Aus der Klinik mit Schwerpunkt Klinische Immunologie und Rheumatologie
der Medizinischen Fakultät Charité - Universitätsmedizin Berlin

eingereicht über das Institut für Tierschutz, Tierverhalten und Versuchstierkunde
des Fachbereichs Veterinärmedizin der Freien Universität Berlin

Evaluating the Pain Management in a Mouse Osteotomy Model - Integrating a Refinement Approach in a Basic Research Study

Inaugural-Dissertation
zur Erlangung des Grades eines
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vorgelegt von
Mattea Sophie Durst
Tierärztin aus Bruchsal

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Gewidmet meiner Familie.

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Abbreviations

3D	Three dimensional
3R	Refinement, Replacement, Reduction
BORIS	Behavioral observation research interactive software
BUP	Buprenorphine
BUPDW	Animals only receiving buprenorphine
BUPOP	Operated animals receiving buprenorphine
BUPSham	Sham animals receiving buprenorphine
BV	Bone volume
DW	Drinking water
FCM	Fecal corticosterone metabolites
GV-SOLAS	German Society of Laboratory Animal Science
ISAP	International Association for the Study of Pain
i.p.	Intraperitoneal
MGS	Mouse Grimace Scale
MOMo	Mouse osteotomy model
M1	O-desmethyltramadol (M1 metabolite)
NSAID	Nonsteroidal anti-inflammatory drug
OCT	Optimal cutting temperature
OP	Osteotomy surgery
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
POC	Proof-of-concept
Rel. BV	Relative bone volume
ROI	Region of interest
RT	Resting time
s.c.	Subcutaneous
SD	Standard deviation
Sham	Animals with anesthesia, sham surgery and analgesia
Thigh	Tramadol in the high dosage (1 mg/ml)
ThighDW	Animals only receiving tramadol in the high dosage

ThighOP	Operated animals receiving tramadol in the high dosage
ThighSham	Sham animals receiving tramadol in the high dosage
TierSchG	TierSchutzGesetz
TierSchVersV	TierschutzVersuchstierVerordnung
TINT	Time to integrate into nest test
Tlow	Tramadol in the low dosage (0.1 mg/ml)
TlowDW	Animals only receiving tramadol in the low dosage
TlowOP	Operated animals receiving tramadol in the low dosage
TlowSham	Sham animals receiving tramadol in the low dosage
TV	Total volume
In vitro µCT	In vitro micro computed tomography
ZF	Zona fasciculata
ZG	Zona glomerulosa

1 General Introduction

Welfare of laboratory animals plays an increasingly important role in the research community. In 1959 Russell and Burch first described the 3Rs [1]. Besides the reduction and the replacement of animal experiments, the refinement of animal experiments was additionally pointed out in order to increase the animals' welfare [1]. Researchers are obliged by law to take the animals welfare into account when conducting animal experiments (TierSchutzGesetz (TierSchG) [2], TierschutzVersuchstierVerordnung (TierSchVersV) [3]). The refinement of animal experiments contains e.g. the enrichment of housing conditions, careful and stress-free handling and the optimization of experimental procedures to minimize pain for example with analgesia. A suitable pain management protocol is an essential prerequisite for an optimal analgesic care that should be adequate for a specific animal model.

There are many reliable recommendations for researchers on how to avoid and minimize pain in their animal experiments. Unfortunately, they are often not evidence-based and specific recommendations regarding a specific animal model are rarely available. A specific pain management for one model is superior to a more general protocol, preventing the animal's pain even more precisely. Such standard protocols for each model would also lead to a reduced variability between experiments applying the same models.

The mouse osteotomy model is a commonly used animal model in basic research to assess bone healing after inducing a defined gap by osteotomy and stable fixation. The procedure goes along with moderate pain and animals are treated with a variety of analgesics mostly opioids and different application routes. In some experiments, the analgesia is given in the drinking water to reduce handling stress in the animals. However, for this model, there are no specific recommendations available. The influence of many analgesics on bone healing and therefore on the read outs is not finally clarified. Sometimes possible side effects of analgesic medications are disregarded. In a frequently used German recommendation, "Pain management for laboratory animals" by the Society of Laboratory Animal Science (GV-SOLAS) general recommendations for mice are provided. Concerning tramadol, an analgesic that has been often used in the mouse osteotomy model, the dosage suggestion for the application via the drinking water for mice was increased by forty times between 2010 and 2015. This increase was not due to new results on analgesia studies, but mostly due to the fear of underdosing.

To answer open questions concerning drug, dosage, application route and side effects of analgesia in the mouse osteotomy model, this work evaluates three different pain management protocols in a mouse osteotomy model commonly used in our laboratory. For

that purpose, the refinement study is integrated into a study on bone healing using the mouse osteotomy model. The previously mentioned tramadol in two dosages as well as buprenorphine in the drinking water are tested. The aim of this study is to provide researchers with evidence-based recommendations on the pain management for the mouse osteotomy model.

This general introduction is followed by a review of the literature in chapter 2 concerning the research question. Chapter 3 highlights the aims and objectives of this work. In chapter 4 the materials and methods are described and chapter 5 lists the results. In chapter 6 these results are discussed and compared to findings of other studies. The conclusions and recommendations from the gained results follow in chapter 7.

2 Literature Review

2.1 Pain in Animals

2.1.1 What is pain?

The International Association for the Study of Pain (IASP) defines pain as *"An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage."* [4]. This definition was developed for humans but is also applied for animals. Differentiated from pain is nociception. Defined by the IASP as *"Pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors."* [4]. The two definitions differ therefore between the term pain as an actual feeling or sensation of pain versus the pure realization and processing of a noxious stimuli on a neuronal basis.

2.1.2 Pain in animals used in biomedical research

Pain plays an important role in the setting of biomedical research where laboratory animals are used. Animals can be subjected to mild, moderate or severe pain during an experiment, possibly resulting in distress and a reduced wellbeing. There are ethical, juristic and scientific reasons why pain in animals should be reduced in an experimental setting. Ethical reasons include a humane way of doing animal testing that can be achieved with experimental conditions exposing animals to as little pain as possible. Based on legal requirements researchers are obliqued to keep pain in animals to a minimum. Additionally, animal experiments must be approved by governmental institutions. The approval is based on the ethical justification of these experiments. A harm-benefit-analysis weighing the burden of animals against the potential positive outcome for the society is done. All factors increasing the burden of an animal shifts the balance towards the harm side of such a harm-benefit-analysis. The chance of a successful approval of an animal experiment therefore depends in part on the effort of the researcher in reducing the burden on laboratory animals. Especially important for researchers are the scientific reasons for avoiding pain in laboratory animals. Pain alters the physiology, behavior and neurobiology in animals [5]. The GV-SOLAS lists the pathophysiological effects of pain on several systems in the body [6]. Pain could therefore possibly depict a confounding factor in animal experiments as Sneddon explains. With the relief of pain an investigator can *"ensure that responses measured are to the experimental treatment itself, rather than to the pain"* [7]. Untreated pain or varying pain relief protocols in one model could lead to a higher variability of gained results and therefore reduced reproducibility between experiments and between laboratories. In conclusion, the performed studies could suffer from diminished

quality and the effects of an experiment could possibly not be detected. Thus, there can be a risk that more animals are used for an experiment and wrong conclusions are drawn.

2.1.1 Regulations on pain and welfare in laboratory animals

The legal basis for pain prevention and welfare in animal experiments is stipulated in several laws. The German Animal Welfare Act regulates the interaction with animals including livestock, laboratory and companion animals. The overall principle of this law is to protect an animals' life and wellbeing. Without a reasonable cause, nobody can inflict pain, suffering or harm on an animal (§1, TierSchG [2]). The act specially deals with laboratory animals in §7 where it determinates that pain, suffering and harm must be minimized to an imperative amount. The European legislation on laboratory animal welfare is represented by the Directive 2010/63EU [8]. This directive must be implemented in the union member's legislation. In Germany, the implementation is achieved by the Laboratory Animal Welfare Regulation (TierSchVersV [3]). Besides the regulations on e.g. housing conditions, killing methods and severity assessment, the regulation also focuses on the reduction and elimination of pain for example by using appropriate analgesia.

2.2 Pain Assessment

2.2.1 Assessing pain in laboratory mice

In 2015 68% of the used laboratory animals were mice [9]. The reason for their frequent use lies in the many benefits that the species offers for scientific research. Mice can be used to study complex biological systems and the interactions between different organ systems. The animal's genome has many similarities to humans which allows the study of genetic diseases for example by manipulating the mice's genome [10]. Mice are convenient and cost effective to house. They multiply quickly with a short generation time and lifespan enabling the researchers to study long disease courses in a short time. Taken together, mice represent a very important model in scientific research with high animal numbers used in laboratories every year. Many mice will therefore benefit from proper pain assessment strategies and pain management protocols.

Before the implementation of an appropriate pain management protocol, pain must be detected and evaluated. The assessment of pain in laboratory mice is often difficult due to their behavior. These animals tend to hide symptoms of reduced wellbeing and pain [11]. The chosen methods therefore must be sensitive enough to detect early changes in the animal even though signs may not be obvious to an observer. When clear signs of pain are observed in mice they are often already in severe pain or even moribund. Another challenge is the differentiation between pain and other physical and mental states or con-

ditions that reduce wellbeing. The question is what changes in the animal are due to a reduced wellbeing and what is actually pain-specific. Detecting painful states in animals is further complicated by the fact that pain and other factors like wellbeing and stress influence each other. Ideally, measurements that specifically measure pain should be applied to evaluate the pain in an animal. These indicators unfortunately are very scarce. Additional, difficulties in detecting pain occur when the experienced pain is ranging from rather mild to moderate severity, but recognizing and possibly fighting these lower levels of pain already plays an important role to ensure animal welfare. Only the proper assessment of pain can lead to a sufficient pain management.

2.2.2 Physiological parameters

Simple measurements such as the body weight, water and food intake are used to assess general health and wellbeing of animals during experiments. These parameters can be influenced by pain and are part of many pain assessment protocols [12, 13]. It should be kept in mind, that these measurements have a great influence on each other. A reduced water and food intake will lead to a reduced body weight and a weakened animal may further decrease its food and water intake. Attention has to be payed when applying body weight, food and water intake as these parameters are not solely affected by pain but also by other factors such as analgesia and anesthesia [12, 14, 15].

Pain leads to physiological changes in the body in order to adapt to the stressor. Among others it influences the endocrine system [16]. The sympathetic nervous system is activated affecting the cardiovascular system. Thus the measurement of heart rate and heart rate variability can be used to assess pain [11]. In a study by Arras et al. mild to moderate pain could be identified that would have been missed with simple observation. To gain the telemetric data a greater effort is needed as the transmitter must be implanted in an additional procedure. Besides these objective parameters there are also subjective assessments. A score that displays changes in the animals appearance (coat condition, piloerection) or posture can be included in the assessment [17].

2.2.3 Behavioral parameters

Pain affects the behavior of laboratory animals and influences their daily activities. This fact can be used by the investigator to assess painful states in an animal. An overall approach to quantify behavioral changes is to measure an animal's activity, with assessing mobility e.g. in a running wheel or time of sleeping. The activity is generally decreased due to reduced wellbeing or pain [14, 18]. With the presence of pain spontaneous and abnormal movements can be observed in the animal [17]. In some painful models specific

behaviors, for example stretch and press, can be observed and used in following studies to identify pain [19].

Laboratory animals are highly motivated to perform behaviors, belonging to the normal behavioral spectrum of the animal. For mice these tasks include nest building [20, 21], burrowing [22, 23] and the time-to-integrate-into-nest-test [24], all useful pain assessment tools. In animals experiencing pain there is a reduced willingness to perform such activities. These animals show reduced nest building activity and fail to build proper nests [11, 20]. The burrowing behavior also is reduced or not existing [23, 25]. Animals in pain fail to integrate new nesting material in their nests or have a prolonged latency [26]. One could say that due to pain animals are shifting their behavioral spectrum to activities that are more viable to them. Attention must be payed to the fact that the motivation to perform these tasks is not only influenced by pain itself but also affected by the animal's wellbeing [22, 24].

As the previous mentioned parameters are not solely specific to pain there is a need for pain-specific measurements. Researchers must differ between simply measuring nociception versus assessing the actual experience of pain. The assessment of pain sensation focuses more on the emotional experience and sensory processing. For example, the mouse grimace scale, developed in 2010 from Langford et al. [27] is used to grade the facial expression of pain in mice. It is frequently used in many models to evaluate pain of different kinds [28, 29]. Recently the MGS is controversially discussed because studies showed that the allegedly pain-specific parameter is influenced by anesthesia and analgesia [30, 31], sex, strain and whether it is observed live or retrospective [32].

Another approach to identify the sensation of pain is to test for the rewarding experience of pain relief in the animals. It is expected that animals suffering from pain show a preference for analgesia or increase their intake. One method is the conditioned place preference test where the animals' preference to an environment linked to pain relief is tested [33, 34]. A self-administration setup can be used where the analgesia intake for example in the drinking water is measured. It can be used to detect painful procedures or identify refinement measures [35, 36].

2.3 Pain Management

2.3.1 Available pain management recommendations

There are many sources from which researches can gather information on pain management for laboratory animals. For example, the GV-SOLAS gives recommendations on analgesia and anesthesia [6]. Other organizations like the American College of Laboratory

Animal Medicine provide the community with recommendations as well [37]. Besides these recommendations one can also find standard operating procedures from research facilities [38] and local animal welfare officers can consult researchers on specific questions. More pain management protocols are delivered e.g. by Flecknell in the book “Laboratory Animal Anaesthesia” [39] or can be found in different publications [40-42]. Though there are many recommendations and studies on usable drugs and strategies to relief pain in general, researchers often feel like protocols to manage pain in specific models are lacking [43].

2.3.2 General recommendations on tramadol and buprenorphine

Two opioids often used in the research setting for laboratory animals are tramadol and buprenorphine. Tramadol is used for treating moderate to severe pain. The used dosages on visceral pain highly varies from 1.25 to 80 mg/kg [44, 45]. In a bone cancer model tramadol in higher dosages (50-100 mg/kg) was needed to relief pain [46]. In the diet, nut paste, dosages of 20 to 40 mg/kg were effective [47]. For tramadol in the drinking water the used dosage often found in the literature is 0,025 mg/ml [48, 49]. The recommendation of the GV-SOLAS on tramadol dosing in the drinking water is currently 1 mg/ml [6], but was 0,025 mg/ml in 2010.

Buprenorphine is often used to treat moderate to severe pain in the veterinary medicine, also postoperative pain in laboratory rodents. While often used the recommendations on the dosage and the actually effective dosages range from 0.05 mg/kg to 3 mg/kg [14, 17, 18, 50-55]. Though providing effective analgesia negative side effects like changed behavior or increased body weight loss are noted with dosages of buprenorphine from both ends of this spectrum [14, 18]. No recommendations on the use in orthopedic research can be found. Besides s.c. and i.p. injection, analgesia in the diet, buprenorphine can also be applied s.c. as a sustained-release formula. Unfortunately, this product is not available in Europe. The opioid can also be given continuously via a subcutaneous osmotic pump. Adversely an additional surgery for implantation is needed here.

In tramadol there are several studies indicating no effect of the drug on bone healing [56, 57]. No literature on the influence of buprenorphine on bone healing was found. Studies on morphine in treating fracture pain show a reduction of callus strength and bone healing as well as an increase in bone loss and spontaneous fractures in a murine model of bone cancer [58, 59]. However, opioids have side effects that could impact bone healing indirectly. For example sedation with an occurring reduced mobility and thus a decreasing muscle and bone mass [60]. In the basic research study on bone healing that is the base of the current refinement study 0.1 mg/ml of tramadol is used. Here, the water intake was

adequate and no pain-related issues were observed. The influence of tramadol on bone healing was not assessed until now.

2.4 Research on Fracture Healing

2.4.1 Fracture healing

Fracture healing can either proceed as direct or indirect healing. Direct healing occurs with a rigid fixation and when a correct anatomical reduction of the fracture gap is given [61]. In the mouse osteotomy model of the underlying study an indirect fracture healing is intended.

