Institut für Labortierkunde
der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. Kurt Bürki

Arbeit unter wissenschaftlicher Betreuung von
PD Dr. med. vet. Margarete Arras

Assessment of post-operative pain by nest complexity scoring in mice

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Thea Fleischmann

Tierärztin
von Nürnberg, Deutschland

genehmigt auf Antrag von

Prof. Dr. Kurt Bürki, Referent
Prof. Dr. Rolf Graf, Korreferent

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ABSTRACT

Pain in laboratory mice is difficult to detect with conventional methods as mice do not obviously show symptoms of mild to moderate pain. Here we investigated the feasibility and reliability of nest building performance under various conditions as a method to detect mild to moderate post-operative pain in laboratory mice and aimed to standardise this method for the routine use.

Female mice were randomly allocated into control (anaesthesia +/- analgesia) and surgery groups (minor laparotomy +/- analgesia) in two housing conditions. Animals were observed before (baseline) and after treatments (experiment). The nests were scored at seven time points with a numeric scoring system and latency of nest building was measured as well as consumption of the nesting material.

Baseline nest scores were always higher than experimental scores and a reliable discrimination was found at three to nine hours after the start of the measurements. A clear graduation in nest complexity was seen after experiments, with higher nest scores in control groups than in surgery groups. Latency in the baseline was always shorter than in the experiment and latency in control groups was shorter than in surgery groups during experimental measurements. Pair housed mice had a slightly higher consumption of the nesting material than individually housed mice.

In conclusion, scoring of nest complexity at three to nine hours after laparotomy was useful to identify post-operative impairment, caused by moderate pain.

1. INTRODUCTION

1.1. Welfare in laboratory animals

In 2008 over twelve million animals were used in the EU states for experimental or scientific purposes such as drug testing or basic and biomedical research. The most used species today are mice and rats, due to the wide range of genetically modified strains availably (Malakoff, 2000). In 2008, 60% of the animals used were mice and a significant increase in these animals in laboratory research has been observed (Kommission, 2010). The growing demand for high-standard animal models, together with an increasing critical view of the use of animals in experimental research led to the development of animal-welfare strategies. In general, welfare of laboratory animals aims at combining a high quality of research with the improvement of the animal's specific needs. Satisfying the environmental needs of laboratory animals, prevention and treatment of distress or pain and providing adequate methods of anaesthesia and analgesia can lead to physiologically and psychologically more stable animals (Baumans, 2005b) and thus supports the validity of the scientific outcome.

A useful approach to the well-being of animals are the "Three R`s", introduced by Burch and Russell in 1959 in their book "The principles of humane experimental technique": Replacement, Reduction and Refinement. These principles are guidelines to reduce the number of laboratory animals, to optimize or refine the techniques used and minimize the amount of distress on the animals or even to replace animals by other testing systems (Burch and Russell, 1959), such as in vitro systems or computer modelling. Following these guidelines animal experiments should only be accomplished when no alternative method is available and when the benefit of the experiments outweighs the suffering of the animal. Scientists who carry out animal experiments are bound by law to limit pain, suffering or damage in the animals used to an indispensable degree (TSchG, 2005). Furthermore scientists also have an ethical responsibility to reduce pain or discomfort in their animals (Foltz and Ullman-Cullere, 1999). This implies that the animals live in an environment as suitable as possible for their species-specific needs. It further implies that pain, suffering or damage must be clearly recognised to be able to minimize or to avoid them.

1.2. Definition of pain and distress

Pain, stress and discomfort during animal housing and experiment obviously have a great impact on the well-being of laboratory animals. The secondary – mostly unrecognized - biological effects like endocrinological or immunological aberrations also can influence experimental study outcomes substantially (Moberg, 1985; Moberg and Mench, 2001). However stress is part of every animal's life and animals have developed biological strategies to cope with stress. Animals in wildlife regularly experience stress when being threatened by a possible predator and fleeing from it. Once the

stressful experience is overcome the animal will return to the prestress condition. When the stress factor is severe and persisting over a longer period of time, e.g. permanent fighting with cage mates and no possibility to escape, the animal's biological functions alter and turn into a state of distress with considerable impact on the well-being (Carstens and Moberg, 2000).

The definition of pain by the International Association for the study of Pain is „pain is an unpleasant sensory and emotional experience associated with actual or potential damage or described in terms of such damages" (LeResche, 2005). As this statement is of limited use in non-verbal animals the working definition of pain by Molony and Kent is "pain is an aversive sensory and emotional experience representing awareness by the animal of damage or threat to the integrity of its tissues. It changes the animal's physiology and behaviour to reduce or avoid the damage, to reduce the likelihood of recurrence and to promote recovery" (Molony and Kent, 1997).

Pain can be physiologic, pathologic or neurogenic; acute or chronic; visceral or somatic. The sensation of pain is caused by noxious stimuli and arises through nerve fibres via nociception to the brain (Liebeskind and Paul, 1977). Acute pain-associated behaviours in animals are withdrawal reflexes or other nocifensive reactions such as escaping, attacking or biting and scratching of the stimulated body part (Carstens and Moberg, 2000). Pain normally serves as a protective function to warn the animal of impending danger and the animal will show an immediate pain response (Baumans et al., 1994).

This normal perception of pain however differs from the emotional and individual feeling of pain or distress an animal might suffer either from an unsatisfactory environment, social stress or pain caused by surgical interventions. Chronic pain normally develops over time, resulting in a persisting sensation and slowly causing distress in the animal. Suffering is the consequence of pain or distress and is reached when pain or distress is of such intensity that it is no longer tolerable for the individual animal (Baumans et al., 1994). "Pain is a complex, subjective and emotional experience" (Vinuela-Fernandez et al., 2007) and objective measurements are required for its assessment.

1.3. Conventional animal models in pain research

Established animal models are based on nociceptive, inflammatory, neuropathic or disease trials. The reaction of an animal to a painful stimulus is used to determine pain sensitivity, hyperalgesia and the efficacy of analgesia. In inflammatory pain models it has been shown that rats react to administered alogenic substances like formalin, capsaicin or mustard oil (Joshi and Honore, 2006) in a paw with lifting, licking and guarding of the injured limb. The tail-flick test is a nociceptive test measuring the pain response and the effectiveness and dose rate of analgesics. A radiant heat from a strong source of light is directed onto the rodent's tail and time is measured

until the animal reflexively flicks away its tail (D`Amour and Smith, 1941). The hot-plate test also measures the pain response and the effectiveness of analgesics by observing the reaction to pain caused by heat (Woolfe and Macdonald, 1944). The animal is placed on a hot plate at 55°C and the time is measured until the animal reacts. End-points are forepaw or hind-paw licking, lifting the feet away from the hot plate or so called escape jumping (the animal jumping upwards and away from the heat source).

Numerous environmental factors like welfare and housing enrichment or laboratory environment and procedures also have a great influence on the pain sensitivity in animals and can therefore alter the pain reaction in analgesiometric tests (Baumans et al., 1994; Chesler et al., 2002). Furthermore nociceptive thresholds can vary in laboratory mice depending on the physiological or behavioural state (Callahan et al., 2008) and in one study it could be found that inducing pain in a mouse altered the pain sensitivity of a cage mate that observed the manipulation (Langford et al., 2006). Therefore the same painful treatment may cause different reactions in each animal (LASA, 1990).

Although the behavioural response to noxious stimuli can be reliably and objectively scored, the tests rely on simple reflexes or simple innate behaviours only and can hardly be used for the assessment of stress and chronic or persistent pain in clinical situations. It has also to be considered that provoked withdrawal responses measure hypersensitivity rather than pain itself (Mogil, 2009). Acute stimulus-evoked pain can not be compared with the complex sensation of spontaneous or even persistent pain, which an animal might suffer from potentially painful and stressful procedures or in post-surgical conditions.

1.4. Pain assessment

1.4.1. Clinical investigation

Clinical investigation in laboratory mice is mostly based on the examination of outer appearance, posture and spontaneous movements of the animal. Clinical investigation can also include assessment of coat and skin condition, wound healing after surgery and movement or activity. It is the most common method for assessing the general condition of an animal.

Locomotion in the home cage can give an insight on possible lameness, injury, ataxia or any other changes in gait. In constitutive studies Liles & Flecknell examined the depressant effects of surgery on locomotion, water and food consumption and body weight in rats (Flecknell and Liles, 1991; Liles and Flecknell, 1993). Overall activity usually decreases with pain, but pacing and restlessness may also indicate pain.

Further investigation contains physiologic parameters like body weight, food and water consumption, body temperature, heart rate and respiratory rate and blood pressure. These components can also give an impression on the general condition (Hawkins, 2002). Objective measures like heart rate, respiratory rate and body temperature alone might be unreliable in the assessment of pain (Conzemius et al., 1997), as they are influenced by many other factors such as stress. Arras et al. could show with telemetric recording that surgical treatment without pain relief affected the heart rate and heart rate variability in mice for 24 hours (Arras et al., 2007).

In summary clinical investigation is the primary method for obtaining an overview of an animal's general condition and health status. For the detection of pain clinical signs can be useful additional parameters in connection with further, more specific assessment methods.

1.4.2. Biochemical Parameters

Biochemical signs such as alterations in blood chemistry or increase in corticosteroids and catecholamines could also be used to assess an animal`s general condition. Corticosteroids and their metabolites can be detected in blood, feces or urine and it is suggested that a rise in corticosteroids (especially glucocorticoids) signifies pain.

In a few studies some authors used corticosteroids as an additional parameter for assessing pain in animals (Molony and Kent, 1997; Wright-Williams et al., 2007). However all hormonal factors change in such complex and quick ways, that they are of only poor diagnostic value, as the correlation with pain is highly imprecise. As sample taking is known to be a stressful procedure itself (Flecknell et al., 2007), a rise in corticosteroids therefore not necessarily means that the animal is in pain, but rather is experiencing stress during the handling and changes in it environment.

Hence, biochemical parameters may be helpful when integrated into a pain scoring system, but are of limited use when used alone as an indicator of pain severity (Flecknell, 1999).

1.4.3. Ultrasonic and audible vocalization

Various species, including rodents, audibly vocalize but also emit ultrasonic frequencies above the human hearing range. Vocalization has been observed during animal pain models, suggesting that acute pain may trigger some ultrasonic or audible calls.

Rats with induced arthritis did not show spontaneous ultrasonic vocalization in a chronic pain model (Jourdan et al., 2002). In one study neither audible nor ultrasonic vocalizations provided a reliable tool for the assessment of acute pain in laboratory mice (Williams et al., 2008). Also results in a study with non-human primates showed

that vocalization is not an appropriate measure of pain and hyperalgesia (Cooper and Vierck, 1986).

Despite the fact that stimulus-evoked ultrasonic vocalizations have successfully been recorded for testing hypersensitivity in rats (Han et al., 2005), it is not a sensitive method for assessing pain, because they are also emitted in non-painful situations e.g. general communication, mating etc. Many animals communicate verbally and no study could so far demonstrate reliable vocalization in animals with persistent or chronic pain. Vocalization in general seems not specific to pain or stress nor is it obvious that animals in pain do necessarily vocalize.

1.4.4. Self-administration of analgesics

Laboratory animals can be trained to self-administer drugs in drinking bottles or in special food jellies. This seems a good and objective method for the assessment of acute post-operative pain in animals.

Rats with adjuvant-induced, chronic arthritis have been shown to develop a preference for anti-arthritic medication and to self-administer non-steroidal, anti-inflammatory drugs (NSAIDs) or opiates (Colpaert, 1987; Colpaert et al., 1980; Colpaert et al., 1982). Similarly, a study assessing the need for pain relief during post-operative recovery showed that mice self-administered themselves more of the analgesic Ibuprofen after surgery than mice from control groups (Pham et al., 2010).

Implementation of these models in laboratories however requires a long conditioning period and as laboratory animals tend to have a decreased food and water consumption post-operatively, efficacious blood levels of the analgesics are hard to achieve. Opiates are also known for their quick addictive effect and it is therefore difficult to differentiate between animals in pain and animals with a higher consumption rate because of addiction.

1.4.5. Behavioural pain assessment

The behaviour usually is assessed from a distance in the animal's home cage without disturbing the animal or after provoking a reaction, e.g. handling or placing the animal in a new environment. The assessment can contain observation of social interaction or the exploratory and species-specific spontaneous behavioural repertoire of an animal. Mice as social animals feel most comfortable when living in groups and it is known that social housing can positively influence post-operative recovery and the response to stress (Pham et al., 2010; Van Loo et al., 2007). The behavioural repertoire of mice is ranging from sleeping to grooming, exploring, interaction with cage mates, digging, nest building etc.

A study of Lloyd et al. revealed that most laboratories rather use subjective measures like general condition and spontaneous or provoked behaviour for the assessment of pain in their animals (Lloyd and Thornton, 2000). Any changes in general (e.g. neglected grooming) or social behaviour can be reactions to pain or stress in animals. Rat behaviour (e.g. twitching, staggering) has been analysed successfully for assessing post-operative pain and estimating severity and duration of post-laparotomy pain (Roughan and Flecknell, 2001).

