nature methods*, 7(6), pp.447–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20453868 [Accessed July 9, 2014].

Lascelles, B.D.X., Butterworth, S.J. & Waterman, A.E., 1994. Postoperative analgesics and sedative effects of carprofen and pethidine in dogs. *Veterinary Record*, 134, pp.187–191.

Leach, M.C., Klaus, K., et al., 2011. Are We Looking in the Right Place ? – Implications for Assessing Pain. *ALTEX proceedings of WC8*, 1/12, pp.493–495.

Leach, M.C., Coulter, C.A., et al., 2011. Are We Looking in the Wrong Place ? Implications for Behavioural-Based Pain Assessment in Rabbits (Oryctolagus cuniculi) and Beyond ? *PloS one*, 6(3), p.e13347.

Leach, M.C. et al., 2009. Behavioural effects of ovariohysterectomy and oral administration of meloxicam in laboratory housed rabbits. *Research in Veterinary Science*, 87(2), pp.336–347. Available at: http://dx.doi.org/10.1016/j.rvsc.2009.02.001.

Leach, M.C. et al., 2012. The Assessment of Post-Vasectomy Pain in Mice Using Behaviour and the Mouse Grimace Scale. *PloS one*, 7(4), pp.1–9.

LeResche, L., 1982. Facial expression in pain: A study of candid photographs. *Journal of Nonverbal Behavior*, 7(1), pp.46–56.

LeResche, L. & Dworkin, S.F., 1984. Facial expression accompanying pain. *Social science & medicine*, 19(12), pp.1325–1330.

LeResche, L. & Dworkin, S.F., 1988. Facial expressions of pain and emotions in chronic TMD patients. *Pain*, 35, pp.71–78.

Lichtenberger, M. & Ko, J., 2007. Anesthesia and analgesia for small mammals and birds. *The veterinary clinics of North America. Exotic animal practice*, 10(2), pp.293–315. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17577554 [Accessed September 28, 2014].

Mason, G. & Mendl, M., 1993. Why is there no simple way of measuring animal welfare. *Animal Welfare*, 2, pp.301–319.

Matsumiya, L.C. et al., 2012. Using the Mouse Grimace Scale to reevaluate the efficacy of postoperative analgesics in laboratory mice. *Journal of the American Association for Laboratory Animal Science : JAALAS*, 51(1), pp.42–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3276965&tool=pmcentrez&rendertype=abstract.

Mayer, J., 2007. Use of behavior analysis to recognize pain in small mammals. *Lab animal*, 36(6), pp.43–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17519944.

McCulloch, S.P., 2012. A Critique of FAWC's Five Freedoms as a Framework for the Analysis of Animal Welfare. *Journal of Agricultural and Environmental Ethics*, 26(5), pp.959–975. Available at: http://link.springer.com/10.1007/s10806-012-9434-7 [Accessed October 16, 2014].

Mellor, D.J., Cook, C.J. & Stafford, K.J., 2000. Chapter 9: Quantifying Some Responses to Pain as a Stressor. In M. G.P. & M. J.A., eds. *The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare*. Wallingford UK: CAB International 2000, pp. 171–196.

Miller, A.L. & Leach, M.C., 2014. Using the mouse grimace scale to assess pain associated with routine ear notching and the effect of analgesia in laboratory mice. *Laboratory Animals*, 0(0), pp.1–4. Available at: http://lan.sagepub.com/lookup/doi/10.1177/0023677214559084.

Mogil, J.S. et al., 2010. Hypolocomotion, asymmetrically directed behaviors (licking, lifting, flinching, and shaking) and dynamic weight bearing (gait) changes are not measures of neuropathic pain in mice. *Molecular pain*, 6, p.34.

Moller, H. & Alterio, N., 1999. Home range and spatial organisation of stoats (Mustela erminea), ferrets (Mustela furo) and feral house cats (Felis catus) on coastal grasslands, Otago Peninsula, New Zealand: Implications for yellow-eyed penguin (Megadyptes antipodes) conservatio. *New Zealand Journal of Zoology*, 26(3), pp.165–174. Available at: http://www.tandfonline.com/doi/abs/10.1080/03014223.1999.9518186 [Accessed November 26, 2014].

Molony, V. & Kent, J.E., 1997. Assessment of acute pain in farm animals using behavioral and physiological measurements. *J Anim Sci*, 75, pp.266–272.

Moors, P.J. & Lavers, R.B., 1981.) at Pukepuke Lagoon, New Zealand. *New Zealand Journal of Zoology*, 8(February 2015), pp.413–423.

Nederlandse Voedsel- en Warenautoriteit, 2013. *Zo doende 2013*,

NKCA, 3V Alternatieven. Available at: http://www.nkca.nl/3v-alternatieven/ [Accessed March 20, 2015].

Norbury, G.L., Norbury, D.C. & Heyward, R.P., 1998. Space use and denning behaviour of wild ferrets (Mustela furo) and cats (Felis catus). *New Zealand Journal of Ecology*, 22(Wardle 1991), pp.149–159.