The indirect bone healing process can be divided into overlapping, consecutive phases [62], starting with an acute inflammatory response in the initially formed fracture hematoma. The fracture hematoma is followed by the building of cartilaginous callus, the formation of hard, mineralized callus and ends with the remodeling of the hard callus into a lamellar bone structure. In detail, after the trauma, a hematoma is built in the fracture gap. This hematoma forms a template for callus formation. Immune and endothelial precursor cells are recruited and angiogenesis promoted by tumor necrosis factor-alpha and different interleukin factors. Mesenchymal stem cells infiltrate and proliferate to the hematoma area. They differentiate into osteogenic cells. The hematoma is replaced by a fibrin-rich granulation tissue, where endochondral formation occurs. The cartilaginous tissue forms a soft callus which gives the fracture a stable structure. The soft callus is revascularized, rebuilt and mineralized forming the hard callus. The last phase of indirect fracture healing is the remodeling of the hard callus into a lamellar bone structure with a central medullary cavity. Osteoclasts dismantle the hard callus and osteoblasts build lamellar bone. A medullar cavity is build. The fracture healing is finished and the fracture gap is bridged by stable and functioning bone material [61, 63].

Several kinds of disorders can appear during the healing of a fracture in humans. In the USA about 5-10% of the annually occurring fractures show complication in the union process [64]. These disorders range from a delayed union and mal-union to a non-union in the affected bone [65, 66]. To find the underlying mechanisms and possible therapies bone healing and fracture disorders are extensively studied.

2.4.2 Animal models in fracture healing research

Besides *in vitro* research on fracture healing, many research questions are addressed in animal models. Here mostly rodents, but also other animals like rabbits, dogs or sheep are used. In the time from 1970 to 2001 mostly mice and rats were used in orthopedic research. Martini et al. showed that in 21500 examined studies 62% of the used animals

were rodents [67]. A reason for the extensive use of mice is the availability of genetically modified mouse strains on which to study on. These animals can be used as models of human diseases. Questions on specific biological mechanisms and therapeutic options can be addressed. More advantages of using mice in fracture healing research are the low husbandry effort and the possibility to house many animals in rather little space compared to large animals. Therefore, the costs of using this species are lower. Additionally, mice produce offspring very quickly, providing the researcher with big animal numbers in short time.

2.4.3 Mouse osteotomy model

There is no classic mouse osteotomy model. Instead, this model comes in various characteristics. Uncontrolled fracturing of the animal's bone resulting in no straight gap by the so called Einhorn device [68] is in contrast to the osteotomy. With this device, a weight is dropped from a defined height on to either the animal's blunt bone, or on the covering skin or muscle. In the contrary, the osteotomy model always contains a controlled fracture with a defined gap by using e.g. a surgical scissor, bone saw or a wire [69-71]. Secondly, the fixation method in the mouse osteotomy model has a wide variation. The osteotomy can be fixated in a rigid way. The stabilization can be achieved by an intramedullary pin, plate or external fixator [72-74]. Each method brings different aspects into the osteotomy model. The appropriate methods are chosen by the researcher concerning the background, scientific questions and aims of the study.

2.4.4 Pain in the mouse osteotomy model

In the Annex VIII of the directive 2010/63/EU examples of different procedures are listed and graded concerning the degree of pain, suffering, distress or lasting harm an animal is expected to experience within the procedure. Three categories are defined: mild, moderate or severe burden. An orthopedic surgery like the osteotomy with an effective stabilization is classified as moderate, while an osteotomy leading to an unstable fracture increases the expected burden and is classified as a severe procedure. Concerning the duration of the burden, Lang et al. considered a moderate severity for three days after surgery and a mild severity for up to the tenth day postoperative [75] in a stabilized osteotomy.

While there are many studies on pain in bone cancer models and fracture pain, studies on pain in mouse osteotomy models were not found.

2.4.5 Pain management in the mouse osteotomy model

Researchers face the problem, that some medications cannot be used in their model as they influence the read-out substantially. An example for that implication are nonsteroidal anti-inflammatory drugs (NSAIDs) in studies on bone healing. The literature review results in contrary conclusions whether NSAIDs inhibit or delay bone healing in animals and humans [76-78]. Therefore, impact of the pain management on a model should be properly assessed to prevent changes in the outcome before changing the pain relief regime. As previously mentioned, tramadol does not seem to impact bone healing and there is no data on the influence of buprenorphine (Chapter 2.3.2). But investigators suggest additional studies on the effect of these drugs on bone healing.

A review by Lang et al. reports morphine, buprenorphine and tramadol as commonly used pain management regimes in bone healing studies in mice. But it also mentions the lack of empirical data on their effectiveness, the high variety between the protocols and the lack of proper reporting [75]. Carbone et al. comes to a similar conclusion reviewing literature on ten surgical animal models [79]. It also shows the wide variety of applied pain management protocols. If pain treatment and medications have an impact on the model itself, knowing the exact pain management protocol applied in a specific model is essential for the comparability of the studies. The lack of properly reported pain treatment leads to a lower reproducibility and is therefore not in line with basic scientific principles.

3 Aims and Objectives of the Thesis

The aim of this project is the evaluation of different pain management protocols in a mouse osteotomy model. This aim is approached by integrating a refinement study into a basic research study on bone healing. The combination of these studies contributes to the reduction of animal numbers. As part of this project three hypotheses were established and tested:

Hypothesis 1: The application of tramadol or buprenorphine via the drinking water represents a continuous, stress-free method of administering analgesia in the mouse osteotomy model.

Hypothesis 2: With the intake of tramadol and buprenorphine via the drinking water no model-specific, unspecific or behavioral changes indicating pain, changes in wellbeing or side effects are observed. The intake is therefore a sufficient pain treatment method.

Hypothesis 3: The intake of tramadol and buprenorphine via the drinking water does not impact the fracture healing in the mouse osteotomy model.

General study design: A refinement project integrated into a basic science research study

In this refinement project, mice with an osteotomy are treated with either one of three pain management protocols. These are tramadol in two different dosages and buprenorphine, all applied via the drinking water. The analgesic efficacy, possible side effects and the impact on bone healing are evaluated. Mice receiving only anesthesia and analgesia in combination or the analgesia solely are serving as control groups (Figure 2). The refinement project is implemented into a currently conducted basic research study (proof-of-concept-study, POC) investigating bone healing under the influence of novel substances. As this refinement project was designed, the concept of integrating a refinement study in the basic research on bone healing was intensely discussed. In the proof-of-concept-study mice are subjected to the osteotomy surgery and euthanized for assessment of bone healing three days to three weeks post-operative at different time points. The mice are routinely checked for any abnormalities in this time but do not undergo any further examinations. With the combination of these two projects animal numbers are reduced, contributing to the 3R principle. On the one hand, the reduction of the animal number is achieved by reusing control mice from the refinement project in the bone healing study. On the other hand, mice subjected to the standard treatment are tested as one group of the refinement project. Data of these animals then enters the results of the original study. In Figure 1 the approach is displayed in more detail.

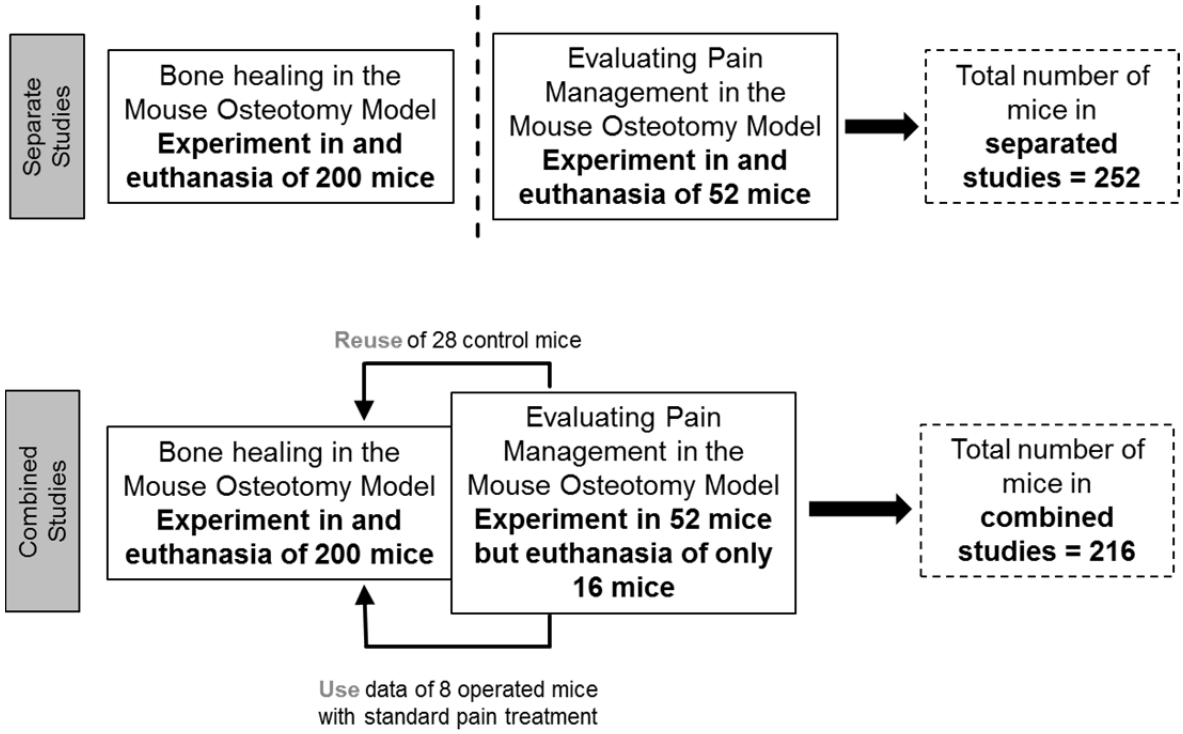

Figure 1: Integration of a refinement project in basic study on bone healing: showing the differences between seprate and combined studies.

Part 1: Evaluation of the pain management using physiological parameters

To test whether tramadol or buprenorphine applied via the drinking water represent a stress-free method of pain management in the mouse osteotomy model, the influence on drinking behavior, food intake and body weight is measured. Additionally, methods to assess short and long-term stress during the treatment with tramadol and buprenorphine in the drinking water are used.

Part 2: Evaluation of the pain management using behavioral and model-specific parameters

The efficacy of three different pain management protocols is evaluated in the mouse osteotomy model. Several parameters detecting experienced pain are used to investigate the actual pain relief with the medication. For this purpose, model-specific parameters like gait analyses are used, where the utlisisation of the operated hind limb is evaluated with several scores. On the other hand, unspecific parameters like a clinical scoring and behavioral testing are applied. In the clinical scoring, two of the originally five Mouse Grimace Scale parameters are assessed together with seven parameters depicting changes in the wellbeing and health of an animal (e.g. body and coat condition). The behavioral testing consists of nest complexity scoring, explorative behavior and activity measurements. The

model-specific and unspecific parameters are combined to detect pain, changes in wellbeing or side effects within the different pain management protocols.

Part 3: Impact of the pain management on fracture healing

An important factor of the pain medication for laboratory animals is the influence on the read-out parameters of the experiment. In the osteotomy model of the proof-of-concept study bone healing is the main read-out. The question here is, if the application of tramadol or buprenorphine leads to dosage or analgesia dependent changes in the fracture healing. The bone healing in this refinement project is evaluated after euthanasia according to the methods used in the underlying proof-of-concept study. These are assessment of the fracture in the micro computed tomography and histological analyses.

Part 4: Side effects and unexpected occurrences

Changes in the animals' livers were histologically analyzed after euthanasia to assess side effects of the pain management protocols on the liver. During the experimental work, several issues came up that needed to be further investigated. One animal had to be euthanized at an earlier time point than indicated in the study plan due to reaching the humane endpoints. A post-mortem examination was conducted. In two mice, wound healing issues occurred after surgery. Additionally, as the minimum effective tramadol concentration in mice is not known the serum levels of operated mice of the proof-of-concept study treated with tramadol in the drinking water is tested. In the current model, the surgical protocol stipulates a subcutaneous injection of buprenorphine 1 hour prior to starting the surgery. The influence of a pre-emptive buprenorphine injection on bleeding during the operation is investigated. These additional observations (serum concentration and bleeding) are conducted in mice of the proof-of-concept study.

4 Materials and Methods

4.1 Materials

4.1.1 Animals

Number	Strain	Age	Sex	Weight	Breeder	Purpose
52	C57BL/6N	10 weeks	female	21.3 ± 1.3 g	Charles River Laboratories, Sulzfeld, Germany	Refinement study
18	C57BL/6N	12 weeks	female	Not assessed	Charles River Laboratories, Sulzfeld, Germany	Serum con-centration
40	C57BL/6N	12 weeks	female	Not assessed	Charles River Laboratories, Sulzfeld, Germany	Buprenorphine and bleeding

4.1.2 Housing

Description	Manufacturer	City, State
1264C Eurostandard Type II	Tecniplast	Hohenpeissenberg, Germany
1264C116 Wire Lid		
1264C400SU Filter Top		
Lignocel FS 14	J. Rettenmaier & Söhne GmbH + Co. KG	Rosenberg, Germany
Enviro-dri	Shepherd Specialty Papers	Tennessee, USA
Standard mouse diet	Ssniff Spezialdiäten	Soest, Germany
Home cage during activity measurements	Self-build University Zürich	Zürich, Switzerland

4.1.3 Medication

Description	Manufacturer	City, State
Temgesic	RB Pharmaceuticals Limited	Heidelberg, Germany
Tramal Drops	Grünenthal GmbH	Stolberg, Germany
NaCl 0.9%	B. Braun Melsungen AG	Melsungen, Germany
Clindamycin	Ratiopharm GmbH	Ulm, Germany
Xylapan	Vetoquinol GmbH	Ismaning, Germany
Narketan	Vetoquinol GmbH	Ismaning, Germany
Fucicort	LEO Pharma GmbH	Neu-Isenburg, Germany
Octenisept	Schülke & Mayr GmbH	Norderstedt, Germany
Douxo Chlorhexidin Pads	Ceva Tiergesundheit GmbH	Düsseldorf, Germany

4.1.4 Surgical procedure

Description	Manufacturer	City, State
Isoflurane	CP-Pharma Handelsgesell-schaft mbH	Burgdorf, Germany
Braunoderm	B. Braun Melsungen AG	Melsungen, Germany
Bepanthen Eye Ointment	Bayer Vital GmbH	Germany
Drill bit 0.45 mm	RISystem	Davos, Switzerland
MouseExFix MountingPin 0.45mm		
Square box wrench 0.70mm		
MouseExFix simple L		
Gigli wire saw		
Lyostypt	B. Braun Melsungen AG	Melsungen, Germany
Prolene (4-0)	Johnson & Johnson Medical GmbH Ethicon	Norderstedt, Germany
Opsite dressing spray	Smith & Nephew GmbH	Hamburg, Germany

4.1.5 Devices

Description	Manufacturer	City, State
Precision scale SBS-LW-300A	Steinberg Systems	Poland
Heraeus Function Line T6 Drying Oven	Thermo Fisher Scientific	Langenselbold, Germany
Vortex Genie 2	Scientific Industries Inc.	New York, USA
Heraeus Fresco 17 Centri-fuge	Thermo Fisher Scientific	Langenselbold, Germany
Tissue processor TP1020	Leica Mikrosysteme GmbH	Wetzlar, Germany
Microtome RM2155		
Nikon Eclipse Ti-E	Nikon GmbH	Zürich, Switzerland
vivaCT 40	Scanco Medical AG	Brüttisellen, Switzerland
Cryostat	Leica Mikrosysteme GmbH	Wetzlar, Germany
LSM 710 confocal microscope	Carl Zeiss Microscopy GmbH	Oberkochen, Germany
Precision scale SBS-LW-2000A	Steinberg Systems	Poland
Observation box (Modular animal enclosure, 10 cm x 10 cm x 14 cm)	Ugo Basile	Varese, Italy
IPhone 5	Apple Inc.	California, USA
IPad 2		
USB 3.0 Monochrome-Camera	The Imaging Source Europe GmbH	Bremen, Germany
Lenovo Yoga 2.13	Lenovo GmbH	Stuttgart, Germany

4.1.6 Staining solutions

Description	Manufacturer	City, State
Xylene	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Ethanol	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Hemalum	Artechemis AG	Zofingen, Switzerland
Acid alcohol	Merck KGaA	Darmstadt, Germany
Eosin	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Acetic acid	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Alcian blue	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Alkaline ethanol	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Weigert's hematoxylin	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Crocein scarlet-acid fuchsin	Merck KGaA	Darmstadt, Germany
Phosphotungstic acid	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Alcoholic safran	Carl Roth GmbH + Co. KG	Karlsruhe, Germany

4.1.7 Equipment for histology

Description	**Manufacturer**	**City, State**
Pertex mounting medium	HistoLab	Askim, Norway
Vitro clud	R. Langenbrinck GmbH	Emmendingen, Germany
SCEM medium	SECTION-LAB Co. Ltd.	Hiroshima, Japan
Superfrost Plus microscope slides	Thermo Fisher Scientific	Reinach, Switzerland
Cover slips 24 x 50 mm		
Cryofilm	SECTION-LAB Co. Ltd.	Hiroshima, Japan

4.1.8 Software and online services

Description	**Manufacturer**	**City, State**
G*Power 3.1.9.2.	Heinrich-Heine-Universität Düsseldorf	Düsseldorf, Germany
PubMed database	National Library of Medicine	USA
EndNote X7	Thomson Reuters	New York, USA
GraphPad Prism V.5	GraphPad	San Diego, USA
Behavioral Observation Research Interactive Software	Olivier Friard, Marco Gamba University of Turin	Turin, Italy
ZEN 2011 software	Carl Zeiss Microscopy GmbH	Oberkochen, Germany
ImageJ	National Institute of Health	USA
Fiji		
NIS Elements BR	Nikon GmbH	Zürich, Switzerland
AxioVision	Carl Zeiss Microscopy GmbH	Oberkochen, Germany
IC Capture 2.4	The Imaging Source Europe GmbH	Bremen, Germany

4.2 Methods

4.2.1 Animals and ethical statement

- **Animals**

The study was conducted with female C57BL/6N mice, delivered to the facility at the age of 10 weeks with a body weight of 21.3 ± 1.3 g. Only female C57BL/6N mice were used in the refinement project based on the POC study. The fact that bone healing in female mice is slower than in male mice is utilized in the POC study to detect differences in the bone healing with different treatements three weeks after the osteotomy. The animals were purchased from Charles River Laboratories, Sulzfeld, Germany. A total number of 52 mice was used for the refinement study. Randomly divided into three sets of 18, 18 and 16 animals. Data of the eight osteotomized animals with the same analgesic protocol were directly incorporated into the basic research study's results. Animals without osteotomy (anesthesia + analgesia, drinking water and control groups) were later reused in the basic research study. The blood serum of 18 mice from the basic research study was used to assess tramadol and O-desmethyltramadol (M1) serum concentrations with tramadol in the low dosage. In 40 mice from the underlying basic research study the effect of preemptive buprenorphine injections on bleeding during surgery was observed.