In an interesting study, veterinarians, who were handling and observing laparotomized rats, could not distinguish whether animals had been treated with saline only or analgesics (Roughan and Flecknell, 2003). Therefore laboratory staff and researchers need to be familiar with the normal behavioural repertoire and characteristics of the animal species in order to recognize and assess any alterations. This might be very difficult as each animal, even within the same species, reacts individually and uniquely (Baumans et al., 1994). Subtle changes in the behaviour of an individual animal may also be difficult to observe in group housed animals. Furthermore behavioural assessment of animals living in opaque cages on large racks often can only be accomplished by moving the cage and disturbing the animals within (Kohn et al., 2007).

It could be shown that the most objective way for the assessment of pain in animals is conducted by measuring behavioural and physiological changes (Baumans et al., 1994; Carstens and Moberg, 2000; Flecknell, 1994). The refinement of the techniques for better assessment of behavioural signs and clinical symptoms – possibly related to pain - is considered a new approach in pain assessment.

Recently a mouse grimace scale was developed by Langford et al.. They introduced a coding system of facial expressions to detect pain or stress in mice. For the evaluation the mouse was placed in a small Perspex chamber and facial expression was observed before and after administering a painful stimulus (Langford et al., 2010). As even handling and restraining causes stress in mice, new approaches are based on behavioural assessment in a non-invasive manner without interfering with the animal.

A novel method is the automated behavioural analysis for collecting data in a standardized and objective manner. Data is collected by video recording and analysed by behaviour-recognition software. This method is considered to be as effective as manual scoring and the system could recognize altered activity levels between surgery and non-surgery groups (Miller et al., 2011; Roughan and Flecknell, 2003). However, a clear identification of pain or the effect of pain killers (e.g. with differences between treated and non-treated groups) has not yet been shown with such commercially available systems.

In a study, burrowing as a spontaneous and highly motivated behavior could be found as an indicator for mild to moderate pain after laparotomy in mice (Jirkof et al.,

2010). Arras et al. found that mice without analgesic treatment post-operatively destroyed their nests and had an unstructured cage area for the following days. Three days after the operation the territory was structured and nests were built again. Simultaneous telemetric measurements could provide evidence that this behaviour could be an expression of distress or pain (Arras et al., 2007).

Most methods for the assessment of pain in laboratory mice like clinical investigation, or general behavioural monitoring, remain unsatisfactory as they are considered too imprecise and assessment is unreliable due to subjectivity between different observers. Species-specific behaviours like hoarding, burrowing or nest building presumably are considered an appropriate method for the assessment of animal welfare and therefore can lead to conclusions regarding the presence of discomfort or pain in the animal's life (Deacon, 2012).

1.5. Pain score systems

Pain rating scales have been established for grading the degree of experienced pain in humans. These scales are descriptive and rate the estimated intensity of pain. A visual analogue scale (VAS) is a one-dimensional scale and consists of a line between two points standing for "no pain" and "worst pain possible". The observer or the patient itself places a mark on the line to indicate the amount of pain the patient believes to be suffering (Flecknell, 1994). Further score systems are the numerical writing scale (NRS), the simple descriptive scale (SDS) and a multifactorial pain scale (MPS). Contrary to numeric or descriptive scales, on binary score systems, clinical signs are simply marked as present or absent. Human self-ratings are generally considered a reasonably reliable tool for pain assessment (Price et al., 1983).

More complex pain scoring systems combine the assessment of the severity of physiological and behavioural changes associated with pain. In human infants and babies that cannot communicate verbally, pain scoring systems have been introduced for better assessment of the degree of pain and the effects of analgesic treatments. The assessment was mainly based on behavioural criteria such as crying, facial expression or posture (McGrath and Unruh, 1987).

The principle of score systems in animals was originally established by Morton and Griffiths. They introduced a system where behaviours and clinical signs associated with pain and distress were assigned numerical scores (usually ranging from zero to five) according to their severity (Morton and Griffiths, 1985). They primarily focused on posture, vocalization, locomotion and changes of physiologic parameters. The authors presented the initial scheme as a prototype that required refinement, acknowledging the difficulties associated with assessing pain in animals. The problems hereby lie in the lack of specific indicators of pain and the subjectivity between different observers (Flecknell, 1994). Over the following years Morton and Griffiths continuously developed a general set of possible observation methods for assessing pain,

e.g. change in body weight, external physical appearance, clinical signs and changes in unprovoked behavior or responses to external stimuli.

More scoring systems have been introduced over the years, e.g. consisting of clinical investigation, physiologic parameters, biochemical changes, facial expression, abnormal activity and response to analgesics (Conzemius et al., 1997). Many of the established pain scoring systems mainly rely on spontaneous behavioural signs and interpretation is very subjective in the individual animal (Flecknell et al., 2007). Another problem is the subjectivity of the assessment system, as each observer tends to estimate signs of pain and distress differently compared to fellow observers. Before adopting a behaviour based scoring system for assessing the degree of pain after certain procedures, the scoring system also should be tested in appropriate control groups to establish the baseline of normal behavior of the animal in a state with no possible pain and stress. Subsequently, experimental groups with no post-operative analgesia or groups with anaesthesia only should be included. Such behavior based scoring systems already have been developed successfully for lambs (Molony and Kent, 1997) and dogs (Firth and Haldane, 1999).

To guarantee the most objective assessment of pain in animals, behavioural and physiological changes, possibly indicating pain, should be combined in a scoring system (Flecknell, 1994). Visual analogue scores, which were based on clinical impression, showed a large variation between different treatment groups and proved to be less accurate than behaviour-based assessment of post-operative pain in rats (Roughan and Flecknell, 2003). The development and refinement of species-specific pain scoring systems seems a continuing process.

1.6. Nest building behaviour in mice

The physiological needs of animals are generally food, water and sleep. Beside those each species also has behavioural needs. For mice they are assumed to be social contact, grooming, exploring, digging or nest building (Baumans, 2005a; Poole, 1998). As these behaviours are performed in the wild and also in captivity, they are considered essential innate behaviours (Baumans, 2005a). According to the innate behaviours nesting material is important for mice and the spontaneous performance of nest building behaviour suggests that nesting material also is a suitable environmental enrichment (Sherwin, 1997). The nest site is used as a sleeping and hiding place and as a place for warming and raising the pups. The nesting material helps keeping up body core temperature (Lynch and Hegmann, 1973). All mice, also male and non-breeding females, regularly build nests and nest building performance is considered to be indicative of good general condition in mice.

In former studies it has been shown that mice will daily build a new nest when the nests are removed repeatedly (Lee, 1973; Lynch, 1977). Several studies have shown that mice are willing to work in order to get nesting material, indicating a high motiva-

tion to perform this behaviour. Mice will pull the nesting material in the cage out of the food hopper or down from the cage lid (Lisk et al., 1969) and they can easily learn to activate some mechanisms such as key pressing (Roper, 1973, 1975). Nest building seems to be such a strong behavioural need that laboratory mice will even overcome aversive obstacles to gain access to nesting material (Sherwin, 1996).

There also seem to be strain differences in nest building in mice (Lee, 1973). As wild mice build dome-shaped and complex nests, C57BL/6J mice are known to build rather flat nests (Hess et al., 2008). Nest building also is affected by a number of maternal and environmental factors. Pregnant or lactating mice build better nests and spend more time for nest building than non-pregnant mice (Bond et al., 2002) and among wild mice pregnant females build the most complex nests (Brown, 1953).

Studies also examined the preferences for nesting material in different mouse strains. Paper, cotton, hay and other natural materials can be easily manipulated and transformed to the mice's needs (Hess et al., 2008; Sherwin, 1997; Van de Weerd et al., 1997). Some mice preferred shredded paper stripes as nesting material (Hess et al., 2008), but most animals seem to combine different kinds of nesting material. This suggests that not only the nature of the nesting material (e.g. paper or cotton) but also the structure (e.g. shredded or as a sheet) plays a role in the choice of preference. Blom showed a preference for shredded filter paper in comparison to smaller particled bedding material (Blom et al., 1996). Beside the preferences for certain materials, as the motivation for nest building behaviour seems to be high, healthy mice will build a nest with any kind of appropriate and available material.

Mice are nocturnal animals and a diurnal rhythm in the behaviour of mice is documented (Aschoff and Meyer-Lohmann, 1954). It was found that the circadian rhythm of nest building is not only constant under light-dark periods but also under constant exposure to a light cycle only (Possidente et al., 1979). Mice are known to repair and rebuild their nest just before dawn (Van Oortmerssen, 1971). The outcome of another study showed that mice build and repair their nests at the beginning, in the middle and at the end of the dark phase as preparation for the light phase when they are sleeping (Roper, 1975).

According to the finding that healthy mice regularly build and modify their nests, any aberration in this natural behaviour could help detecting signs of distress or mild to moderate pain in mice.

2. PROBLEM DESCRIPTION – PAIN ASSESSEMENT IN MICE

Assessing pain in non-verbal creatures is the most challenging task for researchers and staff when working with laboratory animals. To manage pain effectively, it needs to be detected, but identification of pain in animals can be problematic (Stasiak et al., 2003). Each species has its own variety of behaviors for reacting to pain and the pain response also depends on age, sex, environmental or housing factors, health status, genotype and severity of pain (Baumans et al., 1994; Mogil, 2007). As some animals tend to withdraw or become rather passive, some animals tend to become aggressive when in pain. Rodents often react rather passive and become immobile when threatened or in pain (Baumans et al., 1994; UFAW, 1989). Mice usually try to hide any signs of pains, as not to attract themselves to possible enemies (Flecknell, 1999). Animals may react differently and may show a different behavior in a foreign cage or environment than in their home cage and an environment they are used to. Signs of acute pain may be different to signs of chronic pain and the animals may behave completely different again when they are aware that they are being observed. The response to pain is even in the same species highly individual and the same painful treatment may cause different reactions in each animal (Hawkins et al., 2011).

Pain assessment methods are still rather subjective and mainly based on general clinical investigation or short cage-side observations. But assessing the presence and severity of pain correctly can lead to a refined use of analgesics and thus a better alleviation of pain in animals (Flecknell, 1999). As after minor surgical procedures no signs of pain can be observed, analgesia is often withheld or not considered to be necessary (Richardson and Flecknell, 2005). The uncertainty concerning a sensitive assessment and evaluation of pain in mice could also lead to dosage errors as the duration of the analgesic effects are not known exactly.

Symptoms of pain in mice after highly invasive or noxious interventions are obvious: hunching, sunken flanks and neglected grooming. These usually indicate a severely impaired health status and can be easily recognized. But detecting mild to moderate pain is difficult to identify with the standard observations of general condition and general behavioural assessment or by means of clinical investigation. As most standard interventions in research facilities are believed to cause mild to moderate pain, further development of sensitive, reliable and reproducible methods for measuring mild to moderate pain and distress in laboratory animals seems of great importance.

Nest building is a species-specific, highly motivated and spontaneous behaviour of mice. Preliminary studies revealed a correlation of post-surgical pain and nest building performance in laboratory mice (Arras et al., 2007). However, a standardised nest scoring system for the assessment of pain has so far not been developed. Here

we investigated the feasibility and reliability of a quantitative nest complexity score. Under various experimental conditions, the score was evaluated as a method to detect mild to moderate post-operative pain in laboratory mice.

3. AIM OF THE STUDY

The increasing number of mice in research and the growing demand for sophisticated animal models in basic research and testing, has led to animal welfare strategies to combine the high-quality standard of research with the improvement of the animal`s specific needs. Providing good animal welfare and good pain assessment strategies is considered a prerequisite for this.

Pain in laboratory mice is especially difficult to detect with conventional methods as mice do not obviously show symptoms of mild to moderate pain. Nest building in mice is deemed to be a spontaneous, highly motivated behaviour and therefore could be used as a parameter for pain according changes in behaviour.

The aim of the study was to investigate the feasibility and reliability of nest building performances such as nest complexity, latency and consumption of the nesting material as methods to detect mild to moderate post-operative pain in laboratory mice. The impact of mild to moderate pain on the nest building behaviour in mice was evaluated in a clinical situation and was used to differentiate between various treatment groups (surgery with/without analgesia and anaesthesia with/without analgesia).

The outcome of the study should lead to conclusions with regard to the nest building behaviour of the mouse under mild to moderate pain, thus serving as guidance to monitor post-operative pain in mice and to adapt the duration and dosage of analgesia. Finally, this should minimize distress or pain in laboratory mice. To improve animal welfare we aimed at standardising this method for the use in laboratory routine.

4. MATERIALS AND METHODS

4.1. Experimental conditions

4.1.1. Ethics Statement

The animal housing and experimental protocols were approved by the Cantonal Veterinary Office, Zurich, Switzerland, under license number ZH 120/2008 and were in accordance with Swiss Animal Protection Law. Housing and experimental procedures also conform to the Directive 2010/63 EU of the European parliament and of the Council of 22 September 2010 on the *protection of animals used for scientific purposes* and to the *Guide for the Care and Use of Laboratory Animals* (eighth edition, National Academy of Sciences, 2011).

4.1.2. Animals

Female C57BL/6J mice, two to seven months old, were used in the studies. The mice were received from a commercial supplier (Harlan, Horst, The Netherlands) and from our in-house breeding facility.

4.1.3. Health Monitoring

Animals' health status was monitored by a health surveillance program according to FELASA guidelines throughout the experiments. The mice were free of all viral, bacterial, and parasitic pathogens listed in FELASA recommendations, except for Helicobacter species (Nicklas et al., 2002).