Ohl, F. & van der Staay, F.J., 2012. Animal welfare: at the interface between science and society. *Veterinary journal (London, England : 1997)*, 192(1), pp.13–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21703888 [Accessed September 18, 2014].

Olfert, E.D. & Godson, D.L., 2000. Humane endpoints for infectious disease animal models. *ILAR journal / National Research Council, Institute of Laboratory Animal Resources*, 41(2), pp.99–104. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11406703.

Van Oostrom, H., Schoemaker, N.J. & Uilenreef, J.J., 2011. Pain management in ferrets. *The veterinary clinics of North America. Exotic animal practice*, 14(1), pp.105–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21074706 [Accessed October 9, 2014].

Overheid.nl, Wet op dierproeven. Available at: http://wetten.overheid.nl/BWBR0003081/geldigheidsdatum_04-03-2015#1 [Accessed March 4, 2015].

Parr, L.A. et al., 2010. MaqFACS: A muscle-based facial movement coding system for the macaque monkey. *Journal of Physical Anthropology*, 143, pp.625–630.

Pollock, C., 2007. Emergency medicine of the ferret. *The veterinary clinics of North America. Exotic animal practice*, 10(2), pp.463–500. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17577560 [Accessed September 28, 2014].

Poole, T. ed., *The UFAW Handbook on the Care and Management of Laboratory Animals* 7th ed., Blackwell Science.

Poole, T.B., 1978. An analysis of social play in polecats (Mustelidae) with comments on the form and evolutionary history of the open mouth play face. *Animal Behaviour*, 26, pp.36–49. Available at: http://linkinghub.elsevier.com/retrieve/pii/0003347278900064.

Prkachin, M., 1992. The consistency of facial expressions of pain : a comparison across modalities. *Pain*, 51, pp.297–306.

Roughan, J. V & Flecknell, P. a, 2003. Evaluation of a short duration behaviour-based post-operative pain scoring system in rats. *European journal of pain (London, England)*, 7(5), pp.397–406. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12935791 [Accessed October 7, 2014].

Roughan, J. V & Flecknell, P.A., 2001. Behavioural effects of laparotomy and analgesic effects of ketoprofen and carprofen in rats. *Pain*, 90, pp.65–74.

Roughan, J. V & Flecknell, P.A., 2004. Behaviour-based assessment of the duration of laparotomy- induced abdominal pain and the analgesic effects of carprofen and buprenorphine in rats. *Behavioural Pharmacology*, 15, pp.461–472.

Sacerdote, P., 2006. Opioids and the immune system. *Palliative medicine*, 20 Suppl 1(Table 1), pp.s9–s15.

Sadove, M.S. et al., 1971. Analgesic Effects of Ketamine Administered in Subdissociative Doses. *Anesthesia and Analgesia Current Researches*, 50(3), pp.452–457.

Schiavenato, M. et al., 2008. Neonatal pain facial expression: Evaluating the primal face of pain. *Pain*, 138, pp.460–471.

Short, C.E., 1998. Fundamentals of pain perception in animals. *Applied Animal Behaviour Science*, 59(1-3), pp.125–133. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168159198001270.

Sladky, K.K. et al., 2000. Evaluation of epidural morphine for postoperative analgesia in ferrets (Mustela putorius furo). *Contemporary topics in laboratory animal science / American Association for Laboratory Animal Science*, 39(6), pp.33–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11487250.

Sneddon, L.U. et al., 2014. Defining and assessing animal pain. *Animal Behaviour*, 97, pp.201–212. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0003347214003431 [Accessed October 10, 2014].

Sotocinal, S.G. et al., 2011. The Rat Grimace Scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions. *Molecular pain*, 7(1), p.55. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3163602&tool=pmcentrez&rendertype=abstract [Accessed July 9, 2014].

Stafleu, F., Grommers, F. & Vorstenbosch, J., 1996. Animal welfare: evoluation and erosion of a moral concept. *Animal Welfare*, 5(1993), pp.225–234.

Tomlinson, D. et al., 2010. A systematic review of faces scales for the self-report of pain intensity in children. *Pediatrics*, 126(5), pp.e1168–e1198.

Vick, S.J. et al., 2007. A cross-species comparison of facial morphology and movement in humans and chimpanzees using the facial action coding system (FACS). *Journal of Nonverbal Behaviour*, 31, pp.1–20.

Waller, B.M. et al., 2012. GibbonFACS: A muscle based coding system for the hylobatids. *International Journal of Primatology*, 33(4), pp.809–821.

Waller, B.M. et al., 2013. Paedomorphic facial expressions give dogs a selective advantage. *PloS one*, 8(12), p.e82686.

Weary, D.M. et al., 2006. Identifying and preventing pain in animals. *Applied Animal Behaviour Science*, 100(1-2), pp.64–76. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168159106001134 [Accessed August 28, 2014].