- **Ethical statement**

The present study was conducted according to the guidelines of the German Animal Welfare Act and was approved by local animal rights protection authorities ("Landesamt für Gesundheit und Soziales" Berlin, permit number: G0039/16). The underlying basic study was approved under the permit number G0111/13.

4.2.2 Housing and husbandry

Housing was outside the barrier to help develop a functional immune system. Animals were housed in EUROSTANDARD TYP II clear-transparent plastic cages covered with a wire lid with build-in u-shaped feed hopper. Cages were closed with a filter top. Fine wood chips were used as bedding material. Due to possible entanglement with the used external fixator, mice were not provided with houses, pipes or facial tissues as nesting material. Instead, animals were provided with a sufficient amount of shredded paper strips as nesting material (Envirodri®). During the whole study, food and tap water was available ad libitum. Room temperature was constantly between 20 and 22°C with a humidity of 45-50%. The light/dark cycle was a 12/12-hour cycle with the lights on at 6:00 and off at 18:00. Cage changing took place once a week during accommodation phase and was carried out by the experimenter. Mice could habituate to the new surrounding in groups of

8-10 animals for one week. One week prior to testing mice were put into groups of two. These groups were maintained until the end of the trial. After the surgical procedure, all animals were given soaked soft food pellets on the floor to ease food intake. A refining method especially for those animals potentially struggling to reach up to the food hopper with an operated leg. In the experimental period of two weeks cage bedding was not changed. Nesting material was clean and dry during baseline and testing period and was therefore not changed. Animals were handled by tail. As the study progressed the mice were more used to the handler and cup handled.

4.2.3 Study design

For this refinement study three different analgesia protocols were tested in three experimental sets (Figure 2). Group one and two received tramadol in different concentrations (tramadol low: 0.1 mg/ml (Tlow) versus tramadol high: 1 mg/ml (Thigh)) in the drinking water. Group three received buprenorphine with a concentration of 1 mg/kg (BUP) in the drinking water. Each set consisted of an osteotomy group, a sham group with anesthesia and analgesia only and a group with analgesia in their drinking water solely. Additionally, two groups of naïve animals with neither surgery, anesthesia nor analgesia in the drinking water were used as a control group.

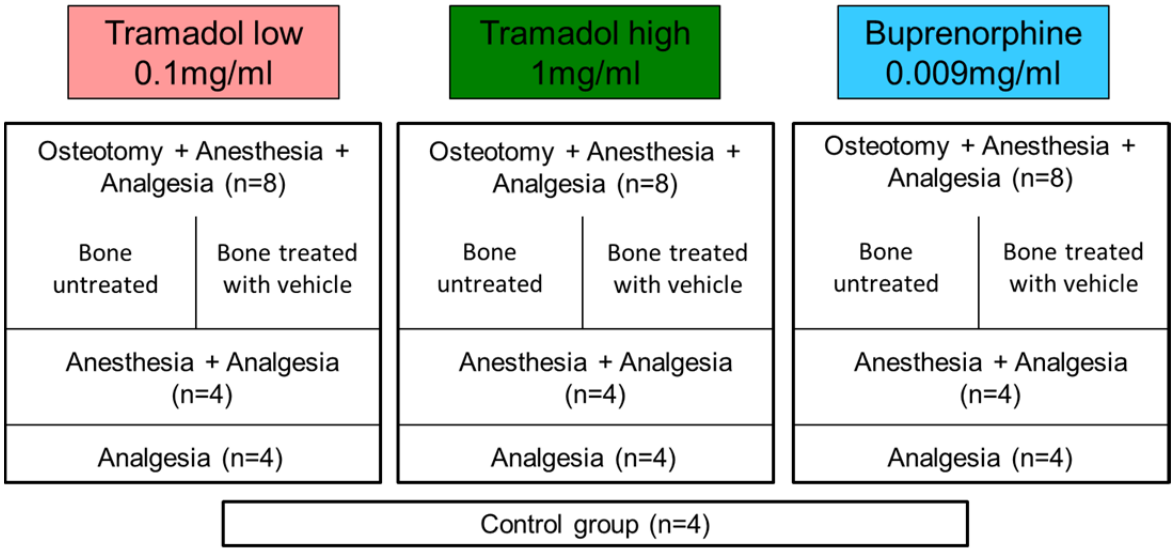

Figure 2: Experimental groups. Each set of analgesic treatment contains three groups with different experimental conditions. An untreated control group is examined in addition.

This refinement project is integrated into a basic research study (proof-of-concept) on bone healing (Figure 1). The data of the mice with an osteotomy receiving the standard pain management protocol (Tlow) are used in the POC study (see also Chapter 3: Aims and Objectives of the Thesis). Additionally to the efficacy of pain treatment, the impact of

the used analgesic medications on bone healing will be assessed. To be able to integrate results on bone healing into the POC study and compare bone healing between the three analgesia groups, the mice in the osteotomy groups undergo the same bone treatment as the animals with osteotomy in the POC study. Specifically, the fracture gap is left untreated or is filled with a vehicle (Lyostypt).

Mice were randomly assigned to a cage, with two animals per cage. The cages were randomly assigned to either one of the three treatment groups and in the sets to one of the previously mentioned groups. Depending on the applied test the single animal or the cage with two animals is observed as an experimental unit.

The experimental design is outlined in Figure 3. After an acclimation time of one week, groups as delivered from the breeder were separated into pairs. Baseline measurements took place in the week prior to surgery. For every postoperative test, a baseline measurement was conducted on the same time of the day. The surgery took place at 8:00. On the day of surgery, mice were tested 1, 6 and 12h postoperative. Home cage video recording started at the beginning of the dark phase and lasted for 48 hours. Testing continued once a day at 9:00 for five days after surgery. Feces were collected on every time point of clinical scoring in the observation box if animals defecated voluntarily. 14 days post-surgery mice with previous osteotomy were anesthetized with a mixture of ketamine and xylazine (i.p. Ketamine 120 mg/kg, Xylazine 16 mg/kg), blood was collected from the heart once depth of anesthesia was achieved. Euthanasia was conducted by cervical dislocation. The operated left femur was dissected for *in vitro* µCT and histology. Adrenal glands and livers were removed for further histological investigations.

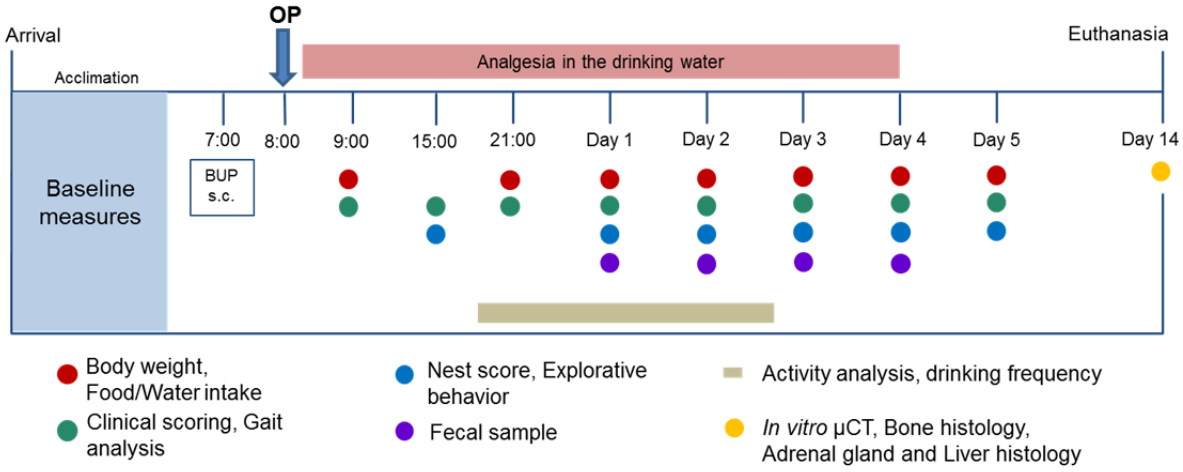

Figure 3: Timetable of study design

The two female researchers assessing mouse behavior at baseline and the time points after surgery were blinded to the groups' treatment. All further methods carried out after euthanasia were done by a single, blinded observer.

Additionally to the refinement study, the blood serum of 18 mice of the basic research study (POC) was obtained after euthanasia three days postoperative during the regular procedure of this experiment. These mice were treated with the low tramadol dosage. The blood serum was analyzed for the tramadol and M1 concentration.

To assess the effect of pre-emptive buprenorphine on bleeding during the surgery, 40 mice from the POC study were observed during the regular osteotomy conducted in this study. Before the surgery this mice were randomly assessed to three groups (Figure 4), injected with buprenorphine according to the group's time point. The bleeding was then observed and graded by the surgeon during the surgery.

Figure 4: Testing of pre-emptive buprenorphine injections. Treatment groups of different injection routes and timepoints to assess bleeding during surgery after preemptive buprenorphine.

4.2.4 Surgical procedure

- **Preparation**

The animals were transferred to the operating room by 7:00. Body weight was assessed. Mice of the osteotomy, sham and analgesia only group were given buprenorphine one

hour prior to surgery as a perioperative pain treatment (s.c. 0.1 mg/kg body weight). The surgery took place at 8:00, done by two experienced surgeons. Anesthesia was induced in a transparent Plexiglas box at 2.5% isoflurane (Figure 5 – A). Once the animal was asleep and spontaneous breathing was deep and consistent, it was put on a breathing mask and kept in anesthesia with 1.5% isoflurane (Figure 5 – B).

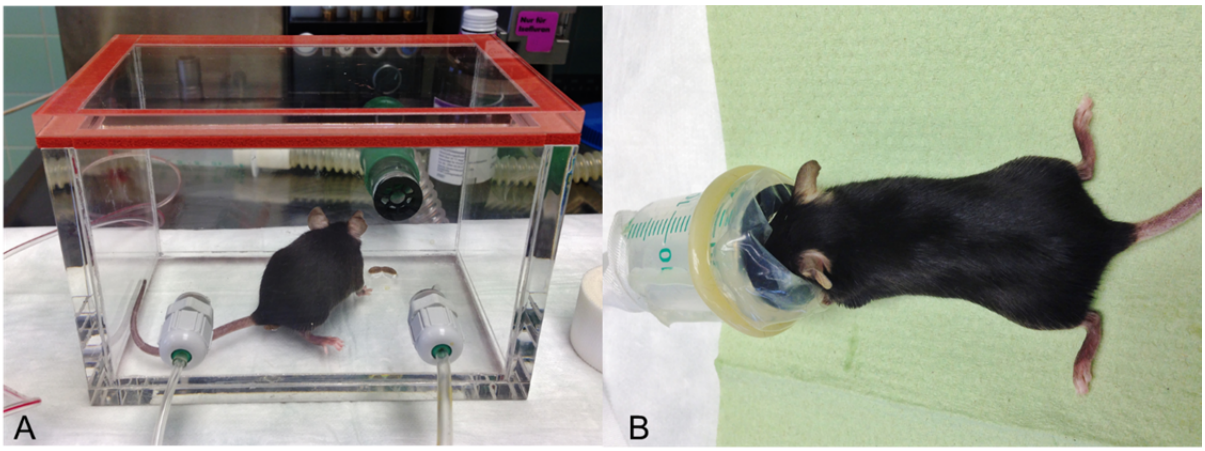

Figure 5: Inhalation anaesthesia with isoflurane. A) Mouse in the induction box, B) Maintenance of anesthesia with breathing mask

Clindamycin (0.02 ml s.c.) for antibiotic prophylaxis was injected. To prevent eyes from drying they were covered with moistening ointment. The operation area over the left femur was shaved and disinfected with an alcoholic iodine solution. Anesthetic depth was checked with the withdrawal reflex in toes and tail. Once withdrawal reflexes were absent surgery was performed on a heating mat (37°C) under aseptic conditions.

- **Osteotomy**

After palpation of the femoral bone, a skin incision of approximately 2 cm was performed along a line from the knee to the hip joint. Dissection of the Fascia lata and blunt preparation of the muscle layer exposed the femur. Care was taken to spare the sciatic nerve. A fine hand drill (diameter: 0.45 mm) was used to drill the first pin hole proximal to the distal metaphysis of the femur, perpendicularly to the femoral axis and cortical surface. The first pin was placed through the first hole of the external fixator in the distal hole. The next pin hole was drilled at the proximal end of the femoral bone with guidance of the holes in the external fixator. The second pin was placed in the proximal hole. Following, the remaining two pin holes were drilled and pins were placed resulting in a parallel fixation of the external fixator and the femur. Subsequently, a 0.70 mm osteotomy was performed between pin two and three with a Gigli wire saw. The osteotomy gap was either stuffed with a vehicle (Lyostypt) soaked with PBS or left untreated (control). The wound was then closed

with single stitches of the skin (Prolene® 4-0, monofilament, non-resorbable). Figure 6 shows the single steps of the surgery from incision to closure of the skin.

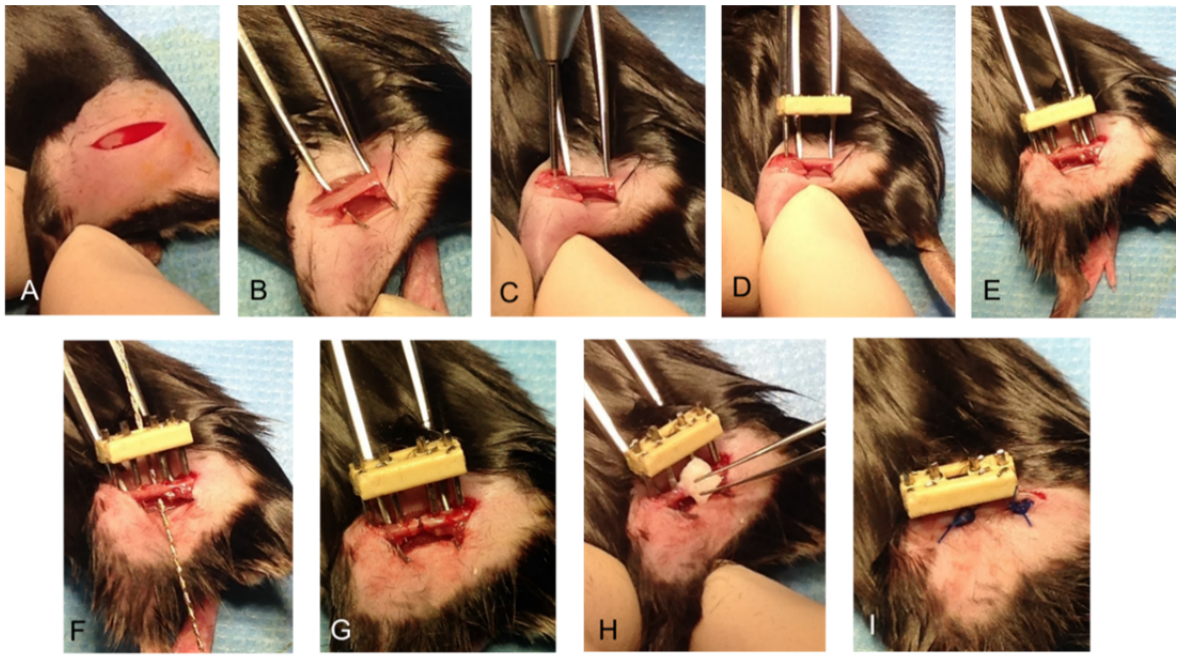

Figure 6: Osteotomy surgery. A) Skin incision, B) Femoral bone, C) Drilling of first hole, D) Placement of first pin, E) Fixated external fixator, F) Creating of osteotomy, G) Osteotomic gap, H) Inserting of vehicle (Lyostypt), I) Skin closure around the external fixator.