4.1.4. Housing environment

Data were acquired in the same room, where the animals were housed. The animal room provided standardised housing conditions with a mean room temperature of 21 ± 1°C and with a relative humidity of 50 ± 5% and 15 complete changes of filtered air per hour (HEPA H 14 filter). The room was insulated against electronic or other noise. Visitors or other experimental procedures were not allowed during the study. The light / dark cycle in the room was a 12 / 12 hours cycle (lights on at 15:00, lights off at 03:00) with artificial light at approximately 40 Lux in the cages.

All animals were kept in Eurostandard Type III open-top and clear-transparent plastic cages (425 mm × 266 mm × 155 mm, floor area 820 cm; Techniplast, Indulab, Gams, Switzerland) with autoclaved dust-free sawdust bedding (80 to 90 g per cage; LTE E-001 Abedd, Indulab). The animals had unrestricted access to sterilized drinking water and ad libitum access to pellets in the food hopper (Kliba No. 3436, Provimi Kliba,

Kaiseraugust, Switzerland). A standard cardbox house (Ketchum Manufacturing, Brockville, Canada) was provided as a shelter, except during the experiments.

Mice were housed in groups of four to eight animals prior to the experiments. During the experiments mice were housed either individually or in pairs (i.e. groups of two females). Pair housed mice were living together for at least three weeks before the experiments begun and remained together during the experiments. Throughout the experiments the mice were living in the same cage (i.e. their home cage); this cage was not cleaned and bedding was not changed during the running experiments.

4.1.5. Nesting material

As nesting material one nestlet per cage (about 5 cm x 5 cm) consisting of pressed cotton fibres was used (Indulab AG, Gams, Switzerland).

4.1.6. Technical equipment

The animal room was equipped with four standard video cameras, suspended over four cages. The video recordings were analysed with the software ObserverXT® 9 (Noldus, Wageningen, Netherlands).

4.1.7. Experimental setup

Prior to the experiments all mice had an adaptation phase of three days. Therefore, one female mouse or a pair (i.e. two females) were placed in a new cage and allowed to habituate for three days. The cage was equipped with clean sawdust-bedding. The cardbox house was omitted to have a better view of the animals during the experiments. At the start of the adaptation phase, one nestlet was placed in the top left corner of the cage. The nestlet was not removed during the adaptation period to give the animals the chance to get used to the nesting material. All necessary husbandry and management procedures were conducted in the room before the adaptation phase.

4.2. Experiment: pilot study

Goal: Determination of the normal circadian rhythm of nest building in order to define time points for nest complexity scoring.

The pilot study consisted of a 24 hour video analysis of naive mice in their home cage for gaining detailed information about the normal time course of construction and destruction of the nest. Forty-eight C57BL/6J mice were used, 16 individually and 32 pair housed mice. After the adaptation phase the old nesting material was removed from the home cage at 15:00 and a new nestlet was placed in the top left corner of the cage. The cage was video recorded for 24 hours with an infrared-

sensitive camera fixed above the cage. At the end of the pilot study 24 hours video sequences of 16 individually housed mice and 32 pair housed mice were analysed.

Nest building was defined as occupation with the nestlet for more than three seconds and was recorded as nest building duration in seconds. Finally, seven time points, where nest building activity was high or where most mice had finished an intense phase of nest building activity and presented a nest, were chosen as measurement points for the main study.

4.3. Experiment: main study

Goal: Nest complexity scoring, latency of nest building and consumption of nesting material under two different housing conditions (individual and pair housing) in combination with various treatments (anaesthesia with/without analgesia and surgery with/without analgesia).

4.3.1. Treatment groups

In total 112 C57BL/6J female mice were used, 48 mice in single housing and 32 pairs (i.e. 64 mice) were tested. The 48 individually housed mice were randomly allocated into six treatment groups and each group consisted of eight mice. In each experimental trial four animals of the same treatment group were tested. The 32 pairs were randomly allocated into four treatment groups. Each group consisted of eight pairs of mice. In each experimental trial two pairs of an anaesthesia group and two pairs of a surgery group were tested.

In individually housed mice three treatment groups served as control groups which underwent anaesthesia only (A), anaesthesia with low dose analgesia (A+5mg) or with high dose analgesia (A+50mg). The three surgery groups underwent anaesthesia and laparotomy without analgesia (S), with analgesia low dose (S+5mg) or with high dose analgesia (S+50mg). In pair housed mice the same regimen was conducted but only with one dose of analgesia (50 mg/kg). All treatment groups in individually and pair housed mice are presented in the following table (Table 1).

Table 1. Allocation of mice in the different treatment groups

Control groups	Surgery groups
n=8 (individually housed mice) n=16 (pair housed mice)	n=8 (individually housed mice) n=16 (pair housed mice)
Anaesthesia with analgesic (A+5mg) Premedication carprofen 5 mg/kg (individually housed mice only)	Surgery with analgesic (S+5mg) Premedication carprofen 5 mg/kg (individually housed mice only)
Anaesthesia with analgesic (A+50mg) Premedication carprofen 50 mg/kg	Surgery with analgesic (S+50mg) Premedication carprofen 50 mg/kg
Anaesthesia only (A) Premedication 2 µL/g phosphate buffered saline	Surgery without analgesic (S) Premedication 2 µL/g phosphate buffered saline

4.3.2. Adaptation and baseline measurements

Nest complexity, latency and consumption of nesting material of each animal or pair was tested before (baseline) and after the treatments (experiment). The baseline values served to compensate for inter-individual variation in nest building performance. At the beginning the mouse or the pair was placed in a new cage with a nestlet in the top left corner. After the adaptation phase (three days), the old nesting material was removed from the cage and a new nestlet was weighed and placed in the top left corner at 15:00. The baseline measurements lasted 24 hours, i.e. from 15:00 to 15:00 (Figure 1). During baseline, the nest was scored visually at the seven time points at 18:00, 20:00, 22:00, 24:00, 09:00, 13:00 and 15:00, established from the pilot study. At the end of the baseline (i.e. at 15:00) the remaining nestlet was weighed again to determine the amount of nesting material which was manipulated

or used for nest building by the animal. Latency until first nest building activity was measured by using a descriptive scale at the end of the baseline.

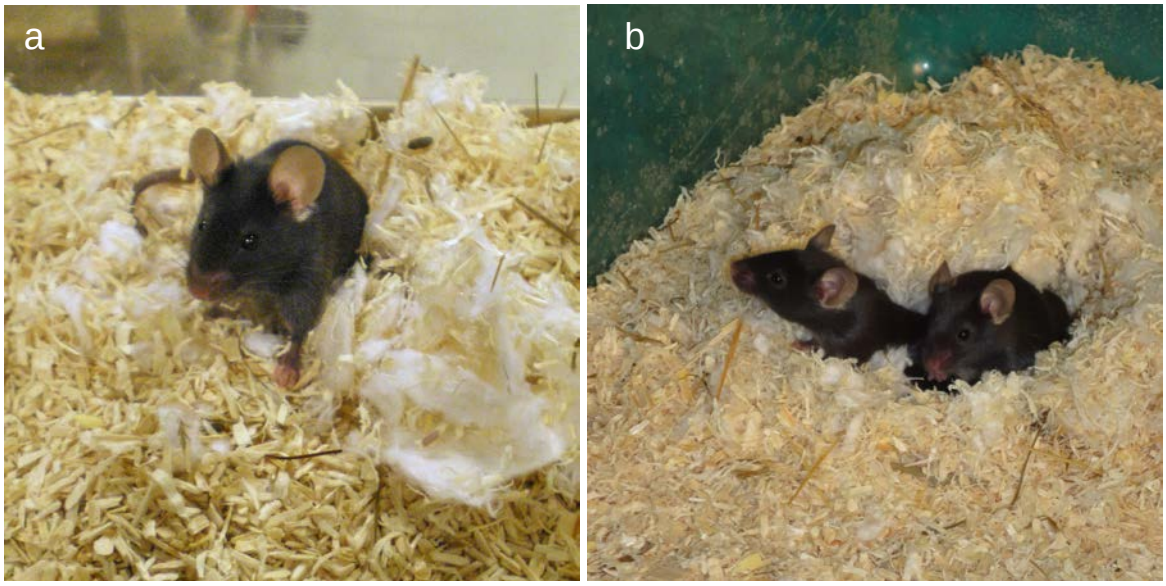

Figure 1: (a) Individually housed mouse and (b) pair housed mice in their nests
Representative appearance at the end of baseline measurements.

4.3.3. Treatments and experimental measurements

Treatments started at 12:30 on the day after the baseline was finished, i.e. approximately 22 hours after baseline measurements were completed. The treatment began with a subcutaneous injection of 2 µL/g body weight of phosphate buffered saline solution (PBS) for the surgery without analgesia (S) and anaesthesia without analgesia (A) groups. In the groups with low dose analgesic (A+5mg and S+5mg) 5 mg/kg body weight of the non-steroidal anti-inflammatory drug carprofen (Rimadyl®, Pfizer Inc., NY, USA) was diluted in PBS and injected as 2 µL/g body weight. In the groups with high dose analgesic (A+50mg and S+50mg) 50 mg/kg body weight of carprofen was diluted in PBS and injected as 2 µL/g body weight.

The injections were performed at 12:30 while animals were in the animal room. One hour after the injection (i.e. at 13:30) the animals were transferred in their home cages to the operation theatre nearby.

Mice were anaesthetised with sevoflurane (Sevorane®, Abbott, Baar, Switzerland) as a mono-anaesthesia. The gas was provided with a rodent inhalation anaesthesia apparatus (Provet, Lyssach, Switzerland) and oxygen was used as a carrier gas. The induction of anaesthesia was carried out in a Perspex induction chamber with 8% sevoflurane and 100% oxygen at a flow rate of 600 mL/min (Figure 2a). After the induction animals were placed on a warming mat (Gaymar, TP500, Orchard Park, NY,

USA) with 39 ± 1°C to keep body temperature constant. Anaesthesia was maintained for 15 min through a nose mask at about 6-7% sevoflurane and 100% oxygen at a flow rate of 600 mL/min (Figure 2b).

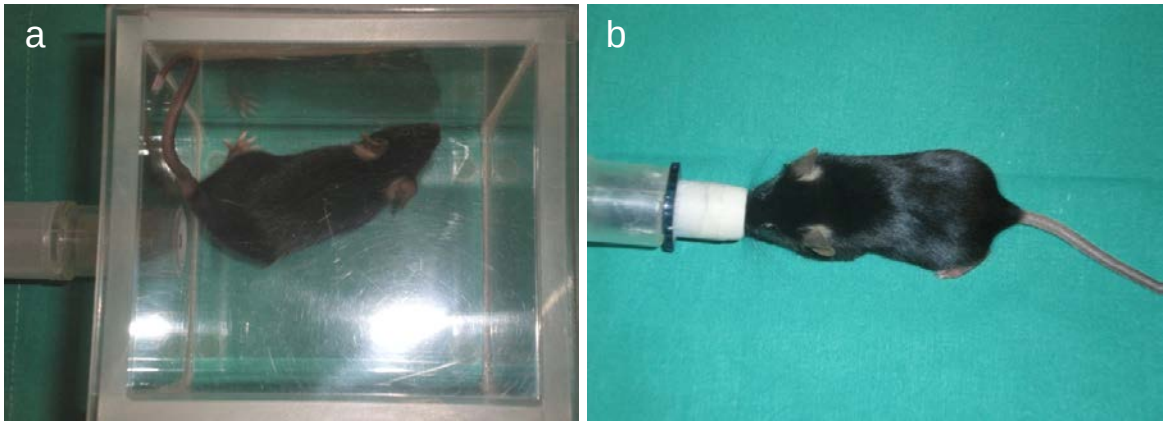

Figure 2: Inhalation anaesthesia with Sevoflurane
(a) Mouse during induction of anaesthesia. The mouse is placed in a Perspex chamber and anaesthetic gas is flowing in.
(b) Mouse during maintenance of anaesthesia. The mouse lies in upright position on the warming mat during the 15 minutes of anaesthesia. Anaesthetic gas is delivered via the nose cone.

Anaesthesia and surgical intervention were standardised beforehand and the procedures were conducted following an already established protocol (Cesarovic et al., 2010). Anaesthesia and surgery were carried out by one person (TF) to minimize side-effects due to different handling or surgical procedure. The surgical procedure had been practiced previously to ensure that exactly the same technique was used and all animals had a quick recovery after surgery.

In mice undergoing surgery during anaesthesia the fur was clipped on the right side of the animal and the operation field was disinfected with ethanol. All mice with surgical intervention underwent a one-side sham embryo transfer, as this method is widely used in laboratory routine. The abdominal muscle wall was closed with two absorbable sutures (Vicryl®, 6/0 polyglactin 910, Ethicon Ltd., Norderstedt, Germany) and the skin was closed with three skin staples (Precise®, 3M Health Care, St Paul, MN, USA). The surgery was completed in all animals within six to eight minutes during the anaesthesia phase.

After the anaesthesia the mice were kept for another 15 minutes on the warming mat for recovery. They were then placed again in their home cage and transferred back to the animal room, where the experimental measurements took place from 15:00 on for the following 24 hours. The old nestlet and pieces of the nestlet were removed

from the cage and a new nestlet was weighed and placed in the top left corner at 15:00 (for detailed time schedule see Figure 3).