Williams, A.C.D.C., 2002. Facial expression of pain: an evolutionary account. *The Behavioral and brain sciences*, 25, pp.439–455; discussion 455–488.

Wolfensohn, S.E. & Lloyd, M.H., 2003. *Handbook of Laboratory Animal Management and Welfare* Third., Oxford: Blackwell Publishing.

Wright-Williams, L. et al., 2007. Effects of vasectomy surgery and meloxicam treatment on faecal corticosterone levels and behaviour in two strains of laboratory mouse. *Pain*, 130, pp.108–118.

Yamane, A., Yuiti, O.N. & Do, T., 1994. Home Range Size and Spacing Pattern of a Feral Cat Population on a Small Island. *J. Mamm. Soc. Japan*, 19(1), pp.9–20.

Yeates, J., 2010. What can pest management learn from laboratory animal ethics? *Pest Management Science*, 66(November 2009), pp.231–237.

Zurlo, J., Rudacille, D. & Goldberg, A.M., 1996. *The Three Rs : The Way Forward*,

APPENDIX

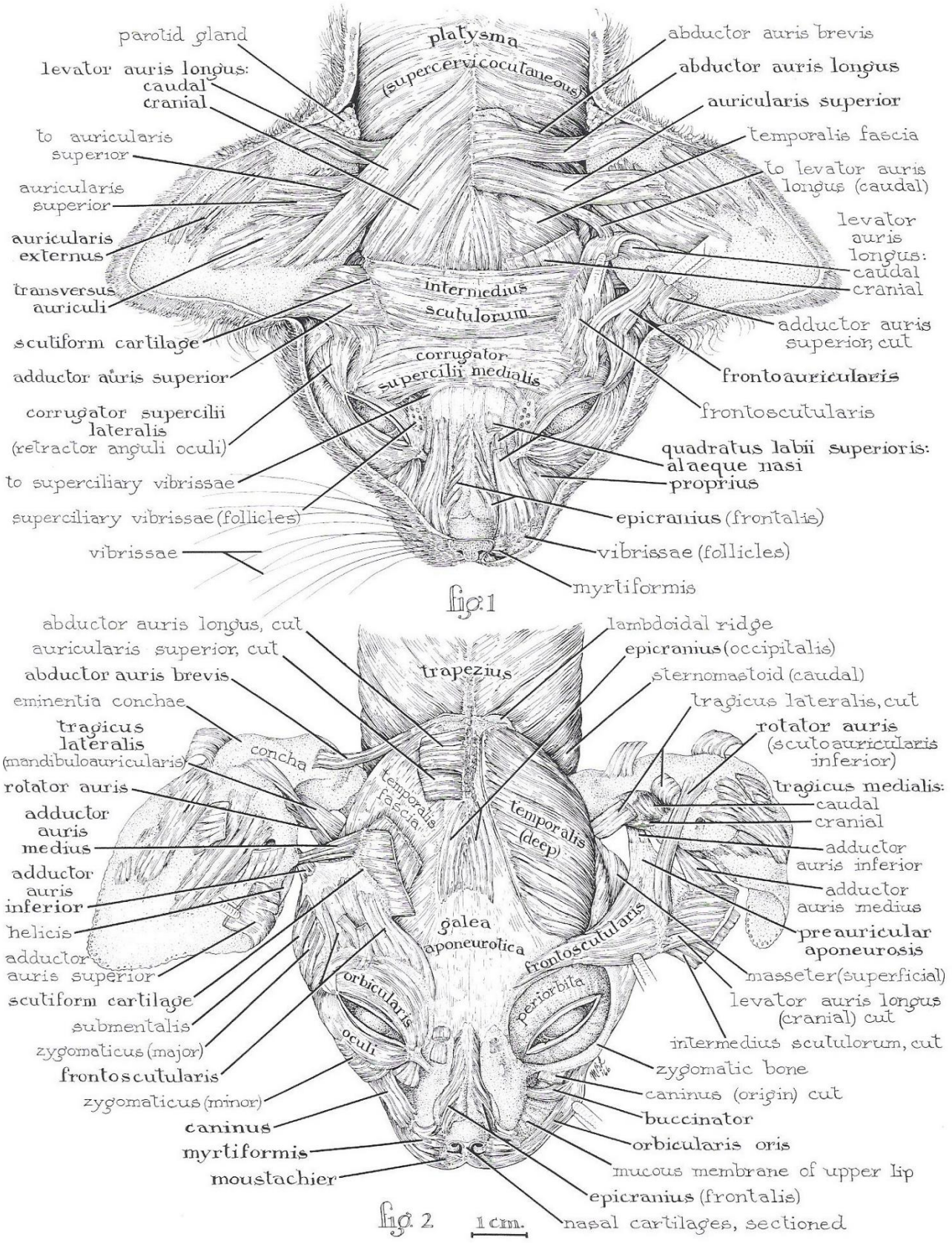


Figure 15. Anatomical drawings of the cat from the *Text-Atlas of Cat Anatomy* by James E. Crouch.