- **Postoperative care**

NaCl (0.1 ml s.c.) for hydration was injected. A permeable spray dressing was applied to the closed wound. Animals were returned to their home cage and put on a soft tissue. Cages were placed under warming lamps in the surgery room and monitored frequently. Cage mates underwent surgery successive and were therefore able to gain consciousness together in the home cage. After approximately 45 min cages were transferred back to the housing room. For postoperative pain management, either tramadol (Tlow: 0.1 mg/ml; Thigh: 1 mg/ml) or buprenorphine (0.009 mg/ml) was administered via the drinking water for three consecutive days.

4.2.5 Evaluating the postoperative pain management

- **Body weight**

Animals were weighed daily starting from six days prior to surgery and continuing until the day of euthanasia. Weight was measured in grams.

- **Food and water intake**

Food and water intake was assessed for each cage with two animals. Prior to the procedures by weighing both water and food every 24 hours. After the intervention, those parameters were assessed every 12 to 24h.

- **Home cage video analysis – Drinking frequency**

To evaluate drinking frequency eight animals of each treatment group (Tlow, Thigh, BUP) were filmed in their home cages for 24h before and 48h after the procedures. Of each treatment group four osteotomized, two animals of the anesthesia and analgesia only group and two animals receiving analgesia only were recorded. Cages were filmed from above (camera distance about 1.5 m) in standard housing cages without grid. To prevent escape the cages had raised walls. Mice were kept with their familiar partner, provided with nesting material, food and water. One mouse in every cage was marked with black stripes on the tail. In the dark phase, an infrared light was turned on to provide proper illumination. Videos were then analyzed manually by one blinded observer with an event logging software (BORIS - Behavioral Observation Research Interactive Software). Drinking frequency of each mouse was assessed manually. The contact of a mouse's mouth with the bottle tip was counted as one drinking event. Continuous drinking for a longer time was counted as one drinking event (see Figure 10).

- **Assessment of short-term stress in cooperation with Prof. Palme (University of Vienna)**

Fecal corticosterone metabolites

Fecal corticosterone metabolites were measured to clarify the stress response of animals in all groups during the time of the experiment.

Sample collection

Feces were collected from the observation box whenever mice defecated during the time being observed. If urine was around fecal pellets it was carefully absorbed with a facial tissue. This sampling technique was non-invasive and posed no additional stress to the animals. Feces were stored at -20°C immediately after collecting.

Sample preparation

The fecal samples were processed according to Palme et al. [80]. The material was dried at 70°C, homogenized with a mortar and weighed. 80% methanol (in ml twice the weight of the fecal powder) was added to the fecal powder and shaken on a vortex for 2 min.

Following centrifugation 2500 x g for 15 min, half of the samples amount was transferred into an Eppendorf tube.

Analysis

Fecal corticosterone metabolites in the samples were then analyzed in the laboratory of Prof. Palme (Vetmeduni Vienna, Austria) using a 5α-pregnane-3b,11b,21-triol-20-one enzyme immunoassay.

- **Assessment of long-term stress**

Hypertrophy and hyperplasia in the adrenal gland

As a response to long-term stress, cells in the zona fasciculata of the adrenal glands cortex increase the production of corticosterone, which comes with hypertrophy and hyperplasia [81]. To assess these structural changes the cortex of the adrenal gland was histologically examined. Note that only mice with an osteotomy were euthanized at the end of the experiment to assess bone healing histologically and with *in vitro* µCT. Sham, DW and control animals were reused in the proof-of-concept-study. In conclusion, only adrenal glands of the osteotomized mice were taken into account for the assessment of long-term stress.

Organ collection

Both adrenal glands were taken from each operated mouse during dissection and transferred into a biopsy cassette. Subsequently, the adrenal glands were fixated in 4% paraformaldehyde (PFA) for 24 hours. Additionally, the adrenal glands of 12 naïve animals were collected. These animals were killed during an unrelated project at our facility.

Embedding

After being fixated in PFA, the samples were watered for one hour and then transferred to a tissue processor (Leica TP1020, preset program “P2”). The adrenal glands were embedded in paraffin.

Microtome

The embedded adrenal glands were cut in a microtome (Leica RM2155). First, cutting to the middle of the sample was carried out. The middle was defined as the area where the cortex and medulla was visible. Slices of 3 µm were cut and put on a microscope slide. The slides were stored in a warming cabinet of 37°C overnight.

Staining

Staining was conducted the next morning. The slices were stained with hematoxylin and eosin. The sections were deparaffinated in two steps of xylene (10 min each) before being moved to a descending ethanol series (three times 1 min of 100%, 1 min of 96% and 1 min of 70% ethanol). The slides were placed under running tap water for 10 min and stained in a solution for 10 min. After a quick rinse in fresh tap water the samples were dipped into acid ethanol three times and washed in running lukewarm tap water for 10 min. 30 secs of staining in eosin was followed by two dips in fresh tap water. The sections were then transferred to an ascending ethanol series, containing three dips of 70% and 96% ethanol each, followed by two times 1 min in 100% ethanol. Subsequently, the slides were put in a fresh xylene bath for 1 min, before being washed in xylene two times for 5 min each. Finally, the stained samples were mounted and covered with a cover slid.

Image analysis

An overview image (4x magnification) of one adrenal gland per animal was taken (Figure 7 – A). A section of the adrenal glands cortex was chosen randomly and enlarged (20x magnification). Five squares (1 cm x 1 cm) each were randomly placed in the zona fasciculata and zona glomerulosa (Figure 7 – B). Cell nuclei were counted in every square. A decrease of the total number of cells was a sign of increased cell size. The zona fasciculata/zona glomerulosa (ZF/ZG-ratio) ratio was calculated, decreased values indicate hypertrophy and hyperplasia in the zona fasciculata due to chronic stress. For an easier comparison values were multiplied with the factor of 100.

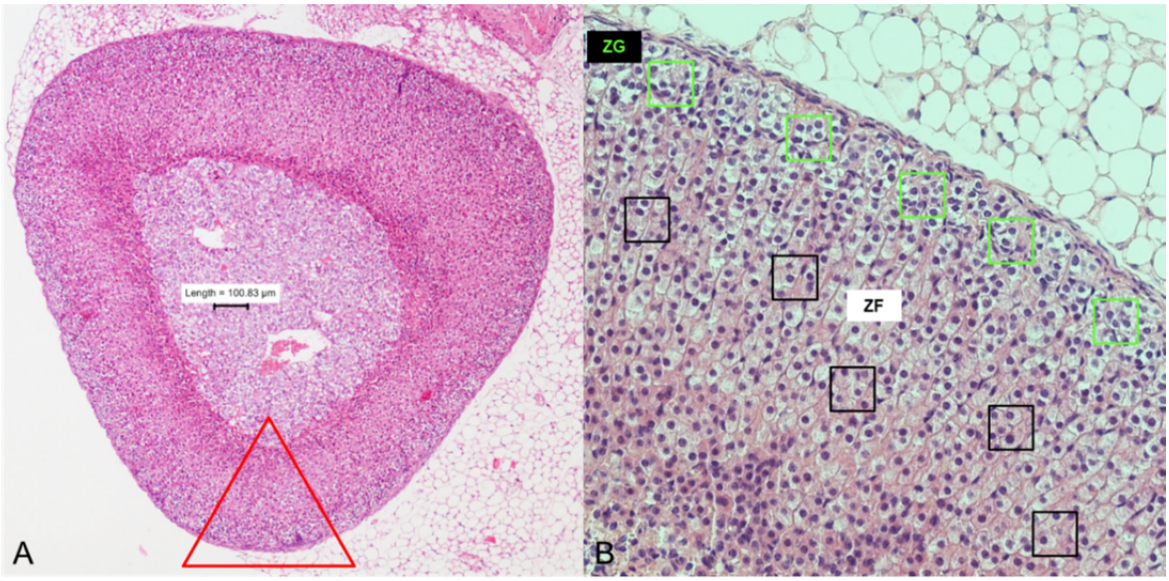

Figure 7: Assessment of changes in the adrenal gland. A) Overview, B) Sector of the cortex with zona glomerulosa (ZG) and zona fasciculata (ZF). Cell nuclei were counted in the five designated areas (ZG green boxes, ZF black boxes).

4.2.6 Clinical and behavioral parameters evaluating analgesic efficiency

- **Clinical score**

To investigate changes in behavior that might hint on pain, reduced wellbeing or impairment of movement, a clinical score was assessed. Mice were individually transferred to a transparent plastic observation box as shown in Figure 8. They could acclimate for one minute and were then observed by a single, blinded female observer for three minutes. Scoring, on the one hand, consisted of parameters based on the MGS. Two of the originally five parameters were included. On the other hand, the general condition was evaluated with seven parameters, indicating changes in wellbeing and health as previously described by Jirkof et al. [17]. Results were noted on score sheets and summed up. In total, a maximal score of 11 could be reached (Table 1).

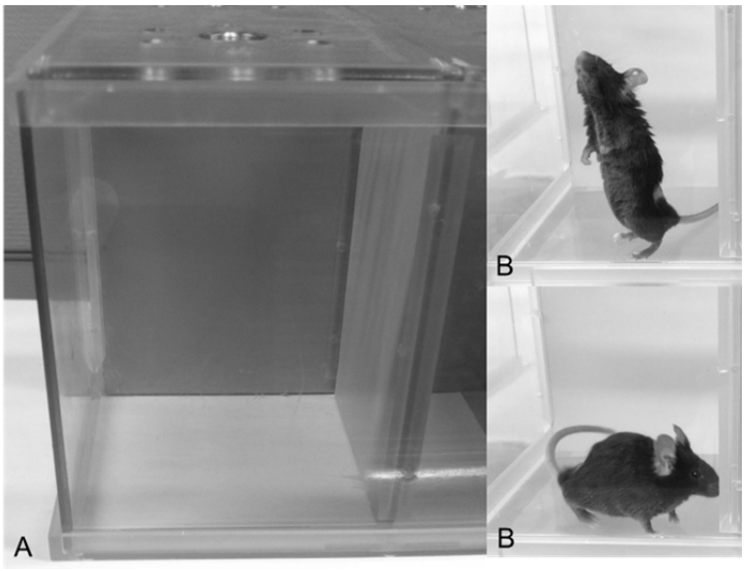

Figure 8: Clinical scoring. A) Observation box, B) Animals during observation.

Table 1: Scoring system for clinical investigation and behavioral based pain assessment

Parameter		**Scores**
Facial expression		
• orbital tightening	• narrowing of the orbital area, a tightly closed eyelid, or an eye squeeze (orbital muscles around the eyes contracted)	not present = 0, moderately = 1, severe = 2
• ear position	• ears pulled back or rotate outwards and/or back, away from the face. space between the ears may appear wider	
General condition		
• spontaneous behavior	• sudden movements, backwards movements, transient involuntary muscular contraction of any body part, kicking with hind paws, licking/biting the wound, highly aggressive, increased vocalization	not present = 0, present = 1
• posture	• hunched, arched back, crouched	
• coat condition	• ruffled, dirty, unkempt, piloerection, hair loss (alopecia)	
• eyes	• discharge	
• body condition	• sunken flanks, swollen areas, ascites	
• wound	• dirty, bloody, uncleaned, signs of self-injury, signs of inflammation or necrosis, i.e., unusual color (e.g., red, pale) or swollen	
• movement	• apathetic, sedated, decelerated, crawling, immobile, lameness, tiptoe gait	

- **Nest complexity score**

The nest-building behavior was used to detect changes in wellbeing that can possibly result from states of pain [20, 22, 82]. To evaluate the nest complexity in the home cage after an osteotomy 20 ± 0.9 g of nest building material (Enviro-dri®, Shepherd Specialty Papers, Tennessee, USA) was provided per cage. The material was given at the time of splitting the groups into pairs. Mice could acclimate with the new material for five days before the first scoring. After that period, baseline measurements were conducted. Following the surgery, complexity was scored at six hours post-surgery, then daily at 9:00 for five

days. Nest complexity was assessed using the naturalistic nest scoring system developed by Hess et al. [82] as seen in Table 2.

Table 2: Nest complexity scoring by Hess et al.

Nest		Score
Undisturbed	Nesting material has not been moved, no sign of interaction or manipulation of the material.	0
Disturbed	Interaction with the nesting material is evident (for example disturbed, chewed, spread around the cage) but has not been gathered to a nest site. If a nest site is present in the regular bedding material, either there is no concentration of the material in the nest site, or the material is merely piled on top of the nest cavity in the regular bedding.	1
Flat nest	Nesting material has been gathered to a form a nest site in the cage, identified by a clear nest cavity in the middle of the material, or between the material and the cage wall. The nest is a flattened saucer shape with no, or incomplete, walls.	2
	Flat nest with 1 side that is less than half of a sphere.	2.25
	Flat nest with 2 sides that are less than half of a sphere.	2.5
	Flat nest with 3 sides that are less than half of a sphere.	2.75
Cup	Nesting material has been gathered to form a nest site in the cage. The nest has identifiable walls that form a ‘cup’ or ‘bowl’ (similar to a shallow soup bowl), such that the walls would not reach the widest point of an imaginary sphere that would fill the nest hollow (‘half of a sphere’).	3
	Cup-shaped nest with 1 side that is half of a sphere	3.25
	Cup-shaped nest with 2 sides that are half of a sphere	3.5
	Cup-shaped nest with 3 sides that are half of a sphere	3.75
Incomplete Dome	Bedding material has been gathered to form a nest site in the cage. The walls reach (and may close back over) the widest point of an imaginary sphere that would fill the nest hollow (‘half of a sphere’).	4
	Incomplete dome with 1 side that is more than half of a sphere.	4.25
	Incomplete dome with 2 sides that are more than half of a sphere.	4.5
	Incomplete dome with 3 sides that are more than half of a sphere.	4.75
Complete Dome	Nesting material has been gathered to form a nest site in the cage. The walls completely enclose the nest hollow. A small (mouse-sized) exit hole may be found on the side or the top of the dome	5

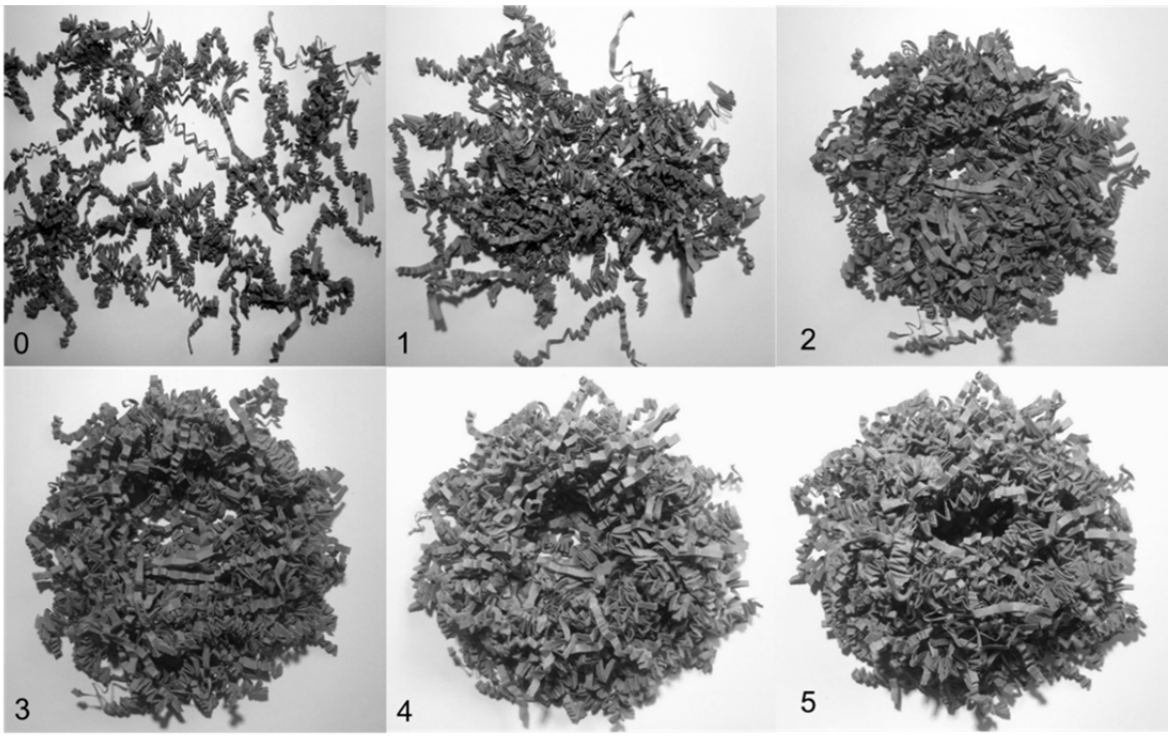

Figure 9: Nest complexity scores. Assessed scores according to the naturalistic nest scoring system developed by Hess et al. Scores from 0 to a maximum of 5 can be reached.