Collection of experimental data started 24 hours after the baseline was completed. The nest scoring was carried out by the same investigator throughout the studies (TF). In the dark phase the nests were scored by torchlight only. No other persons were allowed in the animal room to avoid any disturbances during the experiments. The nest scoring took place at the seven specific time points chosen from the results of the pilot study (18:00, 20:00, 22:00, 24:00, 09:00, 13:00, 15:00).

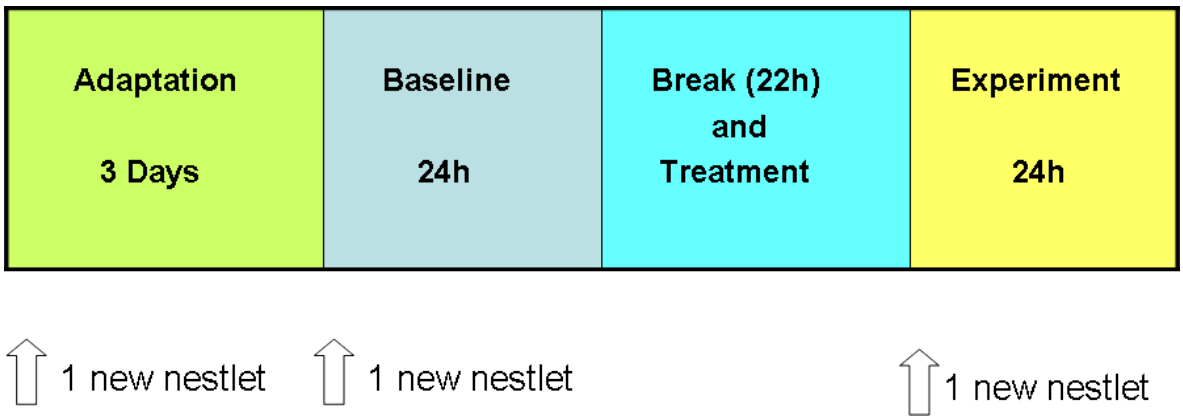

Figure 3: Time schedule during the main study

4.3.4. Nest complexity scoring

A six point scale for rating the quality of the nest was established. During the development process the scoring system published by Deacon (Deacon, 2006) was tried out and his protocol was slightly modified (Table 2). With the present protocol nest complexity can be rated on a moderately wide range with each scoring point defined as precise as possible (Figure 4). Furthermore a score zero was introduced, when mice had not touched the nesting material at the scoring time points or have just dragged the nestlet around the cage.

Table 2. Scale for nest complexity scoring

Nest complexity scoring was conducted in baseline and experimental measurements.

Score 0	nestlet not touched no shreds torn out of the nestlet nestlet just dragged around the cage
Score 1	nestlet slightly touched more than 80% intact some shreds picked out
Score 2	nestlet notably touched more than 20% shreds picked out shreds spread around in defined area
Score 3	identifiable nest site more than 20% shreds picked out shreds are all spread in defined nest area little hollow in bedding mouse starts building walls
Score 4	flat nest with walls hollow in bedding walls higher than the mouse walls encase the nest up to 50%
Score 5	perfect nest more than 50% shreds picked out bowl-shaped nest walls higher than mouse and encase nest for more than 50%

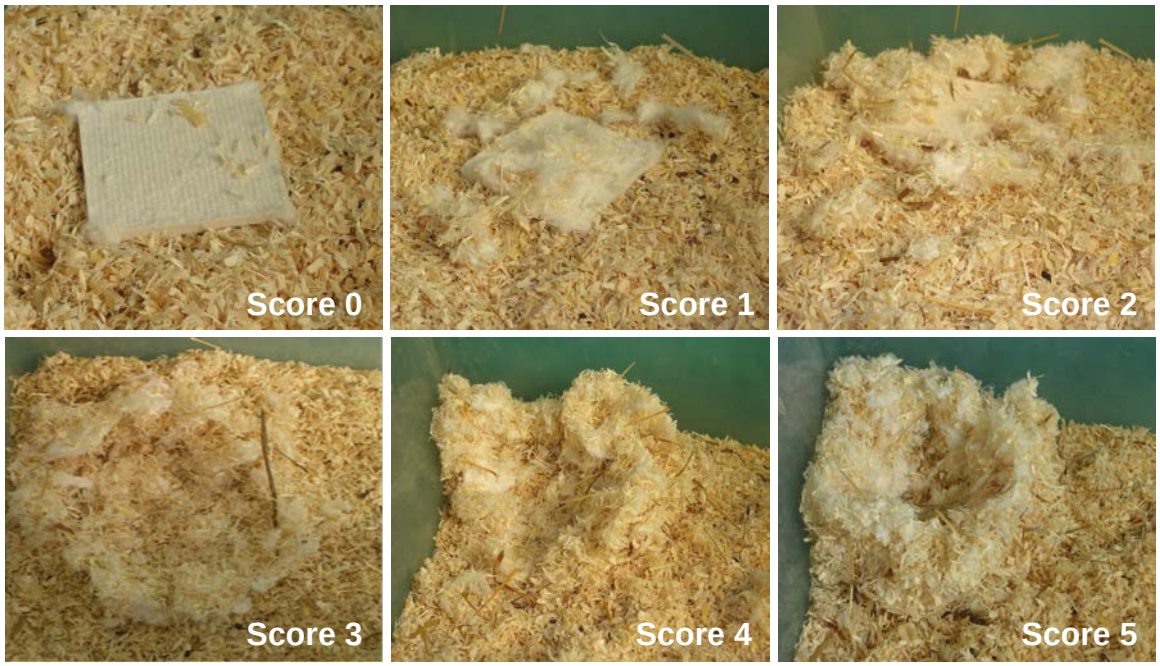

Figure 4: Representative examples of nest scores 0 - 5

4.3.5. Latency of nest building

Latency was defined as the time from the start of the measurements until mice started with nest building activity. Latency of nest building was defined positive when the mouse had started with the manipulation of the nesting material. The data were established at the identical time points when nest complexity scoring took place. As soon as the mouse had reached a nest complexity score above zero latency was defined positive, e.g. when a mouse had started with nest building activity resulting in a nest complexity score ≥1 at the time point 22:00, latency was defined as seven hours, according to seven hours from the start of the measurements (Table 3).

Table 3. Scale for rating latency until first nest building activity

Time points	18:00	20:00	22:00	24:00	09:00	13:00	15:00
Time intervalls	0-3h	3-5h	5-7h	7-9h	9-18h	18-22h	22-24h
Latency: time from start of the measurements (15:00)	3	5	7	9	18	22	24

4.3.6. Consumption of the nesting material

Before placing the nestlet in the cage at the start of the baseline and experimental measurements the nestlet was weighed to the nearest 0.1 g. After the 24 hour measurements the remaining piece of the nestlet was weighed again to the nearest 0.1 g. This method was also adopted from Deacon (Deacon, 2006) to have a better control over the estimated consumption of the nesting material. Only the remaining, intact piece of the nestlet was weighed, whereas any loose or shredded pieces that had been picked out of the nestlet by the mouse, were considered manipulated.

4.4. Statistical Analysis

All statistical analyses were performed with SPSS 20.0 software for windows. Mean (m) and standard deviation (SD) of nest scores were calculated for baseline and experimental measurements of the ten treatment groups. All data was tested for normal distribution and homogeneity of variance (Levene`s test) and all data met the necessary assumptions for parametric analyses.

Comparison of means showed a reliable discrimination between baseline and experimental measurements at three to nine hours after the start of the experiments in all groups. Therefore statistical analysis was concentrated on the time point 22:00 (seven hours after the start of measurements) in baseline and experiment.

Independent t-tests were used for the comparison of individually and pair housed mice. A dependent t-test was used for comparison of nest scores and latency between baseline and experimental measurements at the different time points. To compare nest scores and latency in the different treatments a one way analysis of variance (ANOVA) was used, post hoc testing was conducted with the Tukey test. The effects on nest building performance of two dosages of the analgesia carprofen were tested with an independents t-test. P-values ≤ 0.05 were considered significant.

5. RESULTS

5.1. Pilot study

Investigation of the normal circadian rhythm of nest building in order to define optimal time points for nest complexity scoring. Twenty-four hours video recordings were analysed and several time points for nest complexity scoring were determined.

Analysis of the 24 hours video recording showed that all mice used the offered nesting material for nest building. Three peaks of high nest building activity were found during the 24 hours observation of the circadian rhythmicity in mice (Figure 5).

The first peak was observed immediately after the new nesting material was placed in the cage. All mice were exploring the nestlet by nibbling or tearing some shreds out of it and they already started with some nest building performance.

The second peak of intensive nest building activity took place between the first one to three hours of the daylight phase (15:00 – 03:00). Within 16:00 to 18:00 mice were repeatedly nest building and elaborated a well-built nest for the following sleeping periods during the daylight phase. Completion of a well-built nest was followed by long sleeping phases with short disruptions for locomotion, eating and drinking and short activities for rebuilding or maintaining the nest. With the beginning of the dark phase (start at 03:00) the mice left the nest site and had a long period of locomotion with only short breaks for eating and drinking and occasional resting phases in the nest. In this locomotion phase, the nest was usually destroyed or flattened out in the bedding by constantly running over the nest.

The third peak of high nest building activity was observed from the middle of the dark phase to the start of the next light phase. Mice started to rebuild and restructure their nest and had some longer sleeping phases towards the end of the dark phase.

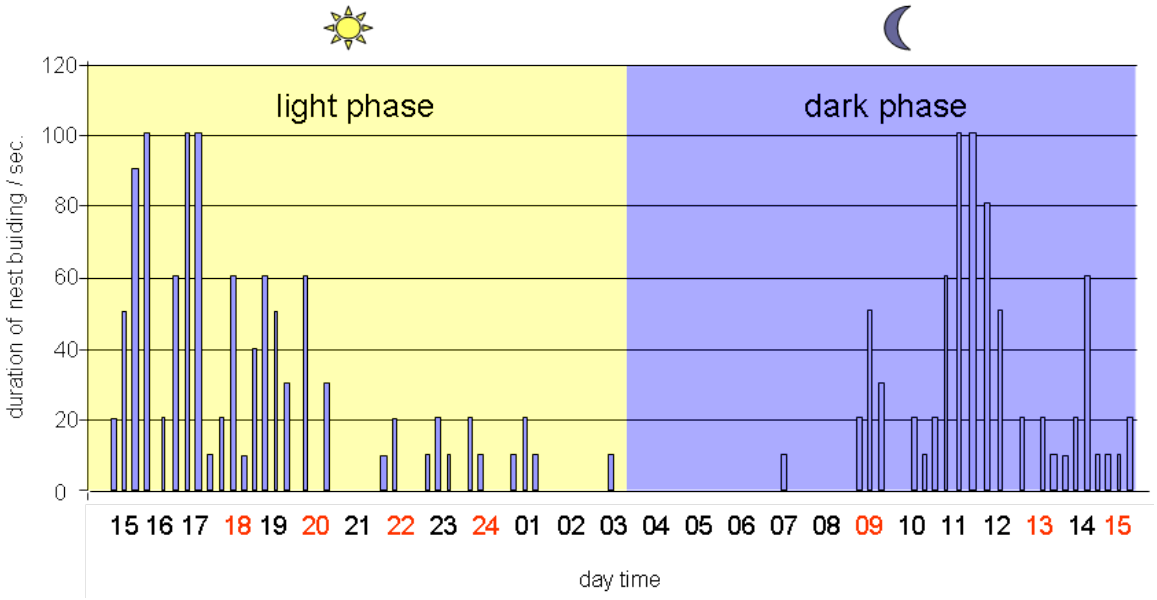

Figure 5: Rhythmicity of nest building activity
Mean values of 16 single and 32 pair housed mice, showing the average nest building activity over a 24 hours course. SD was omitted for better clarity of the figure. Red numbers indicate the seven time points which were chosen for nest complexity scoring during the main study.

The sleeping and nest building periods in the dark phase were altogether not as long as at the beginning of the light phase. From the results of the time point determination study, the following seven time points were selected for nest complexity scoring. At these time points the nest building activity was high or most mice had just finished with nest building and were presenting a nest site (Figure 6).

Day time	18:00	20:00	22:00	24:00	09:00	13:00	15:00
Time from the start of the measurements (start at 15:00)	3h	5h	7h	9h	18h	22h	24h
Light scheme	☀	☀	☀	☀	☾	☾	☾

Figure 6: Time points for nest complexity scoring
The seven time points resulted of the detailed analysis of circadian nest building rhythmicity and were then used in the main study for nest complexity scoring. Four time points lay in the light phase, three time points lay in the dark phase. Corresponding day time and time from the start of the measurements are depicted for each time point.

5.2. Main study

Nest complexity scoring*, latency of nest building and consumption of nesting material were analysed i*n two different housing conditions (individual and pair housing) and under various treatments (anaesthesia with/without analgesia and surgery with/without analgesia).

5.2.1. Nest complexity scoring

In the main study the feasibility of nest complexity scoring as a method to detect mild to moderate pain in mice was investigated. In general all mice used the offered cotton squares for nest building. The highest baseline nest scores were found between 22:00 to 24:00 and 13:00 to 15:00, both periods around the middle of the light and at the end of the dark phase.

The highest difference between baseline and experiment was found between three to nine hours after the start of the measurements, i.e. at 18:00, 20:00, 22:00 and 24:00. After 22 – 24 hours the experimental nest scores increased towards baseline values. The difference between baseline and experimental nest scores was less significant in control groups than in surgery groups (Figure 7).

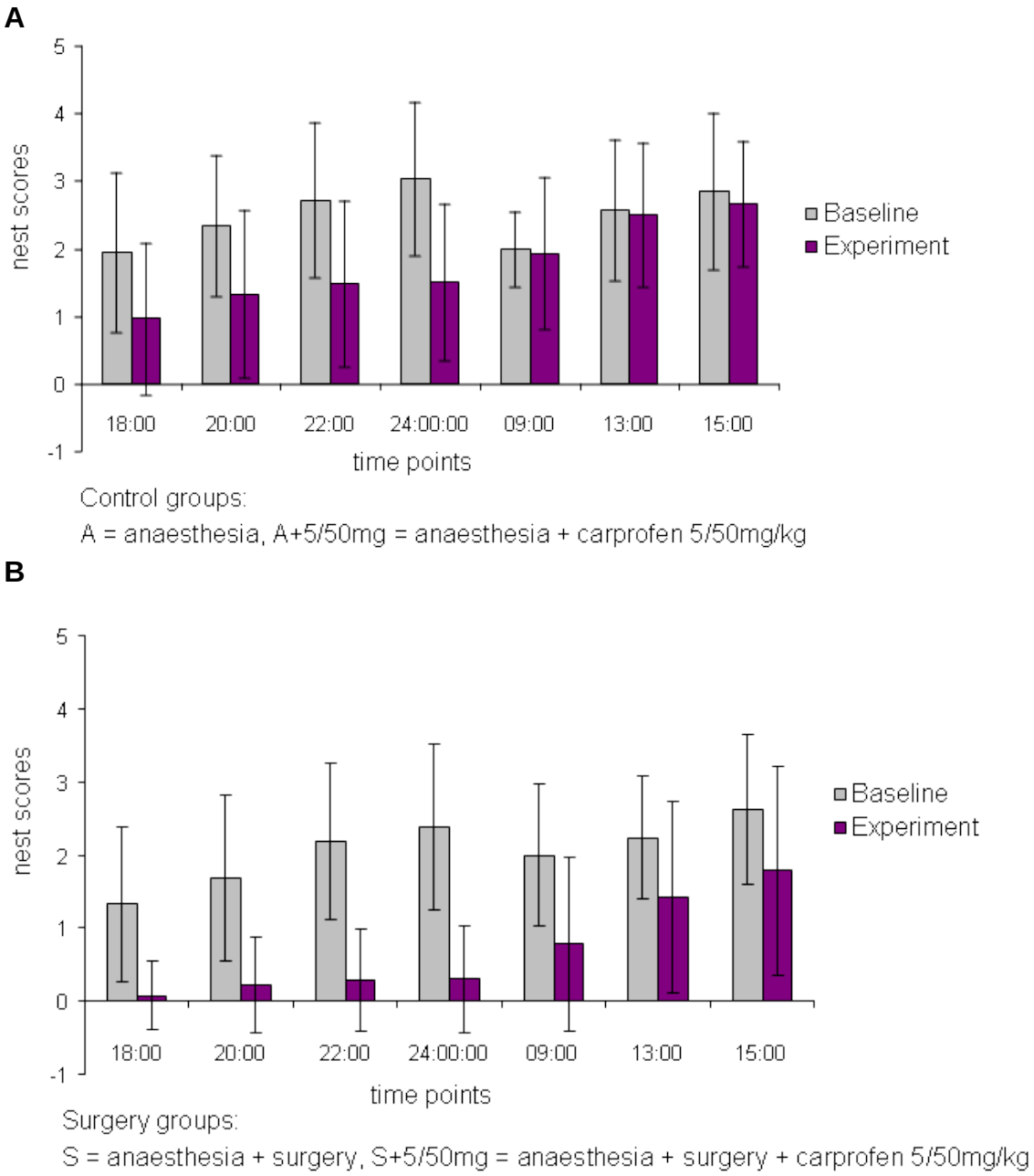

Figure 7: Comparison between baseline and experimental nest scores in (A) control groups and (B) surgery groups
Mean values with standard deviations of nest scores at all time points in individually (n=8/group) and pair (n=16/group) housed mice (n=56).

Nest scores showed to be always higher in the baseline than in the experiment in both housing conditions and in all treatments. A reliable discrimination between baseline and experiment was found in the time period from three to nine hours after the start of the measurements. As the highest difference between baseline and experiment was found between three to nine hours after the start of the measurements, in the following graphics only results at 22:00 (seven hours after the start of the measurements) are shown.

In individually housed mice significant differences between baseline and experimental measurements ($p \leq 0.017$) occurred in all three surgery groups and the anaesthesia group (S: $p=0.001$; S+5mg: $p=0.001$; S+50mg: $p=0.017$; A: $p=0.002$; Figure 8). A clear graduation between treatments could be seen with highest experimental nest scores in control groups, intermediate nest scores in the surgery group with analgesia high dose (S+50mg) and lowest nest scores after surgery with analgesia low dose (S+5mg) and surgery without analgesia (S). Differences in experimental scores were significant between the group surgery without analgesia and anaesthesia with analgesia high dose ($p=0.006$) and also between the group surgery with low dose analgesia (S+5mg) compared to the control group anaesthesia with analgesia high dose ($p=0.009$).

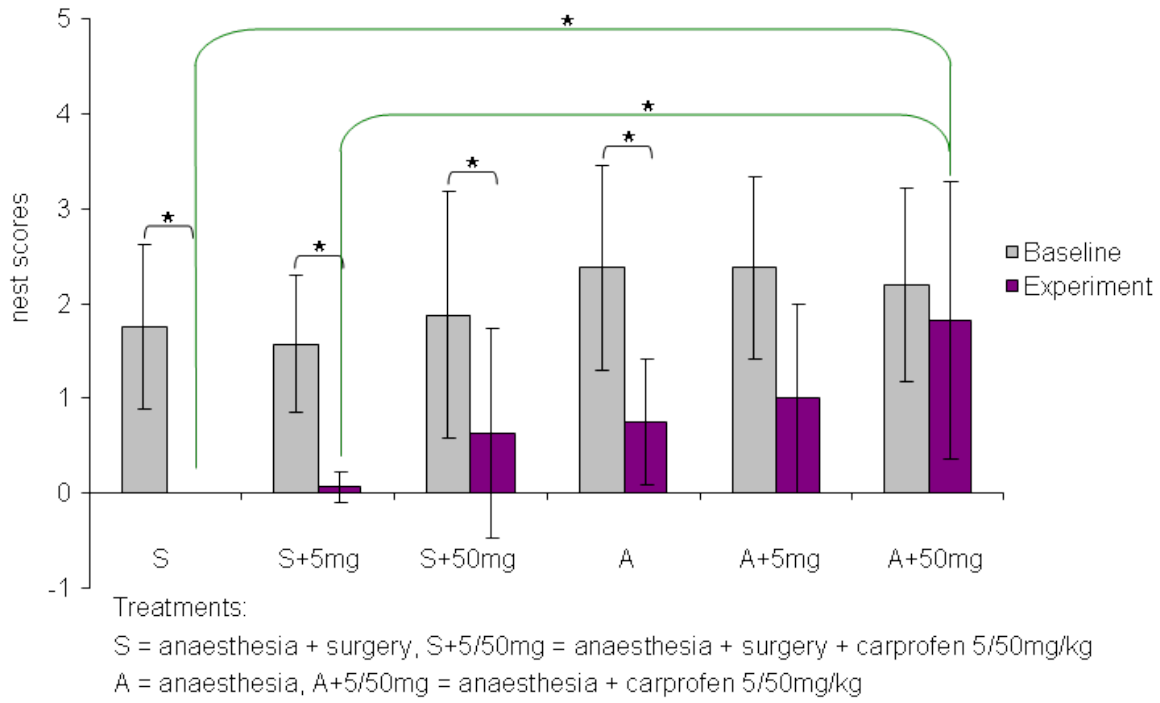

Figure 8: Nest scores in individually housed mice
Mean values and standard deviations of nest scores seven hours after the start of measurements in individually housed mice (n=8/group). Significant differences between baseline and experimental nest scores (marked with black brackets and asterisks) and differences in between experimental nest scores (marked with green brackets and asterisks).

In pair housed mice significant differences between baseline and experimental measurements ($p \leq 0.0001$) occurred in both surgery groups and one anaesthesia group (S: p=0.000; S+50mg: p=0.000; A: p=0.000; Figure 9). Similar to individually housed mice a clear graduation between treatments could be seen with higher experimental nest scores in control groups than in surgery groups. Differences in experimental scores were significant between both surgery groups surgery without analgesia (S) and surgery with high dose analgesia (S+50mg) and both control groups anaesthesia only (p=0.0034) and anaesthesia with analgesia high dose (p=0.025).

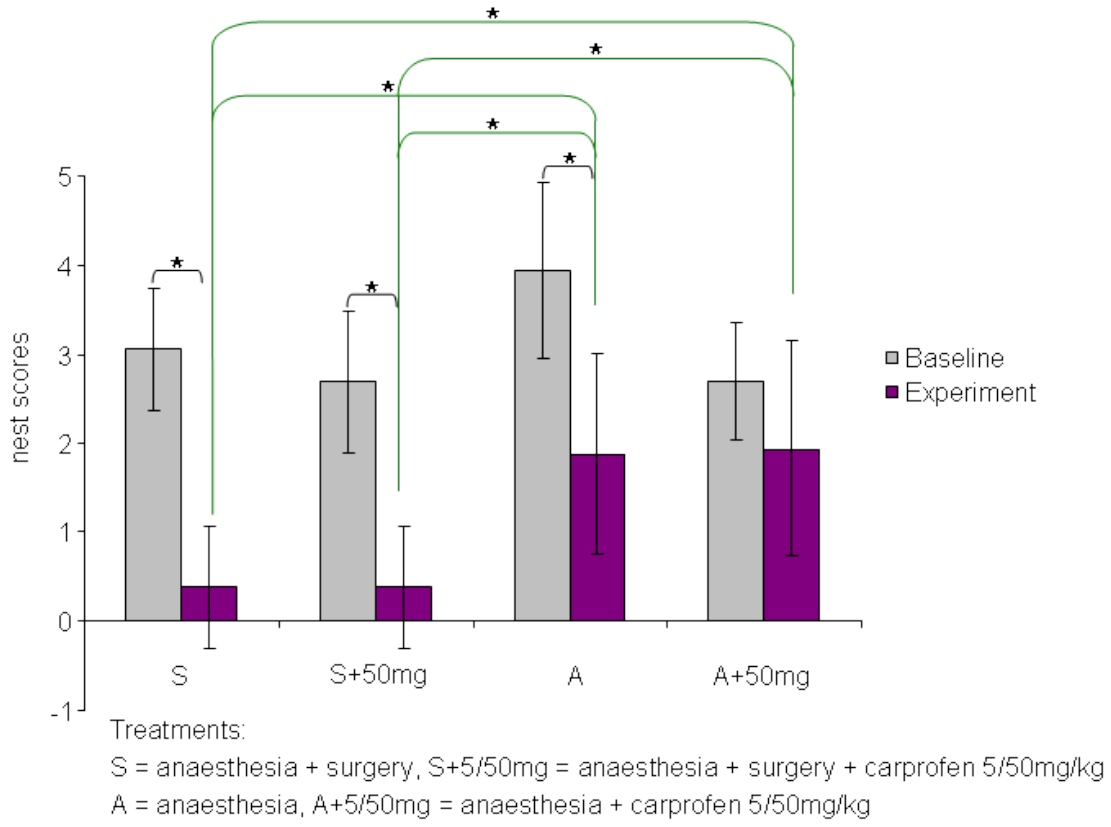

Figure 9: Nest scores in pair housed mice
Mean values and standard deviations of nest scores seven hours after the start of measurements in pair housed mice (n=8 pairs/ group). Significant differences between baseline and experimental nest scores (marked with black brackets and asterisks) and differences in between experimental nest scores (marked with green brackets and asterisks).

Although pair housed mice mostly had slightly higher nest scores (Figure 10), only one relevant difference between the two housing groups could be found in the control group anaesthesia without analgesic (A: p=0.040).