- **Explorative behavior**

Additional, the time to integrate nest material into the nest (TINT) was measured. This test is used to evaluate wellbeing and pain in mice by testing if new nesting material is integrated in to the existing nest [24, 26]. During the first baseline measurements, it turned out, that the animals interacted regularly with the added nest material but never integrated it into their nests, leading to a negative test result in all animals. The protocol was therefore changed towards testing explorative behavior rather than a nest building behavior. The explorative behavior test was used to assess motivation to interact with a new object in the home cage. This motivation was assumed to be decreased in states of pain or reduced wellbeing. The two animals were provided with a novel object, which in this case was a ball of four Envirodri® stripes. Without disturbing the animals, the ball of nesting material was put in the home cage, the lid was closed and the animals were observed for one minute by a blinded observer. The outcome was either negative (score = 0) with no interaction or positive (score = 1) with an interaction with the new object. Interaction was defined as an active dealing with the provided object (carrying, holding with forelimbs, rolling and intensive sniffing). This test was not animal based but cage based.

- **Home cage video analysis - Activity**

To evaluate mice activity the video recordings were used. Like previously described (drinking frequency analysis) eight animals of each treatment group (Tlow, Thigh, BUP) were filmed in their home cages for 24h before and 48h after the procedures (Figure 10). Of each treatment group four osteotomized, two animals of the anesthesia and analgesia only group and two animals receiving analgesia only were recorded. Cages were filmed from above (camera distance about 1.5 m) in standard housing cages without grid. To prevent escape the cages had raised walls. Mice were kept with their familiar partner, provided with nesting material, food and water. One mouse in each cage was marked with black stripes on the tail. In the dark phase, an infrared light was turned on to provide proper illumination. Videos were then analyzed manually by one blinded observer with an event logging software (BORIS - Behavioral Observation Research Interactive Software). The occurrence and duration of time spent in the nest was assessed manually in seconds for each hour as the resting time (RT).

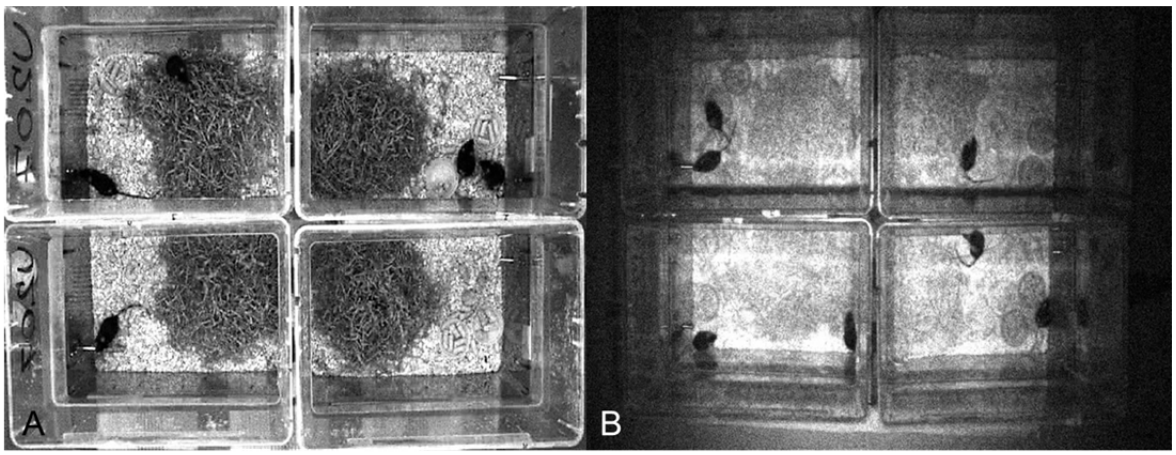

Figure 10: Home cage video analysis. Screenshots A) during light and B) dark phase. Two animals per cage with one animal being marked at the tail.

4.2.7 Model-specific parameters evaluating analgesic efficiency

Mice were individually put in standard cages covered with a filter top. The wire grid was removed to keep animals from climbing during the measurement. They were given an acclimation time of 15 minutes before being filmed for three minutes (IPhone5, IPad2). The cage was filled with regular bedding material but did not contain any nesting material, food or water. The videos were later analyzed by one observer blinded to the treatment. Seven different parameters were recorded (Table 3):

- **Limp score**

The use and weight bearing of the operated left hind limb was evaluated with a subjective pain scale modified to serve the setup of the used osteotomy model [83, 84]. Each animal was assigned a score considering the degree of limping with the operated leg.

- **Dragging score**

Additional to limping, dragging of the left hind limb occurred in some operated animals. Therefore, dragging was graded by assigning a score to the animal. This score is based on our experience of the manifestation of the dragging.

- **Grooming**

The total time an animal spent with grooming the left hind limb was measured during the observation time. Licking, combing and rubbing with the forelimbs was characterized as grooming.

- **Rear up**

The time of being in a rear-up position with weight bearing on both legs was measured.

- **Flinching**

Flinching is a spontaneous pain behavior and is characterized as a rapid, repetitive lifting of the affected limb [85, 86]. Frequency of flinching with the left hind limb was recorded as the total number of flinches.

- **Guarding**

Time of guarding the left hind limb was recorded. Guarding was defined as an active lifting of the left hind leg while pressing it against the torso [85, 87]. The floor was not touched with the guarded leg. Guarding behavior was assessed as total time of guarding in the total recording time.

Table 3: Model-specific pain parameters

Parameter		Scores
Limping	• normal use • sporadic limping/hopping, complete ground contact • limping, constant hopping • partial non-use of limb • complete lack of use	0 1 2 3 4
Dragging	• normal use • sporadic dragging of toes • constant dragging of toes • sporadic dragging of complete leg • constant dragging of complete leg	0 1 2 3 4
Rear up	• time rearing on both legs	seconds
Grooming	• time grooming spend solely towards the operated leg	seconds
Flinching	• number of flinches	frequency
Guarding	• time guarding operated leg	seconds

4.2.8 Assessment of bone healing

- ***In vitro* µCT measurements**

Sample collection and preparation

After euthanasia, the operated left femur was carefully detached from the hip joint and separated from the lower leg by cutting the fibula and tibia slightly distal of the knee joint. The muscles were carefully removed sparing about three millimeters of muscle layer around the bone, which served as a protection around the fracture in the first time of fixation. The external fixator stayed in place during the whole fixation phase. The bones were transferred into a PFA (4%) filled tube and fixated for 24 hours. Following, they were put in an ascending sugar solution of 10%, 20% and 30% for 24 hours each. After fixation, the samples were prepared for *in vitro* µCT by careful removal of the remaining muscle layer. To provide standardized, even images and fixation during *in vitro* µCT imaging, the bones were slid into the lengthways cut narrow part of a plastic pipette which was filled with a 30% sugar solution. The external fixator was outside the pipette. Pins were carefully extracted with a surgical wrench and the external fixator could be removed. Due to the stabilization in the plastic pipette, collapse of possibly unstable fracture gaps was prevented. Four bones were analyzed at once. Care was taken to avoid air between the samples. The plastic pipette containing the samples was then transferred into a 15 ml plastic tube,

filled with 30% sugar solution. The plastic tube containing the bone samples was put in the *in vitro* µCT and analyzed with an isotropic voxel size of 10.5 µm.

Settings

The scan axis was according to the diaphyseal axis of the femur. The osteotomy gap of the bone was magnified six times in the analyzing software and manually defined by the analyzer. The area between the slide where only half of the cortex was still visible and the slide where half of the cortex was visible again was considered as the osteotomy gap (Figure 11). The amount of analyzed slides in the fracture gap was noted.

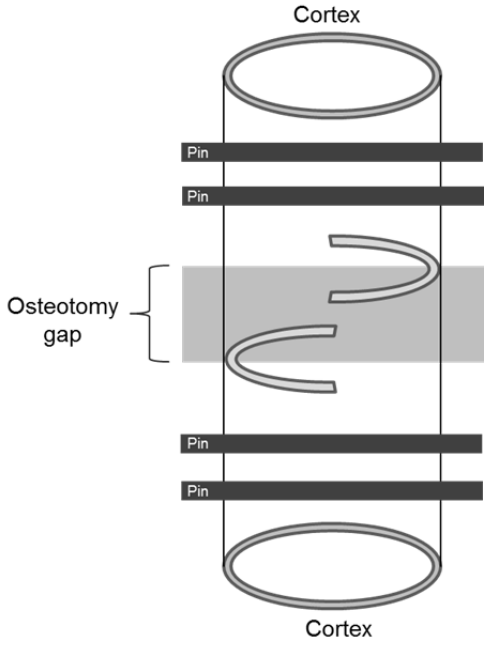

Figure 11: Defining the osteotomy gap in *in vitro* µCT analysis. The four pins enclose the fracture gap. Measurement takes place where the cortex opens up half and where half of the cortex is visible again.

Image analysis

The region of interest (ROI) was manually included while the cortical bone was manually excluded from the ROI. A fixed global threshold of 240 mg HA/cm³ was applied for the automatic 3D callus tissue analysis. The total volume (TV in mm³), the total bone volume (BV mm³) and the calculated relative bone volume (Rel. BV in %) were included in the analysis.

- **Histology**

Preparation and embedding

The bones previously used for *in vitro* µCT were carefully removed from the plastic pipette, individually embedded in a SCEM medium and immediately frozen in liquid nitro-

gen. The resulting blocks of frozen medium with the femur inside were stored at -80°C until cutting.

Cutting

The embedded bones were attached into the cryostat in a horizontal order. Slices were taken off until the middle of the bone was reached. The middle was defined as the area where all four pinholes and the fracture gap were visible. Reaching the designated area was checked microscopically with sample slides during the slicing process. Once this area was confirmed, ten slices of 7 µm each were taken. The slices were picked up with Cryofilms stripes, transferred to a microscope slide and taped down at both ends. The microscope slides were left to air dry overnight and then stored at -80°C until staining.

Movat-Pentachrome staining

The slides were air dried for 30 min, fixated in PFA for 10 min and washed in distilled water for 5 min. 3 min in acetic acid 3%, 30 min in alcian blue and a quick wash in acetic acid 3% followed. Subsequently, the samples were put in distilled water for 3 min before being transferred to alkaline ethanol for 60 min and washed in tap water for 10 min. The water was changed every 2 min. One dip in distilled water and 15 min in Weigert's hematoxylin followed. The next step contained washing in tap water for 15 min, water was changed every 2 min followed by 15 min in crocein scarlet-acid fuchsin. Afterwards the slides were rinsed in 0.5% acetic acid, put in phosphotungstic acid for 20 min and again rinsed in 0.5% acetic acid. The staining process continued with an ethanol series, 100% ethanol three times 5 min each, before 60 min in alcoholic safran. The sections were transferred to the second ethanol series, three times 100% ethanol 2 min each. At last the slides were put in xylene two times for 2 min each, mounted and covered with a cover slid.

Image analysis

Full bone images were taken automatically with a confocal laser scanning microscope in a five-time magnification (Figure 12). The focus during image acquisition was set on the area of the fracture gap (Figure 13).

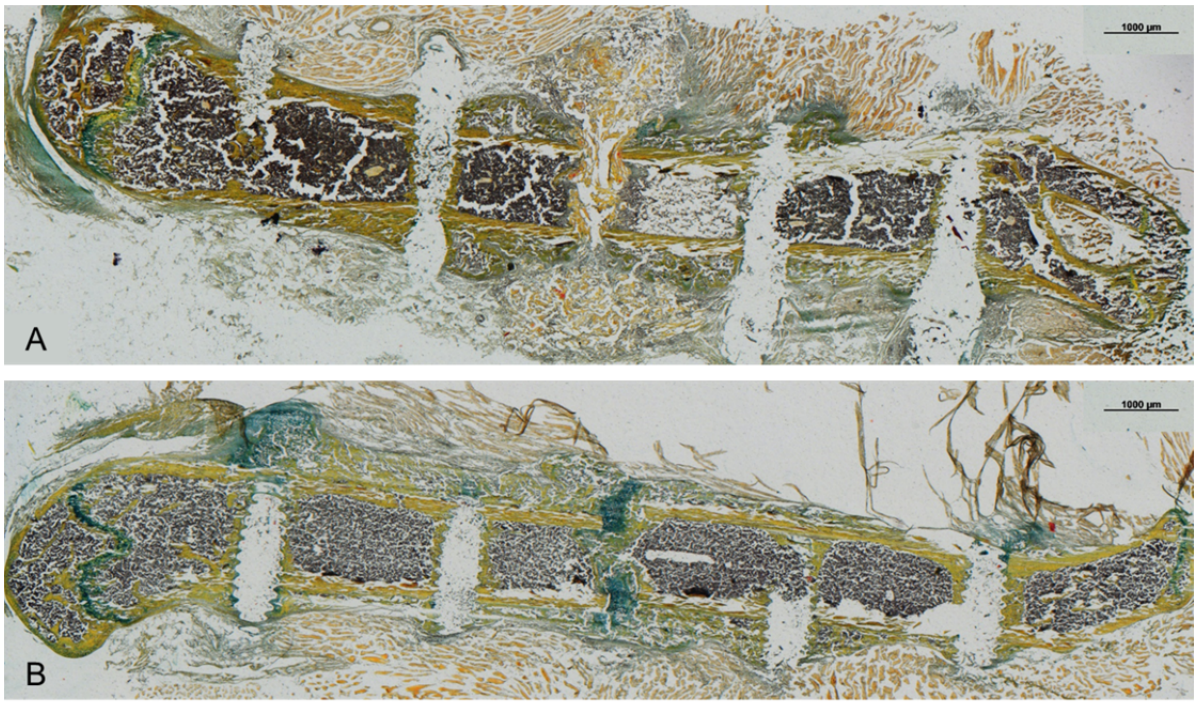

Figure 12: Overview image of an osteotomized murine femur. A) Lyostypt scaffold in the fracture gap, B) Fracture gap without Lyostypt scaffold.