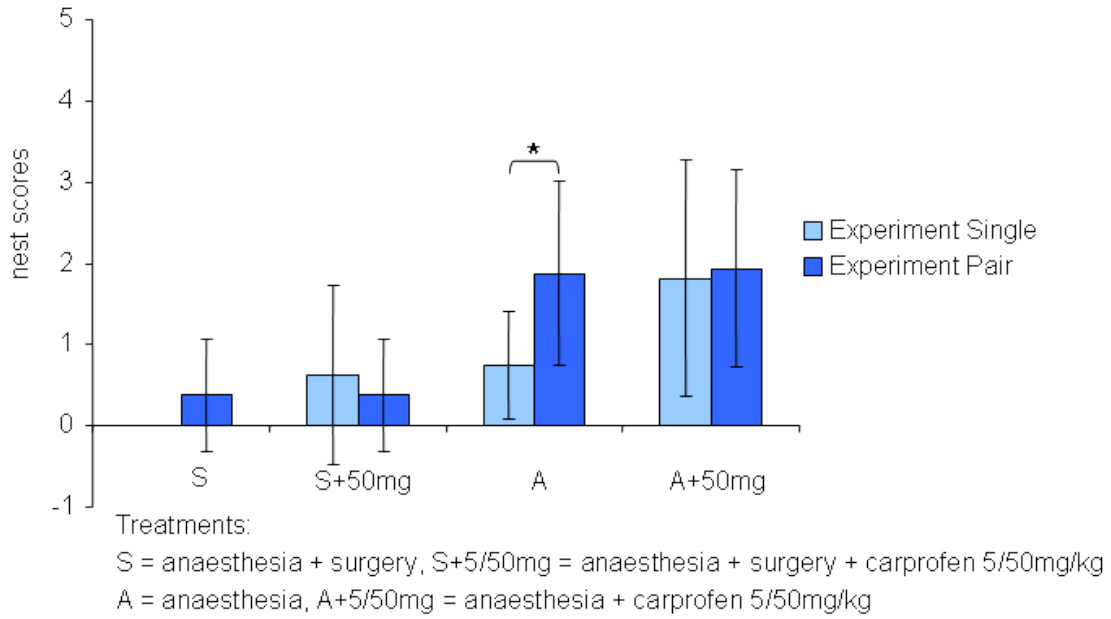

Figure 10: Effects of housing condition on nest scores
Mean values with standard deviation of experimental nest scores in individually and pair housed mice at 22:00. Significant difference is marked with black bracket and asterisk.

5.2.2. Latency of nest building

During baseline measurements more than 90% of the mice showed nest building activity within minutes up to three hours and simultaneously reached nest scores higher than zero at the first time point of nest scoring at 18:00. Pair housed mice always started with the nest building within the first three hours of baseline measurements. In individually housed mice, some mice had a prolonged latency up to seven hours, but the majority of mice also started nest building activity within the first three hours of baseline measurements.

The latency in the baseline was always shorter than in the experiment. The latency during the experiment was always shorter in control than in surgery groups. In the experiment 40% of mice showed nest building activity within the first nine to eighteen hours after the start of measurements. However 60% of mice of both anaesthesia and surgery groups had a delayed latency until up to 18 to 24 hours. No significant differences were found between the housing conditions.

In individually housed mice the latency in the baseline was always shorter than in the experiment (Figure 11). Significant differences between baseline and experimental latency occurred in the groups S+5mg (p=0.003); A+5mg (p=0.028) and A+50mg (p=0.047). During the experiment a slightly shorter latency in control groups and in the surgery group with analgesia high dose (S+50mg) was found compared to the surgery groups with analgesia low dose (S+5mg) or no pain relief (S), however without significant differences.

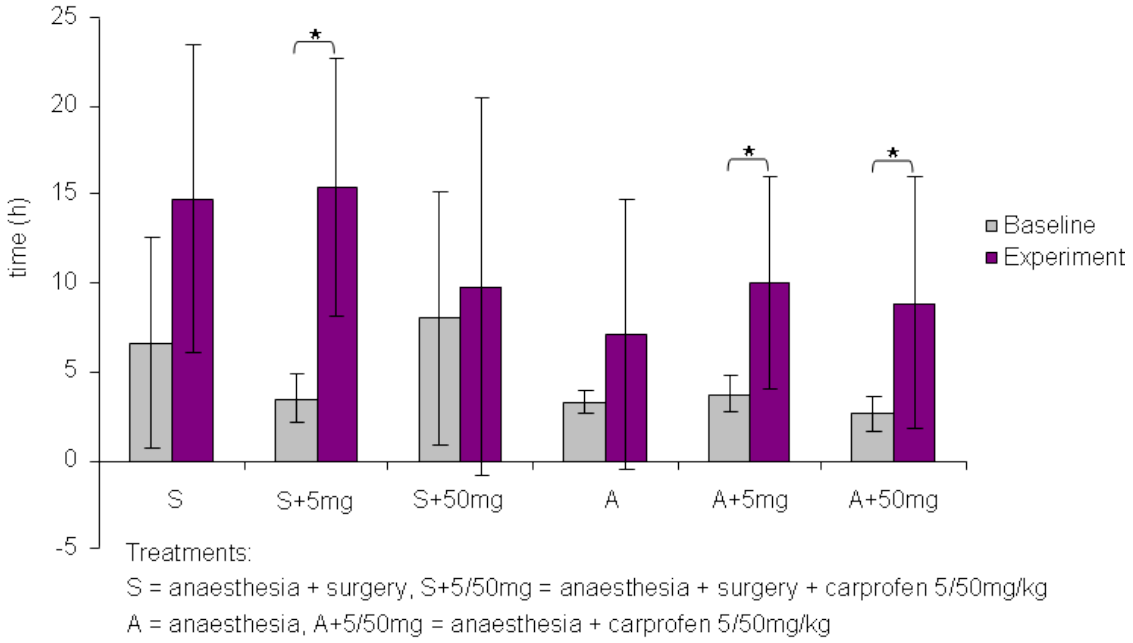

Figure 11: Latency in individually housed mice
Mean values with standard deviation of latency in individually housed mice (n=8/group). Significant differences in latency between baseline and experiment are marked with black brackets and asterisks.

In pair housed mice all mice had a shorter latency in the baseline than in experiment (Figure 12). Significant differences between baseline and experiment were found in both surgery groups S (p=0.001) and S+50mg (p=0.011). During the experiment a clear graduation of the latency could be seen between control and surgery groups. Significant differences occurred between the group surgery without analgesic (S) and the control groups anaesthesia (p=0.015) and anaesthesia with high dose analgesic (p=0.008).

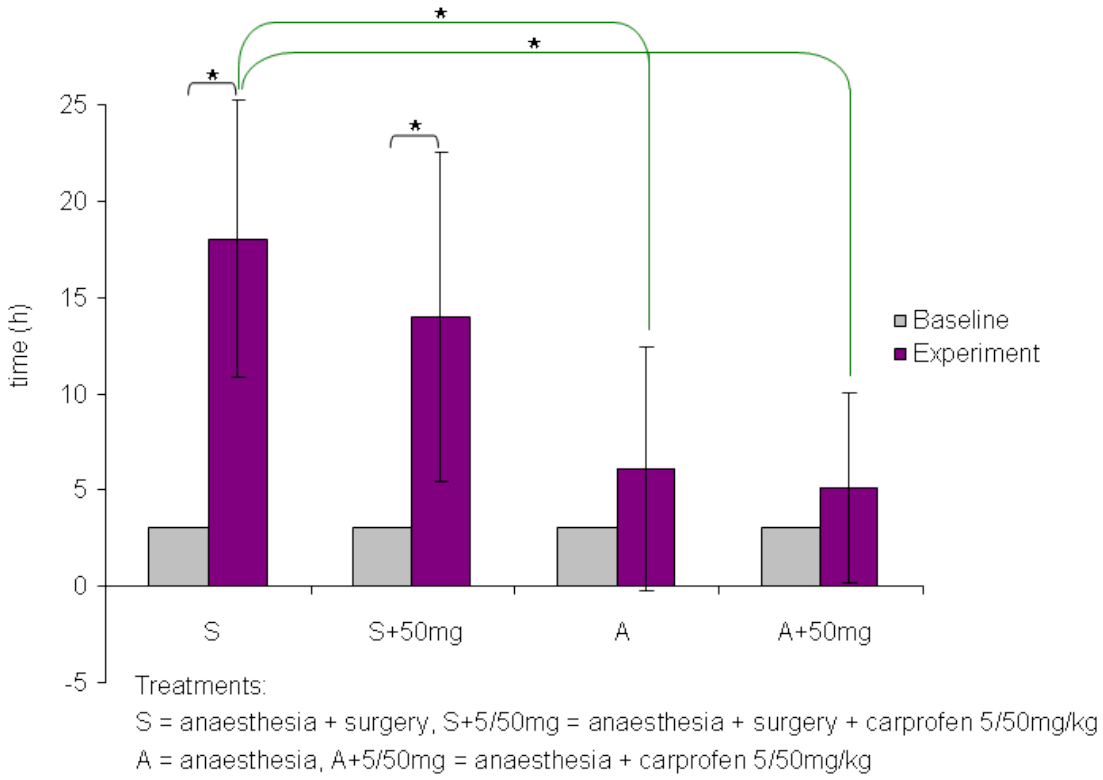

Figure 12: Latency in pair housed mice
Mean values with standard deviation of latency in pair housed mice (n=8 pairs/ group). Significant differences in latency between baseline and experiment (marked with black brackets and asterisks) and differences in between experimental latency (marked with green brackets and asterisks).

In both control groups (A and A+50mg) pair housed mice had a slightly shorter experimental latency than individually housed mice, whereas in both surgery groups (S and S+50mg) individually housed mice had a shorter experimental latency than pair housed mice (Figure 13). Comparing the effect of the two housing conditions on latency, no significant differences could be found.

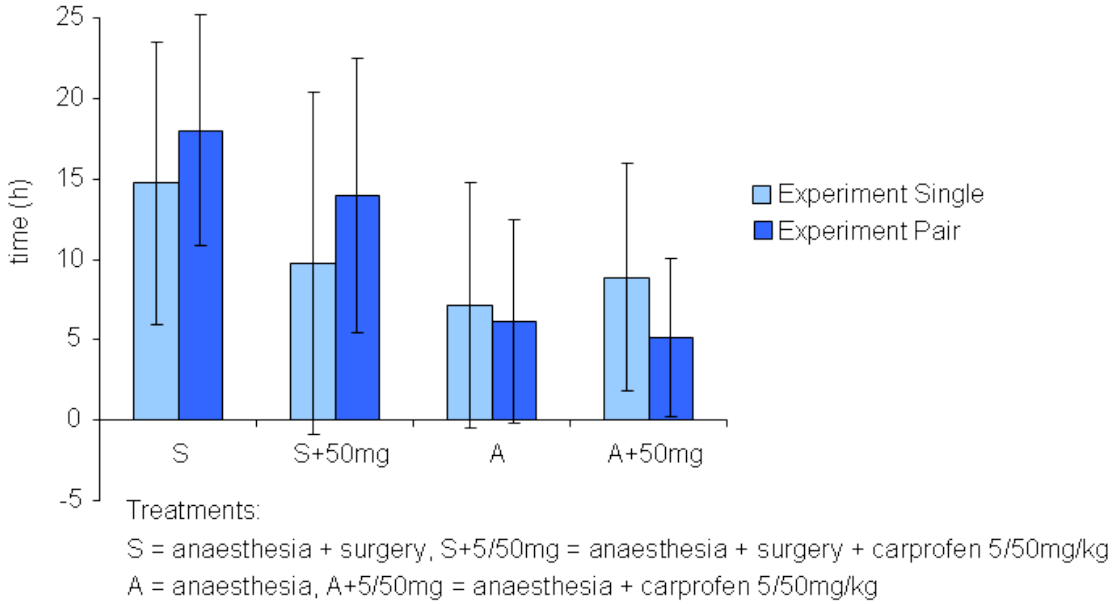

Figure 13: Effects of housing condition on latency
Mean values with standard deviation of latency during experiment in individually and pair housed mice

5.2.3. Consumption of the nesting material

No significant differences in the consumption of the nesting material were found between baseline and experiment in all groups (Figure 14). There also occurred no significant differences between the treatments.

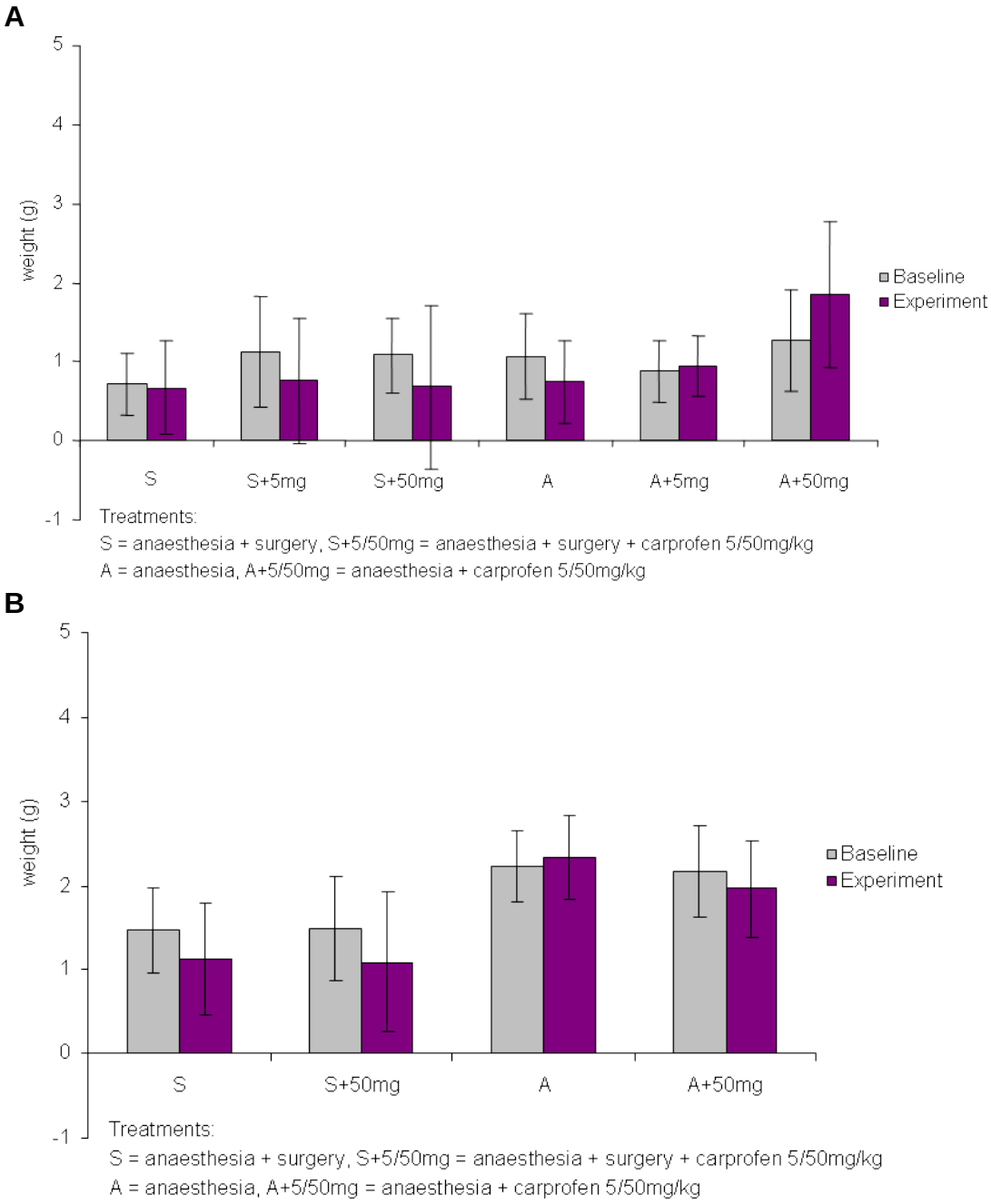

Figure 14: Consumption of nesting material in (A) individually housed mice and (B) pair housed mice
Mean values with standard deviation in individually (n=8/group) and pair housed mice (n=8 pairs/group). Consumption of the nesting material in baseline and experiment and effects of the different treatments on the consumption of the nesting material are presented. No significant differences occurred.

Pair housed mice had a slightly higher consumption of the nesting material than individually housed mice (Figure 15), but only one relevant difference could be found between the two housing conditions (A: p=0.0001).

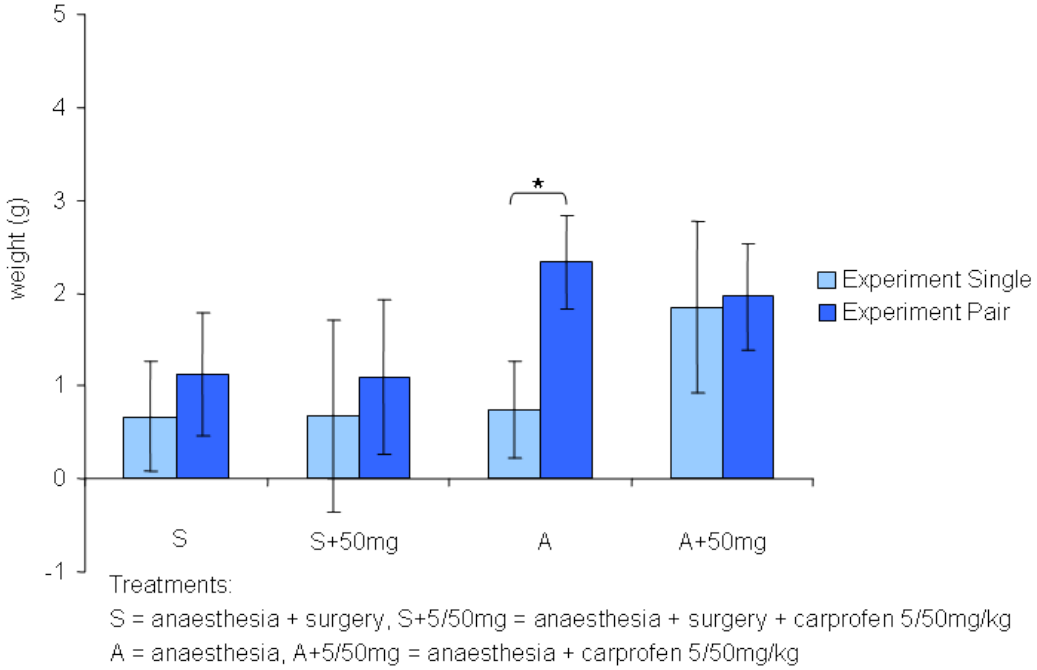

Figure 15: Effects of housing condition on consumption of nesting material

Mean values with standard deviation of the consumption of the nestlet in individually and pair housed mice after experiments. Significant difference in consumption of nesting material is marked with black bracket and asterisks.

6. DISCUSSION

Nest building performance was investigated for its feasibility in the assessment of post-operative pain in a routine laboratory setting. Therefore, in a pilot study, the overall circadian rhythmicity of normal nest building behaviour was determined and a practical procedure for detailed examination of nest building performance was established. Three parameters of nest building performance were used in the main study: nest complexity scoring, latency of nest building, and consumption of nesting material. The validity of these parameters regarding the assessment of post-operative pain was evaluated, with nest complexity scoring being the most reliable method. The procedure and measurements were easy to perform in the animal's home cage, thus, nest complexity scoring was also the most useful method in the laboratory routine.

According to the finding that healthy mice regularly build and modify their nests, any aberration in this natural behaviour could help detecting signs of distress or mild to moderate pain in mice. Nest building in mice can be affected not only by pain, but also by fear, distress, suffering, changes in accommodation or by a generally depressed condition. Mice undergoing surgical intervention without pain relief were seen to have an unstructured cage area with no clear identifiable nests for up to two days (Arras et al., 2007). Deacon could demonstrate that lesions in the hippocampus are inhibiting mice from nest building and he established a protocol for its assessment (Deacon, 2012). Although Deacon used his nest scoring protocol for mice with hippocampus lesions and not for the assessment of pain, we still adopted his protocol. Nest building in mice as a strong behavioural need (Baumans, 2005a) can also be used for the assessment of general condition and also the effects of pain on nest building could be seen in a study (Arras et al., 2007). As we wanted to analyse the effects of post-operative pain on nest building performance in detail, the following treatments and methods were established for the study: measurements were sampled of all animals before the different treatments to gain baseline values. We used control groups to compare between the effect of anaesthesia only and the effect of surgery on the nest building performance. Surgery was combined without analgesic treatment and with analgesia in two dosages to distinguish the impact of pain on the observed nest building behaviour (Table 1). Before and after the treatments measurements in nest complexity scoring, latency of nest building and consumption of nesting material were sampled of all animals.

6.1. Circadian rhythmicity of nest building behaviour

Nest building is a spontaneous and highly motivated behaviour of mice. As a circadian rhythm in nest building activity is documented (Aschoff and Meyer-Lohmann, 1954; Possidente et al., 1979), a pilot study was conducted to determine the detailed 24 hours daily rhythm of nest building behaviours in our laboratory setting.

In the pilot study the highest nest building activity was seen immediately after the start of the measurements, when the nestlet was put into the cage. This peak could be also due to curiosity and exploratory behaviour, as the mice simply investigated the new object by tearing shreds out of the material. Nest building activity also was mixed with high locomotion in the cage and often a nest was not build properly shortly after providing new nesting material in the cage.

The second nest building peak was between one to three hours after providing new nesting material. In our setting, this took place around the beginning of the light phase, in which the mice needed to build a proper nest for their sleeping period during day light.

After the second peak of nest building longer sleeping phases in the nest followed, which were shortly interrupted only by eating, drinking, or by short but high activities for maintaining and improving the already build nest site. All mice slept in the nest during most of the light phase until the beginning of the dark phase.

During locomotion at the beginning until the middle of the dark phase the nest regularly was trampled down or even destroyed by constantly running over the nest site. The third high nest building peak was found in the middle of the dark phase, i.e. after 19 to 22 hours, where the mice rebuild their nests and prepared again for the next (second) light phase. During this third peak the mice quickly rebuild the nest and had well-structured nests at the end of the 24 hours observation (see Figure 5).

These timeframes are similar to other findings of nest building peaks in the circadian rhythm of mice (Possidente et al., 1979; Roper, 1975). Roper recognized a peak in nest building activity just before dawn in order to prepare for the sleeping phase during daytime, which is in close accordance to our observations. Based on these findings time points were selected after high nest building peaks (Figure 6).

Altogether, in the pilot study the circadian rhythmicity of nest building was determined and several appropriate time points were identified for measuring nest building performance in the main study.

6.2. Nest complexity scoring

The seven time points which we chose for nest complexity scoring included all three phases of high nest building activity as well as the phases where most mice had finished an intense phase of nest building and presented a nest. With these time points we could ensure that in the study scoring time was according to the mices` circadian rhythm and that any alterations in this rhythm, due to possible pain-related behaviour, were accomplished. We tried out the published protocol by Deacon in the pilot study and slightly modified this protocol to our needs, e.g. a score zero was established for no nest building activity (see Table 2). Deacon also had a different time

course of nest complexity scoring: the nestlet was placed in the cage at the start of the dark phase and evaluation took place after 12 hours at the beginning of the next light phase. Timing was not considered critical, as it was assumed that most mice had finished with nest building activity at the start of the light phase and were mostly sleeping in the nest. We however decided on a 24 hours time frame for nest complexity scoring with several time points. On one hand we wanted to cover the complete circadian rhythmicity in the nest building activity of mice in both the light and the dark phase. On the other hand we wanted to assess the effects of possible post-surgical pain on the nest building behaviour in mice and to find the best time point for assessing the mice`s actual general condition with nest complexity scoring as well as to determine the time phase when any possible pain-related effects might fade out again.

The highest baseline nest scores were found between 22:00 to 24:00 in the middle of the light phase and between 13:00 to 15:00 at the end of the dark phase. Analysing the nest scoring values, baseline nest scores were always higher than experimental values in all treatments and in both housing conditions. Overall the difference between baseline and experimental nest scores was less significant in control groups than in surgery groups (Figure 7). It is obvious that the treatment had an effect on the nest building behaviour. Experimental values increased again towards baseline values 22 to 24 hours after the start of the measurements and we resume that the effect of the treatment was fading out again at this time. In a similar study, where incorporation time of nesting material into a nest was measured, the nesting material was incorporated into the nest one day after procedures like osmotic pump placement and ovariectomy (Rodriguez et al., 2012). These results confirm our assumption that the effects of mild interventions might fade out after 24 hours and would therefore explain that experimental scores increased towards baseline values at the end of the 24 hour measurements.

The highest difference between baseline and experimental nest scores was found between three to nine hours after the start of the measurements. We presume that the effects of the treatment were greatest in this period. Immediately after placing the animals back in their home-cage after the different treatments, all animals seemed agitated, nervous and were constantly running around the cage. Cinelli could demonstrate with telemetric recording of physiological parameters like heart rate that even handling induces stress in mice up to one hour following the procedure (Cinelli et al., 2007). Thus we presume that the agitated behaviour and increased locomotion in all mice was due to the stress caused by our intervention. After half an hour up to one hour however all animals showed a decreased locomotion and behaviour for several hours after the treatments during experimental measurements. The mice were mainly sitting or resting in some place in a hunched-up position. All animals underwent 15 minutes anaesthesia and any sedating effects of sevoflurane are unknown in this context. Mice of control groups however earlier adopted their normal

behavioural repertoire and altogether had higher nest scores than mice with surgical intervention. We therefore assume that the reduced locomotion and changes in behaviour in our experiments are induced by pain following surgical intervention.

By trend we could see a clear graduation between the treatments. Mice of control groups always had higher experimental nest scores than mice with surgical intervention. In individually housed mice a graduation between the control groups was found with highest scores in the group anaesthesia with high dose analgesia, intermediate scores in the group anaesthesia with low dose analgesia and lowest scores in the group anaesthesia without analgesia. Further a graduation between the surgery groups was found with highest scores in the group surgery with high dose analgesia, intermediate scores in the group surgery with low dose analgesia and lowest scores in the group surgery without analgesia (Figure 8). Similar studies also investigated the effect of an impairment as well as the influence of a potent pain relief with NSAID`s on the behaviour (Jirkof et al., 2010; Miller et al., 2011; Roughan and Flecknell, 2001; Wright-Williams et al., 2007). The sedating effects of the volatile anaesthesia sevoflurane and the stress the animal might be experiencing seem to have an effect on the nest building behaviour of mice, as baseline scores were always higher than experimental scores in control groups. Surgical intervention even had a greater effect on the nest building behaviour than anaesthesia, which could be related to post-surgical pain. As animals, receiving pain relief after surgery had higher nest scores than animals without pain treatment we presume that pain relief ameliorated the well-being of the animals, resulting in higher nest scores. In pair housed mice only one effect of the treatments on nest building behaviour was seen with higher experimental scores in control groups than in surgery groups (Figure 9). However no graduation was found between the surgery group analgesia high dose and the surgery group with no pain relief in pair housed mice.