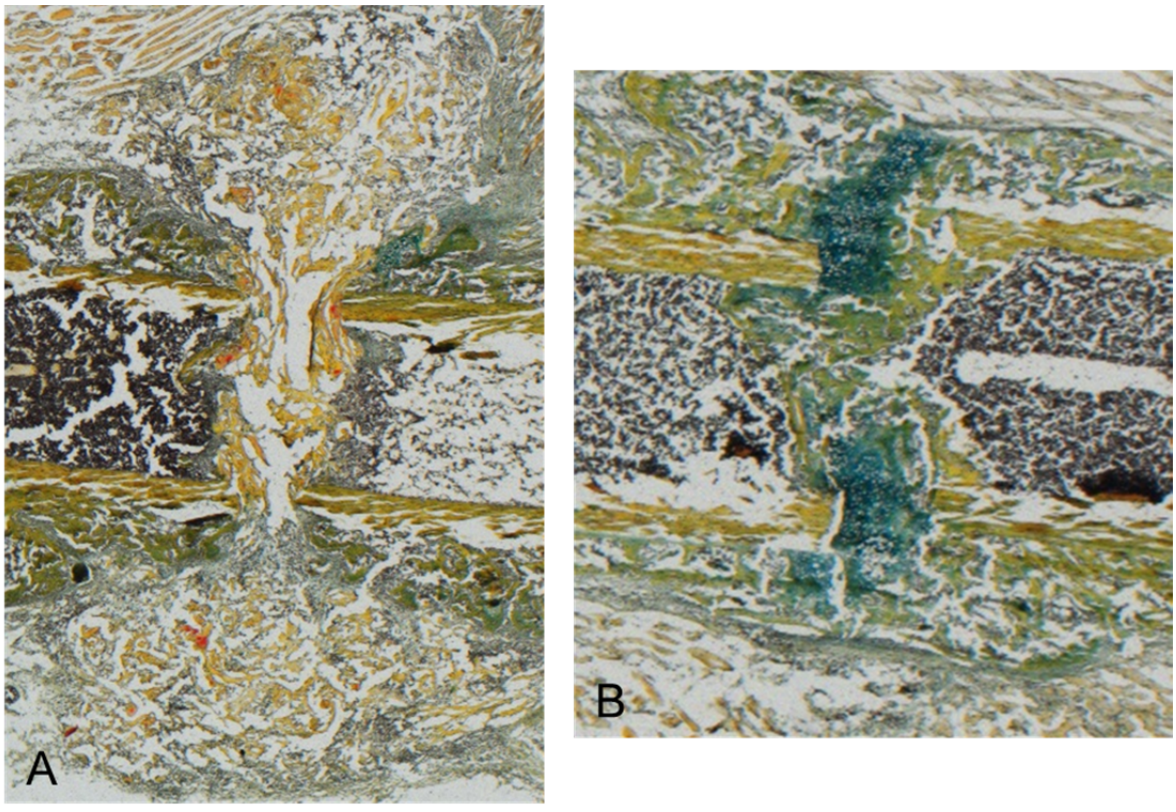

Figure 13: Close up on the fracture gap. A) With and B) without Lyostypt scaffold

Gathered images were then analyzed with the software ImageJ and a self-programmed Makro. At first, the total region of interest (Total ROI) was selected. This region includes the endosteal, intracortical and periosteal area around the fracture gap. The void area was

defined by excluded blank areas via a threshold and manual selection. The ends of the cortices in the total ROI were selected with polygon drawing. In the next step, the area of mineralized bone in the fracture gap was marked by setting a threshold. Again, a threshold was set to define the scaffold in the fracture gap. A manual selection of the bone marrow followed. In the last step, the connective tissue in and around the fracture gap was calculated as the remaining unselected area from the previous steps (Figure 14).

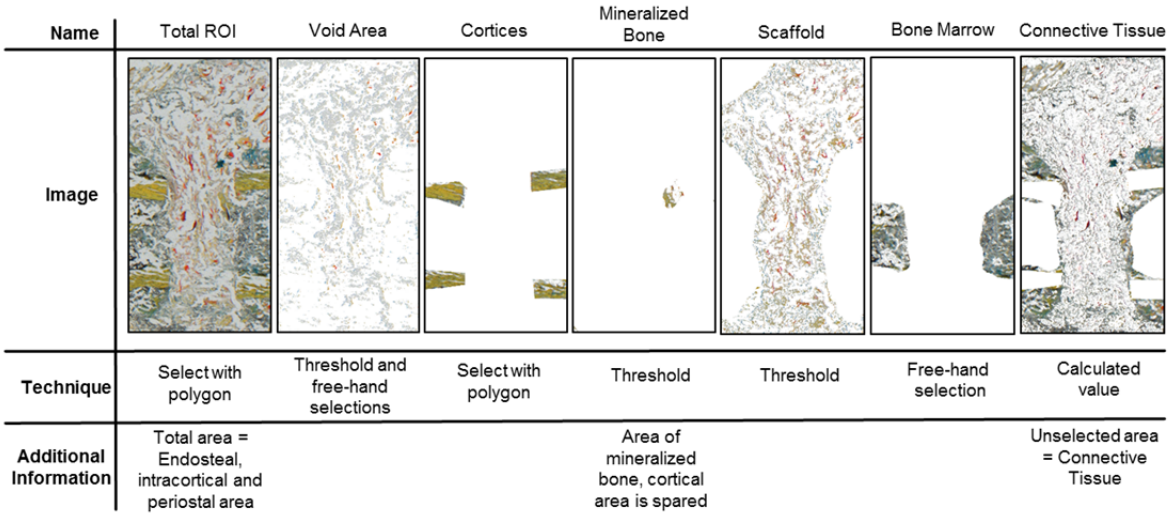

Figure 14: Step-by-step analysis of movat-pentachrome stained bone slides in ImageJ. At first the different tissues are defined manually and automatically with varying techniques. Then their share in total displayed tissue is calculated.

4.2.9 Side effects

- **Liver histology in cooperation with Prof. Klopfleisch (FU Berlin)**

Sample preparation

The liver of each operated animal was removed after euthanasia, transferred to an Eppendorf tube and immediately put on ice. The livers were stored at -80°C. Before embedding the livers were fixated in 4% PFA for 24 hours and then sent to the Institute of Veterinary Pathology at the Department of Veterinary Medicine FU Berlin.

Further processing

Here the livers were embedded in paraffin, sliced in a microtome and stained with hematoxylin & eosin staining. The samples were histologically assessed by Prof. Klopfleisch to evaluate a possible impact of tramadol and buprenorphine on the liver.

4.2.10 Serum analysis in cooperation with TOXILAB Ludwigsburg

To date there is no data on the effective serum concentration of tramadol in rodents. Therefore, blood serum of 18 mice from the POC study on bone healing was obtained after euthanasia three days postoperative. These animals were treated with tramadol (0.1 mg/ml; Tlow) in the drinking water for three days after the surgery. The blood samples were analyzed by TOXILAB Ludwigsburg for tramadol and O-desmethyltramadol (M1) values. Tramadol is metabolized by the liver resulting in O-desmethyltramadol. This M1 metabolite is the main analgesic effective metabolite of tramadol. The amount of tramadol and M1 in the mouse serum was compared to the established minimal effective concentration of the two substances in humans [88].

4.2.11 Impact of pre-emptive buprenorphine on bleeding during surgery

In the underlying POC study on fracture healing the procedure contained a buprenorphine injection during the preparation for surgery. In the current study, the protocol was changed. Buprenorphine was applied one hour prior to the surgery to reach sufficient analgesic levels during the surgery [89-91]. During the first surgeries of the current study, a possible influence of the pre-emptive buprenorphine on bleeding during the surgery was observed. Animals only related to the underlying proof of concept study on bone healing and not included in the refinement project were used to assess the impact of buprenorphine on increased bleeding during osteotomy surgery. These animals were still to be operated at that time point and no additional animals were used. For this purpose, animals were allocated to one of three treatment groups (**Fehler! Verweisquelle konnte nicht gefunden werden.**) and injected according to the group's schedule. The bleeding was assessed during surgery. The operator, blinded to the animals' treatment, graded the bleeding of the skin, pin placement and gave a general bleeding score (Table 4).

Table 4: Scoring of bleeding during surgey.

Skin incision	Pin placement	General bleeding	Score
no – mild bleeding; singular dabbing off the blood was sufficient to stanch the bleeding	no – mild bleeding that directly stopped	no – mild bleeding – common situation during the surgery	1
mild – strong bleeding; two to three times dabbing of the blood was needed to stanch the bleeding	mild – strong bleeding dapping was needed	mild – strong bleeding dapping was needed several times	2
strong bleeding; dabbing was not sufficient; limited view on underlying muscles	strong bleeding; dabbing was not sufficient	strong bleeding; dabbing was mostly not sufficient; limited view on structures	3

4.2.12 Sample size calculation

To determine the animal number necessary to test whether different pain management protocols have an influence on the bone healing an a priori analysis is done. Data on a previously accomplished fracture healing study was consulted. The ratio of bone volume and total volume (BV/TV) of vehicle and non-treated animals 2 weeks after the osteotomy is used. A group size of four animals was used. The BV/TV in vehicle animals mean was 0.31 with 0.08 SD. In non-treated animals mean was 0.56 with 0.02 SD. Effect size was determined as 4.29. Calculation with G*Power with a statistical power of 0.95 and an effect size of 4.29 results in a necessary animal number of three per group.

In the bone healing parameters, an animal number of four animals in the vehicle and four animals in the untreated group is suggested to have a small buffer. A total of eight animals have to be operated.

To test the first hypothesis on analgesia in the drinking water, water intake hast to be assessed. For the animal number necessary data on a previously accomplished study is consulted [17]. The water intake of mice after a laparotomy with buprenorphine was measured. Eight animals were used. Two time points were measured 24h before and after the surgery. The water intake before the laparotomy was at a mean of 4 g per mouse with a SD of 0.2. Postoperative the water intake was a mean of 2.9 g with a SD of 0.5. Effect size was determined as 5.25. Calculation with G*Power with a statistical power of 0.95 and an effect size of 5.25 results in a necessary animal number of three.

Animals in the current study are kept in pairs. The water intake is assessed per cage with two animals. In conclusion, a minimum of three cages should be used to reach a sufficient sample size.

If the pain management protocols have an impact on the wellbeing and behavior of the animals was tested. Necessary animal numbers are determined. Here, another study was consulted for the post hoc computation of the required animal numbers [92]. The activity of mice after a sham-embryo transfer was measured. Eight animals were used in each group. One group of surgery with analgesia and one not operated group was tested. The activity postoperative in the surgery group had a mean of 220 min with a SD of 45 min. The activity in the control group was 114 min in the mean with a SD of 26 min. Effect size was determined as 2.88. Calculation with G*Power with a statistical power of 0.95 and an effect size of 2.88 results in a necessary animal number of five.

In combination, the necessary animals for the bone healing and water intake parameters lead to an animal number of eight animals in each osteotomy group and four animals in the control groups. This calculation is in line with the needed animals for activity measurements which is at least five.

4.2.13 Statistical analysis

All statistical analyses were performed with GraphPad Prism V.5 software for windows. Baseline and experimental measurements are assessed in the same manner and at the same time points during the day. Median with range is used for presentation of the data in the following parameters: body weight, clinical score, nest score, drinking frequency within 6 hour periods, resting time, model-specific pain parameters, short-term stress, bone parameters and bleeding. Mean with SD is used in the parameters food and water intake, serum analysis. Mean only is used in the explorative behavior, and long-term stress. The drinking frequency within 1 hour periods is assessed in total values. All data is tested for normal distribution. As it does not meet the assumptions for parametric testing, non-parametric analyses are used. For statistical analysis, the Kruskal-Wallis test is applied. The Mann-Whitney test is used in the parameters of bone healing. The comparison takes place between the treatment groups not between the time points in the group. P-values of ≤ 0.05 are considered significant.

5 Results

5.1 Body Weight

Body weight of the animals was assessed prae- and postoperative as a measurement of wellbeing and general health. The results are displayed as a percentage change from the baseline weights (Figure 15). Operated animals showed a decrease in their body weight on day 1 after surgery and continuously recovered on the following days. Tlow animals reached the values of control animals on day 4. Thigh operated mice showed the highest decrease (Median: 86.1%, Range: 9.3) and regenerated on day 5. Body weight of BUP operated animals decreased least of all groups (Median: 94.5%, Range: 12.5) and reached control levels on day 3. Three days after surgery, body weight of Thigh and BUP mice differed significantly (Figure 16, p-value < 0.01). Tlow Sham animals showed the largest weight decrease on day 1 after anesthesia (Median: 88.3%, Range: 12,5), followed by an increase to the level of control animals on day 4. Body weight of Thigh sham mice dropped down to the lowest value on day 2 (Median: 85.5%, Range: 10.6), the highest decrease in all animals. On day 7 they reached control values. The weight of the BUP group decreased least and regenerated on day 3. Animals with analgesia only showed the smallest weight decrease in all groups. The highest weight decrease occurred on day 1 followed by a continuous increase. Body weight of Tlow animals increased to control values on day 3. BUP animals had the lowest body weight decrease. Thigh animals showed the highest weight decrease within the drinking water group (Median: 90.7%, Range: 1.6), they regenerated to control values on day 7. In conclusion, in all groups Thigh animals lost the most weight.

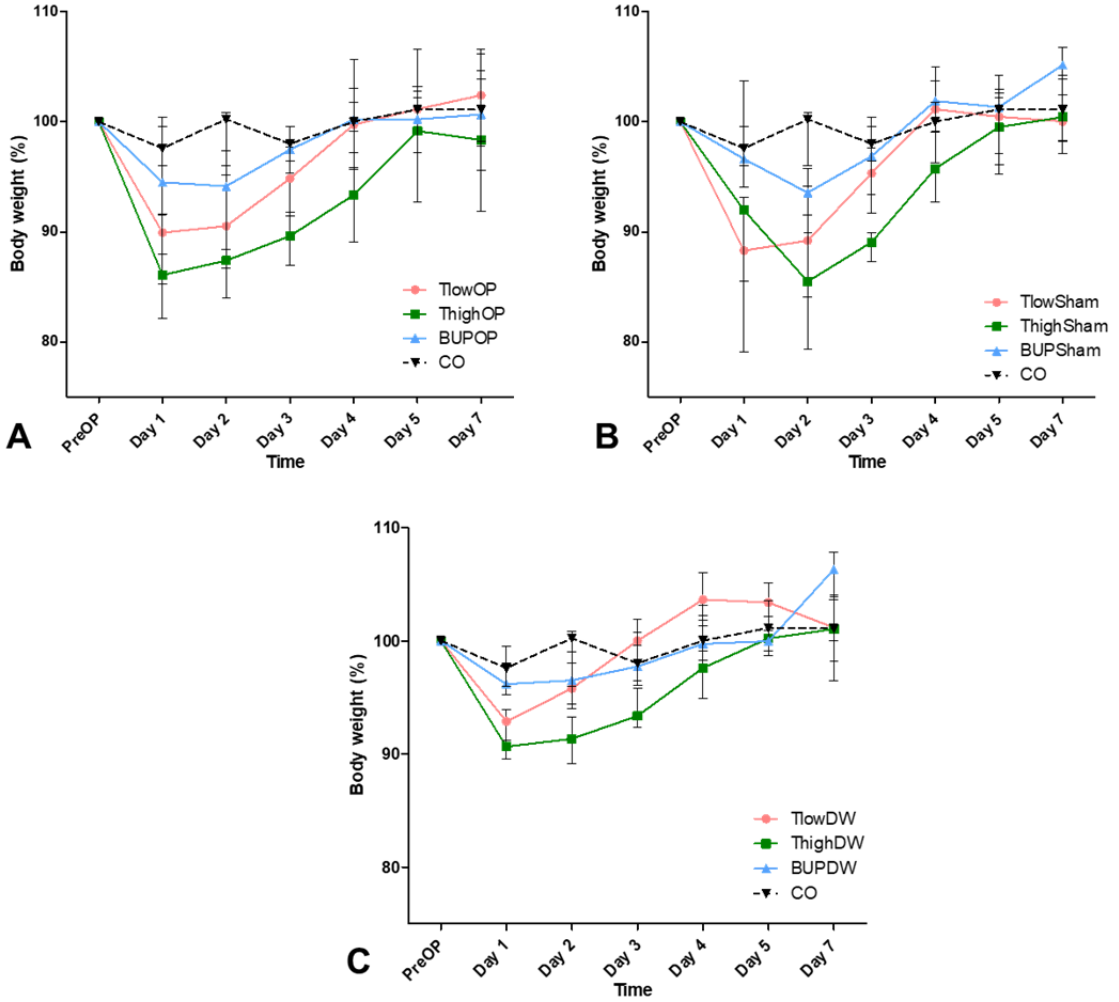

Figure 15: Body weight. Percentage of body weight change over time compared to body weight before surgery. A) Operated and B) Sham animals showed high decreases of body weight after surgery respectively anesthesia. C) Analgesia only animals showed a decreases that was balanced more rapid compared to the operated and Sham animals. (Graphs show Median with Range; TlowOP/BUPOP n=8, ThighOP n=7, others n=4).

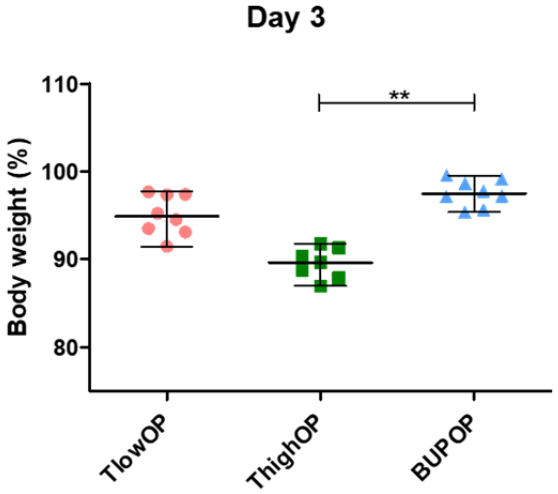

Figure 16: Body weight change on day 3. Significant difference in body weight change in ThighOP and BUPOP on day 3 after surgery (Graphs show Median with Range; TlowOP/BUPOP n=8, ThighOP n=7. Significance tested with the Kruskal-Wallis test with Dunn´s correction, **p-value<0.01).

5.2 Food Intake

Food intake was measured daily before and after surgery to assess changes in the intake affected by the treatment (Table 5). TlowOP showed a slight decrease in their food intake in the 24h after surgery (Mean: 5.1 g, SD: 1.4) while ThighOP values were decreased at 24h and 48h (Mean: 4.4 g/4.6 g, SD: 0.4/0.9). BUPOP had a decreased intake at 72h (Mean: 5.9 g, SD: 1.5). TlowSham showed a reduced intake at 48h (Mean: 4.5 g, SD: 0.5), while the ThighSham had low values 24h and 48h after surgery (Mean: 5.6 g/3.0 g, SD: 0.5/2.5) and BUPSham at 72h (Mean: 3.9 g, SD: 0.4). The animals of the DW groups showed no decreases, besides a slight reduction of intake in ThighDW at 72h (Mean: 6.3 g, SD: 3.6). Taken together, Thigh operated and Sham animals showed high reduction in food intake values.

Table 5: Food intake for each group. Values given in g per cage with two animals each per 24h (Table shows Mean with SD; TlowOP/BUPOP n=4, ThighOP n=3, rest n=2).

	Baseline	24h	48h	72h	96h
Tlow OP	7.8 (1.4)	5.1 (1.4)	8.0 (1.2)	7.7 (2.0)	9.5 (0.8)
Thigh OP	8.8 (0.5)	4.4 (0.4)	4.6 (0.9)	8.1 (0.4)	11.0 (0.8)
BUP OP	8.5 (1.0)	7.1 (1.8)	8.3 (2.0)	5.9 (1.5)	14.1 (3.7)
Tlow Sham	8.0 (1.9)	8.3 (5.9)	4.5 (0.5)	10.1 (4.6)	8.9 (4.4)
Thigh Sham	9.2 (0.7)	5.6 (0.5)	3.0 (2.5)	10.0 (0.1)	10.6 (0.3)
BUP Sham	8.3 (1.0)	9.4 (2.2)	9.8 (2.2)	3.9 (0.4)	8.7 (4.2)
Tlow DW	7.9 (0.7)	10.8 (2.2)	8.4 (0.6)	7.0 (0.5)	8.1 (2.8)
Thigh DW	8.3 (0.7)	8.0 (0.4)	8.5 (2.0)	6.3 (3.6)	10.3 (3.1)
BUP DW	8.3 (1.2)	10.3 (0.4)	10.9 (1.4)	13.0 (1.0)	8.0 (0.8)
CO	7.8 (1.0)	13.9 (4.3)	7.2 (3.3)	6.8 (0.5)	7.1 (0.3)

5.3 Water Intake

The water intake was measured daily before and after surgery to assess changes in intake affected by the treatment (Table 6). TlowOP showed a reduction in the 24h postoperative (Mean: 5.5 ml, SD: 2.9). Values of ThighOP were slightly reduced at 48h (Mean: 6.2 ml, SD: 3.1), while BUPOP levels were not altered. At 24h water intake of only one TlowSham cage could be assessed. In ThighSham water intake at 48h was decreased (Mean: 2.6 ml, SD: 1.7). BUPSham animals drank consistent amounts. In the DW group only BUPDW had a reduction at 24h (Mean: 4.6 ml, SD: 2.4). In summary, water intake values varied strongly, nevertheless means were in general within the normal range and only in the Thigh Sham groups distinctly reduced at 48h.

Table 6: Water intake for each group. Values given in ml per cage per 24h. Note that at 24h postoperative only one cage of the TlowSham group could be assessed. (Table shows Mean with SD; TlowOP/BUPOP n=4, ThighOP n=3, rest n=2)

	Baseline	24h	48h	72h	96h
TlowOP	9.1 (0.9)	5.5 (2.9)	9.3 (2.0)	11.2 (1.1)	8.3 (1.0)
ThighOP	10.2 (1.3)	13.3 (4.3)	6.2 (3.1)	8.9 (4.6)	8.9 (4.6)
BUPOP	8.0 (8.0)	8.3 (3.1)	7.7 (2.6)	11.4 (1.3)	10.0 (0.6)
TlowSham	8.4 (0.8)	*6.6*	8.5 (1.5)	12.8 (1.5)	9.6 (0.0)
ThighSham	9.3 (1.1)	9.6 (3.6)	2.6 (1.7)	10.1 (0.7)	10.5 (0.6)
BUPSham	6.8 (0.8)	7.5 (1.1)	6.4 (0.4)	9.7 (0.1)	8.1 (0.0)
TlowDW	9.0 (1.1)	8.4 (2.1)	9.1 (3.3)	8.9 (0.1)	7.8 (0.0)
ThighDW	9.2 (1.2)	10.9 (6.2)	6.9 (1.6)	7.4 (0.4)	10.5 (0.3)
BUPDW	6.8 (0.6)	4.6 (2.4)	6.4 (1.9)	8.5 (1.2)	6.4 (0.3)
CO	9.8 (1.2)	17.6 (5.3)	9.4 (2.5)	7.9 (0.4)	10.1 (2.0)

5.4 Drinking Frequency

The drinking frequency was assessed manually from video recordings to evaluate the distribution of drinking events throughout the day (Figure 18 and Figure 20) and night (Figure 17 and Figure 19). All animals had a higher intake frequency in the dark than in the light phase.

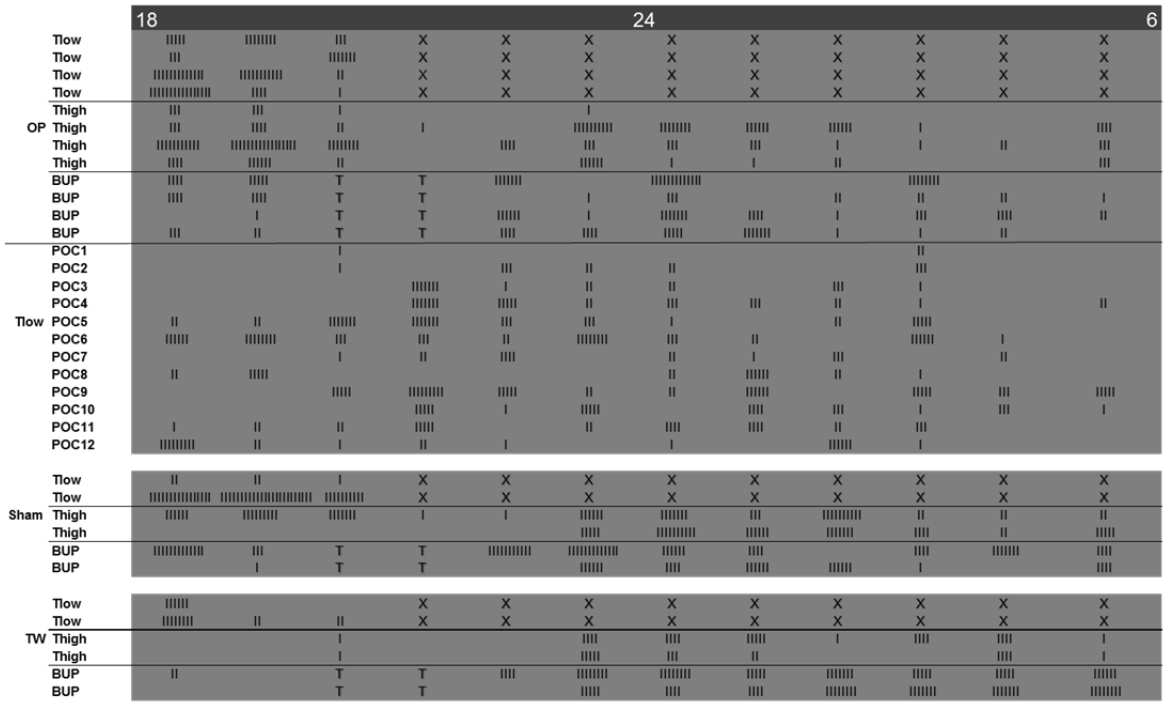

Figure 17: Drinking in the first night. Drinking events that occurred within one hour. From 18:00 to 6:00 starting on the day of surgery. Every row represents one animal and every line one drinking event. POC animals from proof of concept study. (X=data not available due to technical problems, T=animals were tested for clinical score and model-specific parameters for the complete hour).

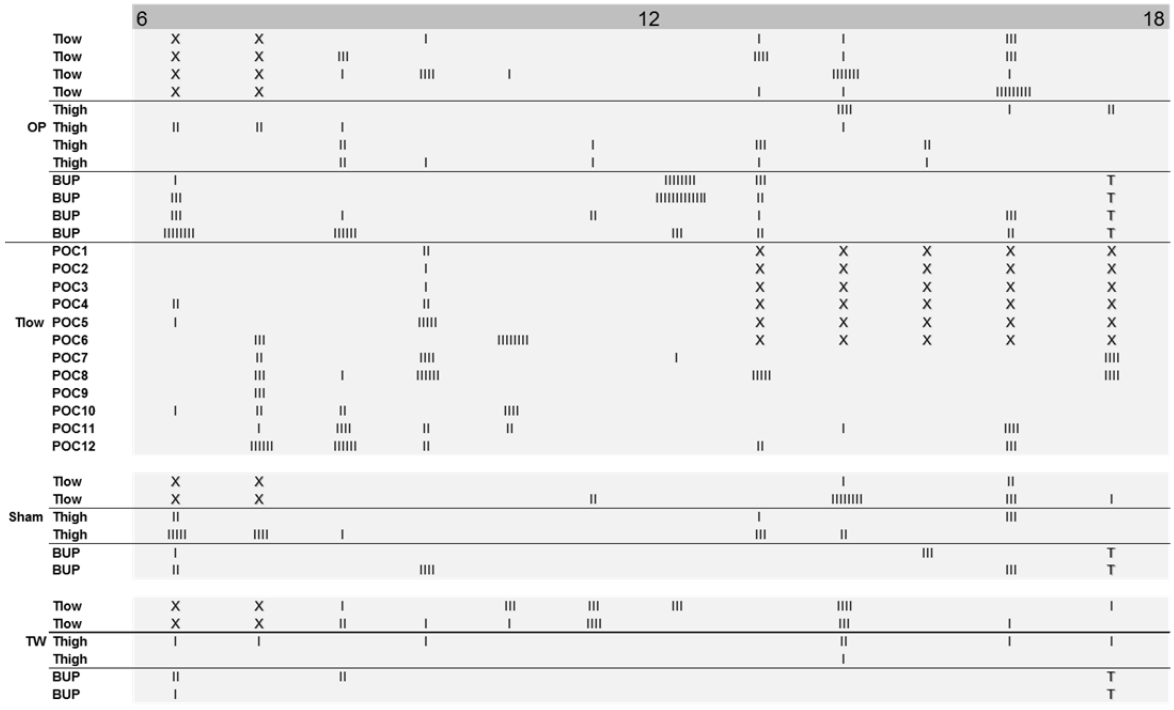

Figure 18: Drinking on the first day. Drinking events that occurred within one hour. From 6:00 to 18:00 starting in the morning on the first day after the surgery. Every row represents one animal and every line one drinking event. POC animals from proof of concept study. (X=data not available due to technical problems, T=animals were tested for clinical score and model-specific parameters for the complete hour).

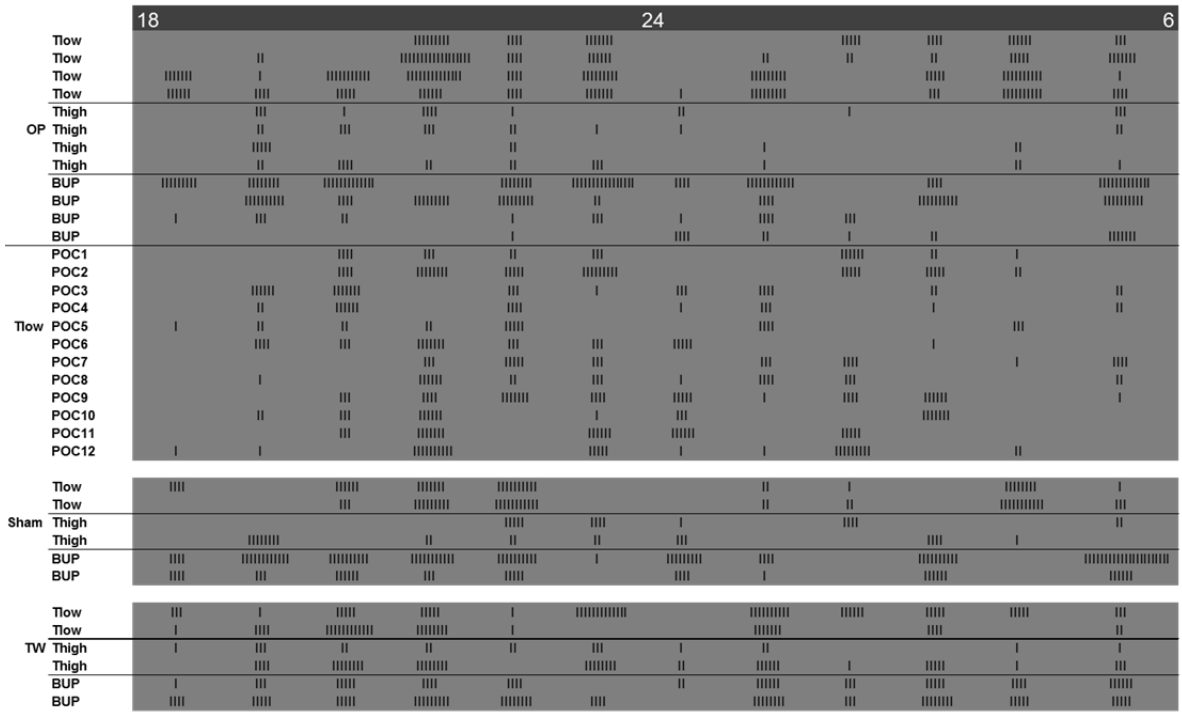

Figure 19: Drinking in the second night. Drinking events that occurred within one hour. From 18:00 to 6:00 starting on the day after surgery. Every row represents one animal and every line one drinking event. POC animals from proof of concept study.

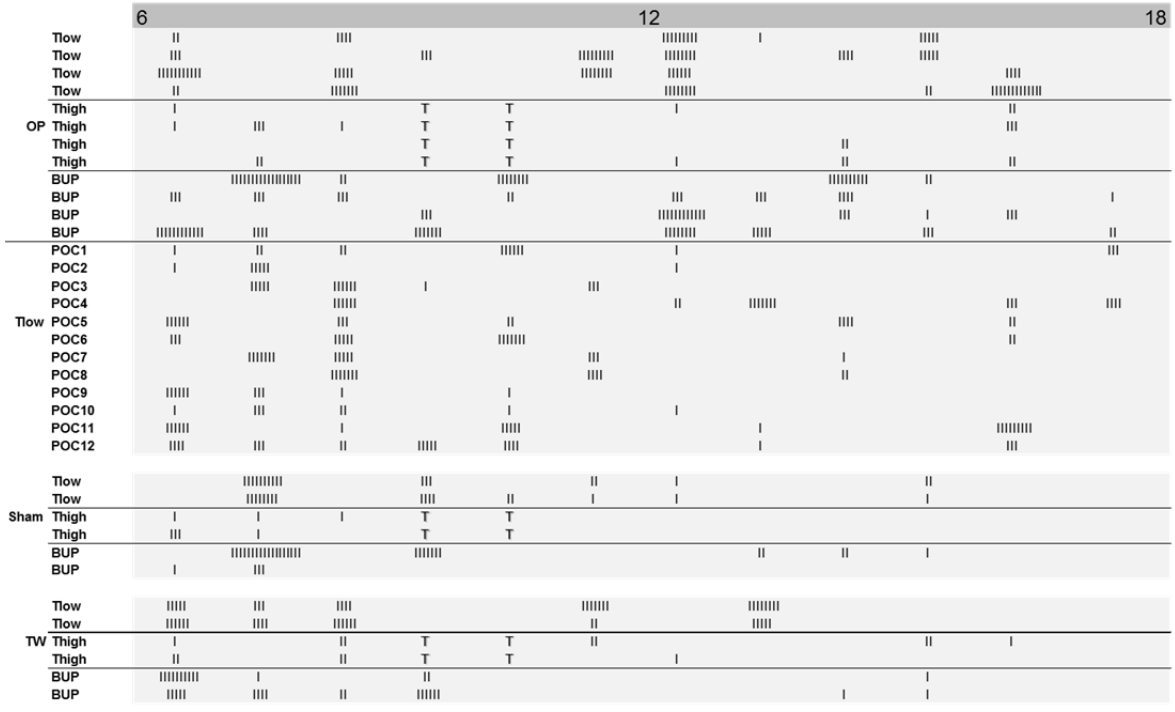

Figure 20: Drinking on the second day. Drinking events that occurred within one hour. From 6:00 to 18:00 starting in the morning on the second day after the surgery. Every row represents one animal and every line one drinking event. POC animals from proof of concept study. (T=animals were tested for clinical score and model-specific parameters for the complete hour).