6.3. Latency of nest building

During baseline more than 90% of the mice generally started with the nest building within minutes up to the first three hours after the nesting material was presented to them. They simultaneously reached nest complexity scores higher than zero at the first time point of nest scoring at 18:00, three hours after the start of the measurements. In preference tests for nesting material the mice also started with the manipulation of the nesting material or the nest building within minutes after the nesting material was introduced to them (Schneider and Chenoweth, 1970; Watson, 1993). Overall the latency during baseline was always shorter than in the experiment in all treatments and in both housing conditions. During experiment latency was always shorter in control than in surgery groups. In experimental measurements 60% of mice in both anaesthesia and surgery groups had a prolonged latency until up to 18 to 24 hours and some mice did not build any nest within the 24 hours timeframe.

40% of mice in experimental groups showed nest building activity within the first nine to eighteen hours after the start of measurements.

In individually housed mice control groups had a slightly shorter latency during the experiment than mice with surgical intervention, but no graduation in between the groups could be seen (see Figure 11). In pair housed mice latency in control groups was clearly shorter than in surgery groups during the experiment (Figure 12). No significant differences were found between the housing conditions.

The results of the latency were in accordance with findings in other studies (Rodriguez et al., 2012), showing a prolonged latency of nest building activity of more than one day in mice after severe procedures such as carotid injury surgery. In our study the majority of mice had a delayed latency until up to 18 to 24 hours during the experiment. This confirms our assumption of a prolonged latency after stressful or painful procedures and also supports the results of the nest complexity scoring. We could not exactly define the time point when mice started with the nest building but only a period of time, as latency was measured at the same time points of nest complexity scoring. When latency is to be defined correctly, either more scoring time points are needed or evaluation has to be done with video recording. Both methods however are considered too time consuming in laboratory routine. The validity of the parameter proved not to be as exact as the nest complexity scoring, as a clear graduation in between the different treatments could be found with nest complexity scoring but not with latency. Feasibility and practicability of measuring post-operative pain are overall easier and more exactly to accomplish with nest complexity scoring.

6.4. Consumption of the nesting material

We decided on pressed cotton squares (nestles) instead of hay, paper stripes or tissue paper, as the material had to be actively manipulated by the mice for nest building purposes. Deacon also used this parameter in studies to examine the effect of hippocampal lesions in mice on the nest building behaviour (Deacon, 2006). In contrast to Deacon each measurement in our study lasted for 24 hours instead of 12 hours, so that mice had a prolonged period of time for consuming and manipulating the nesting material. The nestlets were weighed at the start of the measurements. After the 24 hours measurements any remaining and untorn pieces of the nestlet were weighed again. We adopted this method from Deacon to define the consumption of the nesting material and to clarify if standardisation is possible with this parameter in our setting.

Pair housed mice had a higher consumption of the nesting material than individually housed animals, but without relevant differences (Figure 14). Many mice were able to reach high nest scores in baseline or experiment using only a small amount of the nesting material. Some mice shredded the nestlet completely but only managed a nest of poor quality at the nest scoring time points. All mice used the nestlet for nest

building at least in the baseline and interaction between mouse and nesting material altogether was well. However we could not find any significant differences between baseline and experimental values or between the different treatments. As weighing the nesting material did not correlate with nest scores and quality of the build nest, this method proved rather unsatisfactory in our study.

6.5. Housing Conditions

In laboratory facilities mice are routinely housed in groups. In some experimental designs or after surgical procedures, individual housing also sometimes is required. As mice are usually living in groups, social housing seems the optimal way of housing female mice in the laboratory. In our study we wanted to proof the feasibility of our setting in routine laboratory conditions and also the validity in various preconditions. For this reason we tested the influence of post-operative pain or general condition on the nest building performance in individually and pair housed mice. In our study the possible influence of the housing condition on the nest building performance in female mice was investigated. As mice feel more comfortable when housed in groups, we presumed that pair housed mice would reach higher nest scores and start earlier with nest building activity than individually housed mice in our study.

In another study, telemetric recording of heart rate, body temperature and activity showed that pair housed mice were less affected after abdominal surgery compared to individually housed mice (Van Loo et al., 2007). As this study combined the telemetric recording with evaluation of behaviour and nest scoring, decrease in nest building performance was larger in individually housed mice one day after surgery compared to pair housed mice. However nest complexity scoring took place only once per day in the dark phase and mice were provided with tissue papers as nest material.

In our study pair housed mice mostly had slightly higher experimental nest scores than individually housed mice, but without relevant differences (Figure 10). The nest building behaviour seemed to be more influenced by the impact of the stimulus than by the housing conditions, as a clear graduation of nest scores between the different treatments was found, but not between the housing conditions. Latency of nest building showed no relevant differences between the two housing conditions (Figure 13). The consumption of the nesting material was always higher in pair housed mice than in individually housed mice (see Figure 15). This could be explained by the fact that two mice are able to pick out more shreds of the cotton square than one single mouse. During video observation in the pilot study we discovered that in pair housed mice, most of the time only one mouse was in charge of the entire nest building (data not shown), explaining that nest scores did not greatly differ between the two housing conditions. Although mice are more comfortable when being housed socially, the housing condition seemed to have no influence on the nest scoring in female laboratory mice.

6.6. Relevance and Recommendation

In our study the three parameters nest complexity scoring, latency of nest building and consumption of the nesting material were evaluated for their validity in the assessment of post-operative pain in mice. Nest complexity scoring proved to be the most reliable method and was easily implemented and assessed in our setting.

In our laboratory all mice had an adaptation phase of three days in experimental conditions to get used to the nesting material and a 24 hours baseline was carried out with each mouse before the experiment. Baseline values served to compensate for inter-individual variation and for gaining the individual nest building rhythmicity in our strain. In a pilot study the detailed daily rhythm of nest building performance was determined and several appropriate time points (3, 5, 7, 9, 18, 22, 24 hours) were identified to measure nest complexity scoring in the main study. The best time points for nest complexity scoring in our setting were at five, seven and nine hours after surgery with mild impact. At these time points the highest difference between baseline and experimental measurements was seen.

A constant and reliable nest building performance however may vary between mice due to strain, gender, age or hormonal status. One must also regard that some experimental models (surgery with high impact, infectious or disease models) may result in a different nest building performance during the experiment. In addition, genetic modification *per se* can have impact on nest building performance, and furthermore, development of a phenotype compromising animal behaviour or health can cause gradually growing changes of nest building performance.

Thus for nest complexity scoring, standard values for each specific experimental setting and laboratory conditions (e.g., mouse strain/line, age, sex, kind and invasiveness of the experiment) should be defined before starting the experiment and subsequent nest scorings. Therefore it is recommended that an adaptation phase of approximately three days allows the animals to become familiar with the nesting material. Then, start a 24h baseline with nest scoring at 5, 7 and 9 hours after providing new nesting material to the animals (Figure 16). If nest scoring at these time points is higher than at least score two, the scheme can be adopted in experimental measurements after stressful procedures or surgery. Nest complexity scoring should always be carried out at the same time points and any interfering influences, like husbandry procedures should be completed before baseline and experimental measurements. Throughout the measurements mice should live in their home cages without changing of the bedding. If nest complexity scoring in the baseline is lower than score two at all three time points you should newly define the 24 hours nest building rhythmicity in your strain by repeating baseline measurements or using more, other observation time points. If nest complexity scoring does not proof to be valuable in the assessment of pain (i.e. no difference between baseline and experimental measurements or no nest building activity at the scoring time points during experiment),

further scoring time points could be chosen in the 24h time course after the experiment or the scoring might be extended to 36h or even 48 hours.

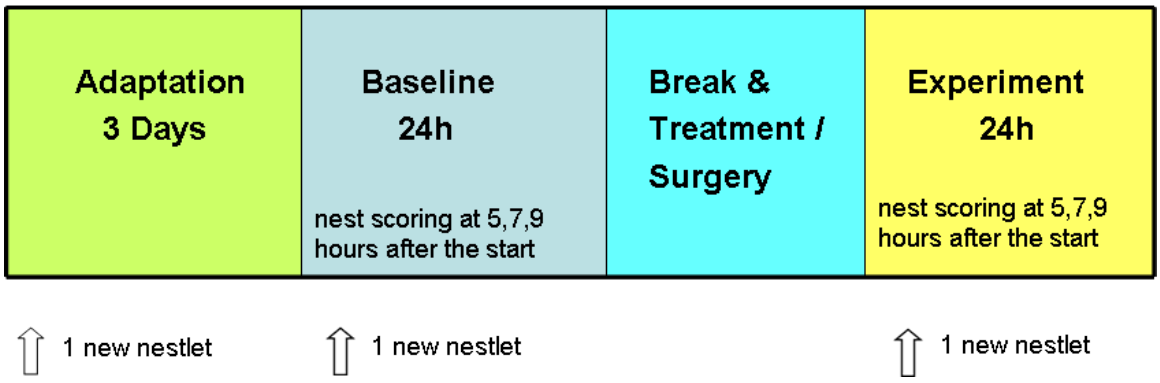

Figure 16: Recommendation for adopting nest complexity scoring in laboratory routine
Arrows indicate the time points when old, used nesting material should be removed and new nesting material be provided.

In genetically modified animals (GMA), an altered approach is recommended as phenotypes in these animals may present itself different, depending on the genotype. Therefore nest building performance should be tested repeatedly and compared to the nest building performance of the wild type (Figure 17).

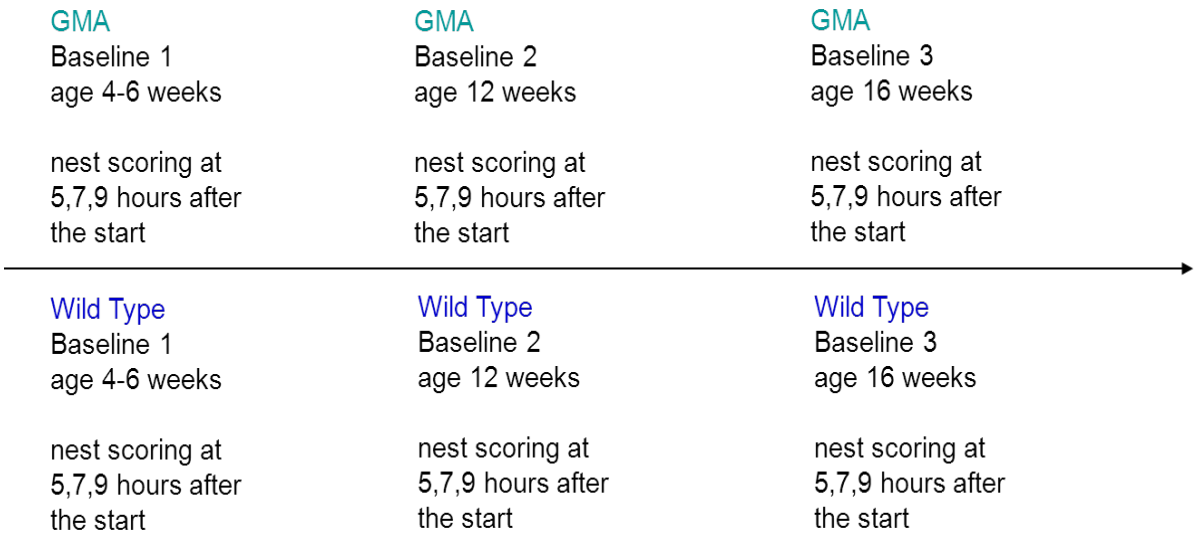

Figure 17: Recommendation for adopting nest complexity scoring in genetically modified animals

Following our recommendations good implementation of nest complexity scoring for the use in laboratory routine can be provided. However due to special experimental settings or laboratory conditions, slight but necessary adjustments may be required.

7. CONCLUSION

The aim of this study was the investigation of feasibility and reliability of nest building performance under various conditions to detect mild to moderate pain in laboratory mice. Nest building as a species-specific and highly motivated behaviour was selected, as any behavioural changes due to stress or pain were assumed to present themselves in poorer performance of this behaviour. We further aimed to standardise this method for the use in laboratory routine if it proved practicable and reliable.

Nest complexity scoring was used to identify significant differences in nest building between baseline and experimental measurements in both housing conditions and between treatments. By trend a clear graduation was feasible in nest complexity during experimental measurements between control groups and surgery groups. Using the analgesic carprofen the effects of surgery on nest building behaviour could be improved and mice with pain relief after surgery reached higher nest scores than mice without pain relief. Latency of nest building proved to identify differences between baseline and experimental measurements, but without graduation in between the treatments. We suggest that the best time point for nest complexity scoring lies between five to nine hours after surgical or other stressful procedures with mild impact.

The study proved to be useful to assess post-operative pain in mice by nest complexity scoring. The described method of nest complexity scoring is easy to implement in laboratory routine.

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9. LIST OF TABLES AND FIGURES

10. LIST OF ABBREVIATIONS

A	anaesthesia
A+5mg	anaesthesia + carprofen 5 mg
A+50mg	anaesthesia + carprofen 50 mg
ANOVA	analysis of variance
C57BL/6J	mouse strain
EU	European
GMA	genetically modified animal
m	mean values
MPS	multifactorial pain scale
n	number
NRS	numerical writing scale
NSAID	non-steroidal, anti-inflammatory drug
OP	operation
PBS	phosphate buffered saline
p-value	value of probability
S	anaesthesia + surgery
S+5mg	anaesthesia + surgery + carprofen 5 mg
S+50mg	anaesthesia + surgery + carprofen 50 mg
SD	standard deviation
SDS	simple descriptive scale
SPSS	statistic program
TF	Thea Fleischmann
Three R`s	Replacement, Reduction, Refinement
VAS	visual analogue scale

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