Taken together, animals of all groups have more drinking events during the night than during the day. Thigh treated animals showed less drinking events in the second night and day after surgery compered to all other groups.

5.5 Stress

- **Fecal Corticosterone Metabolites**

To evaluate short-term effects of stress FCM were assessed with ELISA. FCM values peaked on day 1 in all groups and then dropped immediately on day 2 to lower values than control animals with handling only until the end of measuring (Figure 21). As shown in Table 7 the numbers of fecal samples varied highly especially at 24h and 48h after surgery. To summarize, all animals showed a similar distribution of FCM levels during the five testing days.

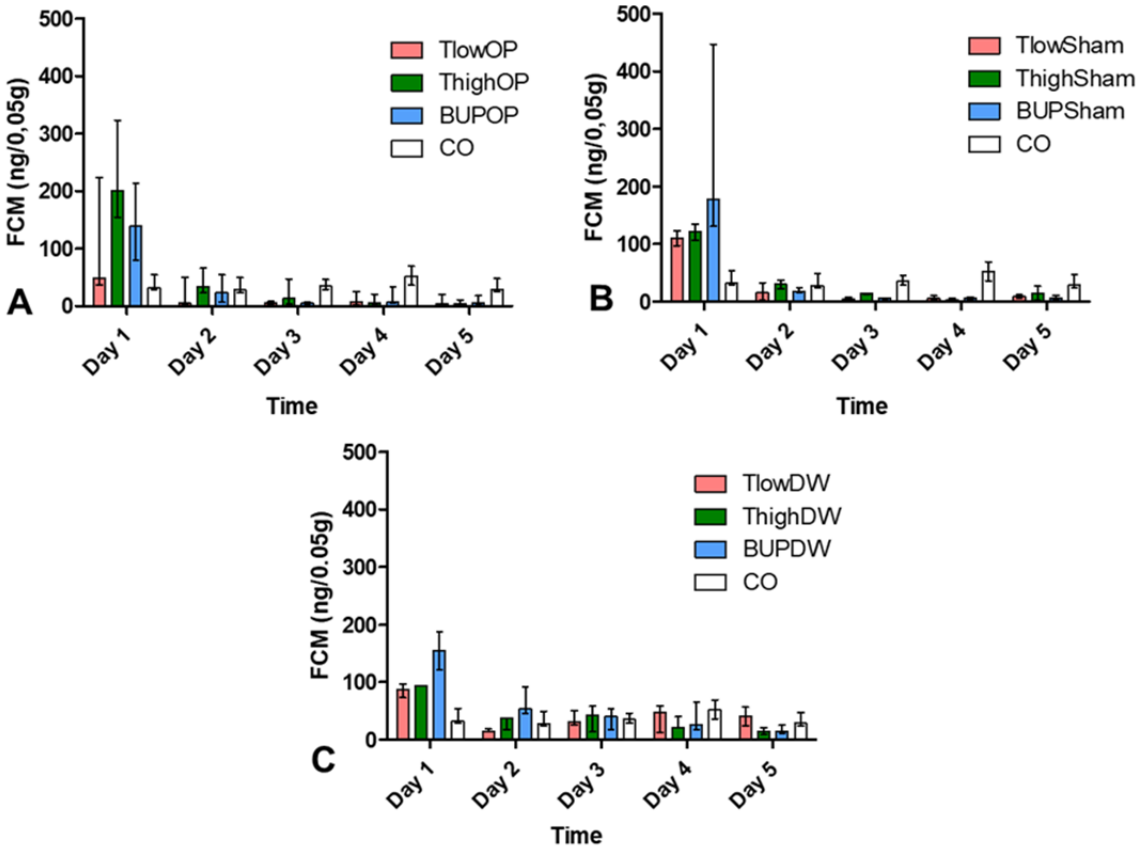

Figure 21: Levels of FCM. A) Operated, B) Sham and C) animals with analgesia only. (Graphs show Median with Range; n-numbers see Table 7)

Table 7: Fecal samples. Numbers of samples of each treatment groups on different timepoints.

	24h	48h	72h	96h	120h
Tlow OP	3	3	7	7	8
Thigh OP	4	5	7	5	7
BUP OP	5	7	8	8	8
Tlow Sham	2	2	4	4	4
Thigh Sham	4	3	4	4	4
BUP Sham	4	3	4	4	4
Tlow DW	3	2	4	4	4
Thigh DW	1	3	4	4	4
BUP DW	3	3	4	4	4
Control	2	3	4	4	4

- **Hyperplasia and hypertrophy in the adrenal glands**

To display potential long-term stress, changes in the adrenal glands of the operated animals are assessed. For comparison, the adrenal glands of naïve animals were evaluated. No significant differences in the ZF/ZG-ratio were found between the operated groups or compared to naïve control animals (p=0.5276) (Figure 22).

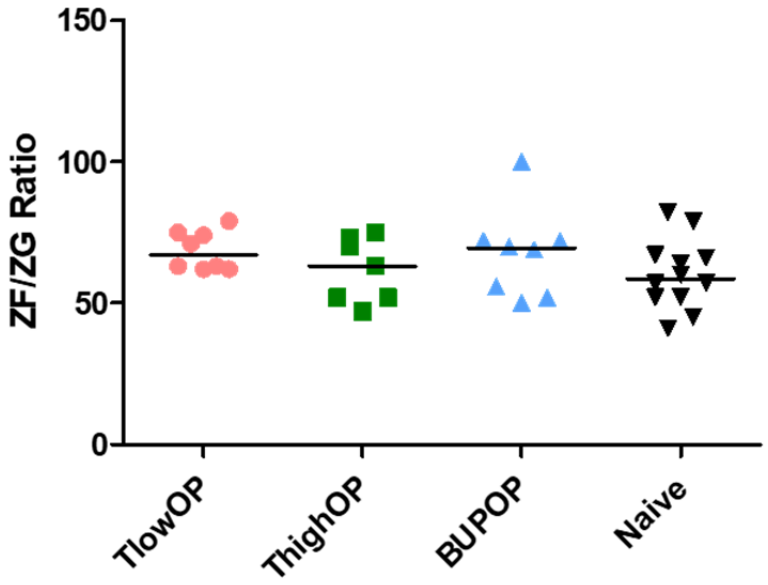

Figure 22: ZF/ZG-ratio in adrenal glands. The ratio shows no differences in adrenal glands of operated and naïve animals. (Graph shows Mean; operated animals n=8, naïve animals n=12; Significance tested with the Kruskal-Wallis test with Dunn's correction)

5.6 Clinical Scoring

Changes in behavior due to pain, reduced wellbeing or impairment of movement were assessed with the help of a clinical score. The results are shown in Figure 23. A maximal score of 11 points is possible. Control animals showed scores of zero. Operated animals from all groups showed a peak one hour after the surgery, then values declined over time. Sham animals reached similar values than the ones with an osteotomy. In the DW groups there were only minimal increased values. In conclusion, elevated clinical scores were seen in operated and Sham animals with no differences between the treated groups.

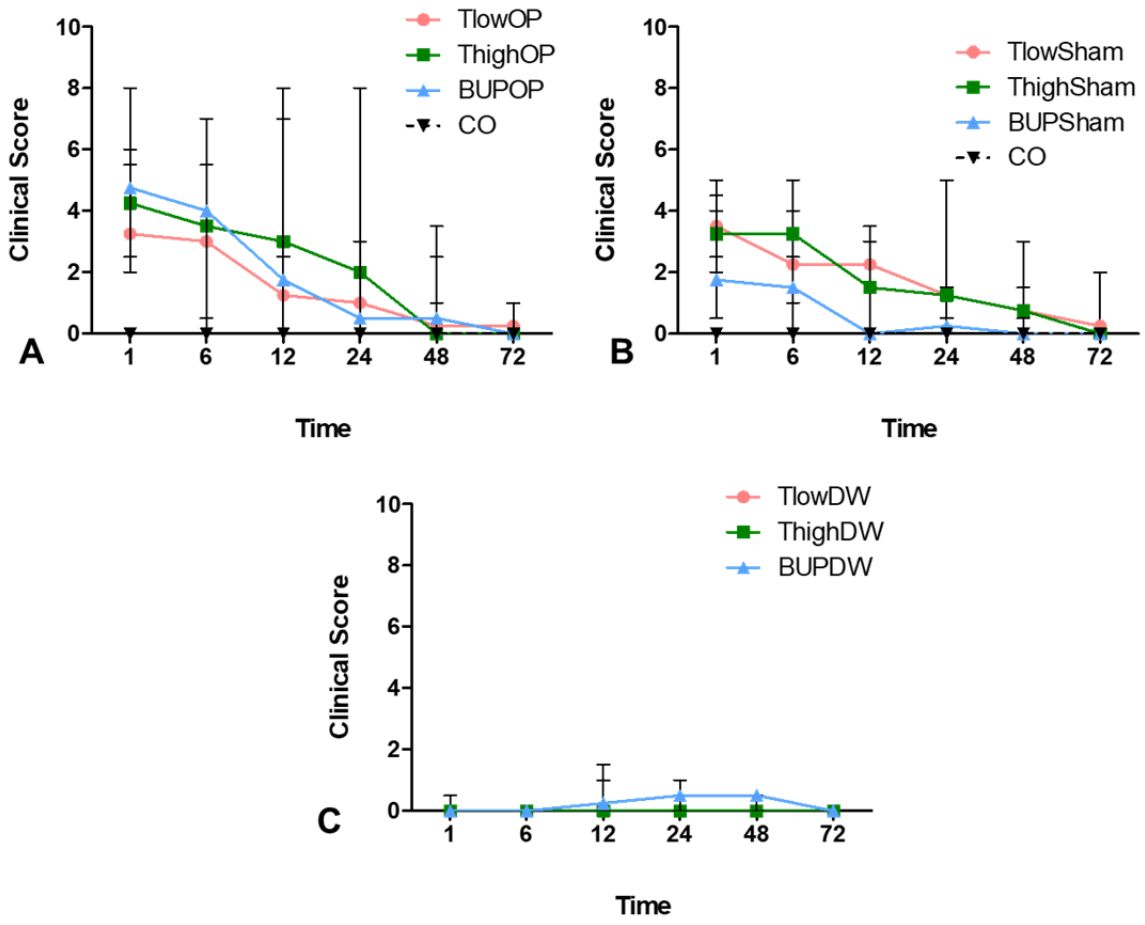

Figure 23: Clinical scoring. A) Operated, B) sham operated and C) animals with analgesia only. (Graphs show Median with Range; Tlow/BUP n=8, Thigh n=7, rest n=4)

5.7 Nest Complexity Score

The nest score was used to detect changes in wellbeing, which can possibly result from pain. It was high in all groups before surgery, reaching for the maximal score of 5 (Figure 24). The nest score of operated animals dropped at 6h after surgery and reached control levels at 48h. TlowOP recovered quickest while ThighOP stayed at low levels until 24h.

Sham treated animals showed a similar drop but recovered faster than operated. TlowDW animals showed a lower baseline value and did not decrease as much as the other groups. ThighDW dropped at 6h and increased rapidly. BUPDW showed low scores for a longer time and recovered more slowly. Taken together, the nest complexity was decreased in all animals after the procedure and recovered afterwards. Thigh operated animals recovered slowest.

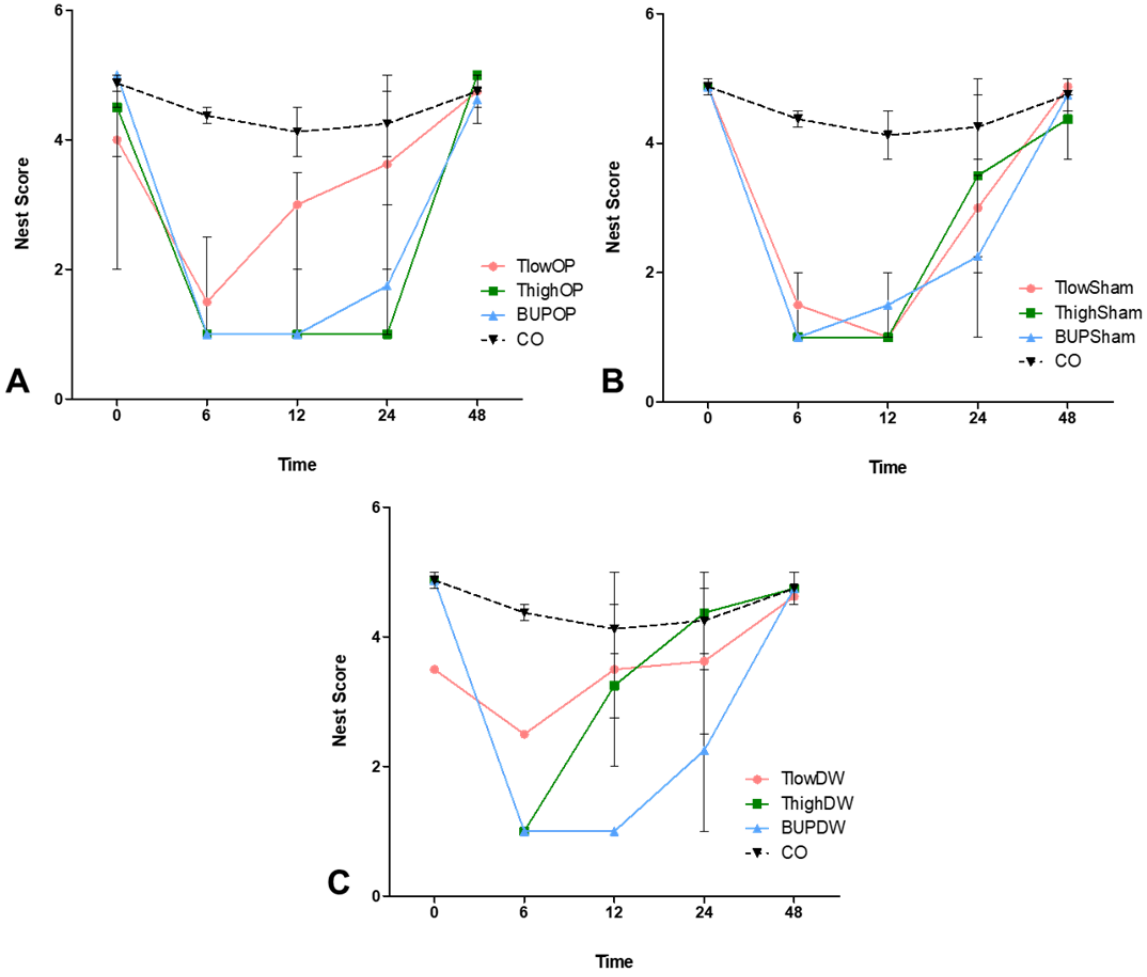

Figure 24: Nest complexity score. A) Operated, B) sham operated and C) animals with analgesia only. The score was tested per cage with two mice each. (Graphs show Median with Range; OP n=4, rest n=2)

5.8 Explorative Behavior

Explorative behavior was assessed as another motivational parameter besides nest complexity. Values were more variable in the operated animals than in the other groups (Figure 25). While all operated animals showed explorative behavior in the baseline measures, results dropped at 6h after surgery. TlowOP recovered to stable values from 24h. ThighOP further declined completely at 24h and slightly recovered at 48 and 72h (Mean: 1). BUPOP recovered faster to variable values. A drop also occurred in the Tlow-

Sham mice at 6h (Mean: 0). ThighSham und BUPSham showed a lower decrease (Mean: 0.5). ThighSham stayed on this level while BUPSham recovered. Consequently, results in operated and Sham animals were reduced after the procedure but recovered over time with TlowOP regaining baseline values the fastest.

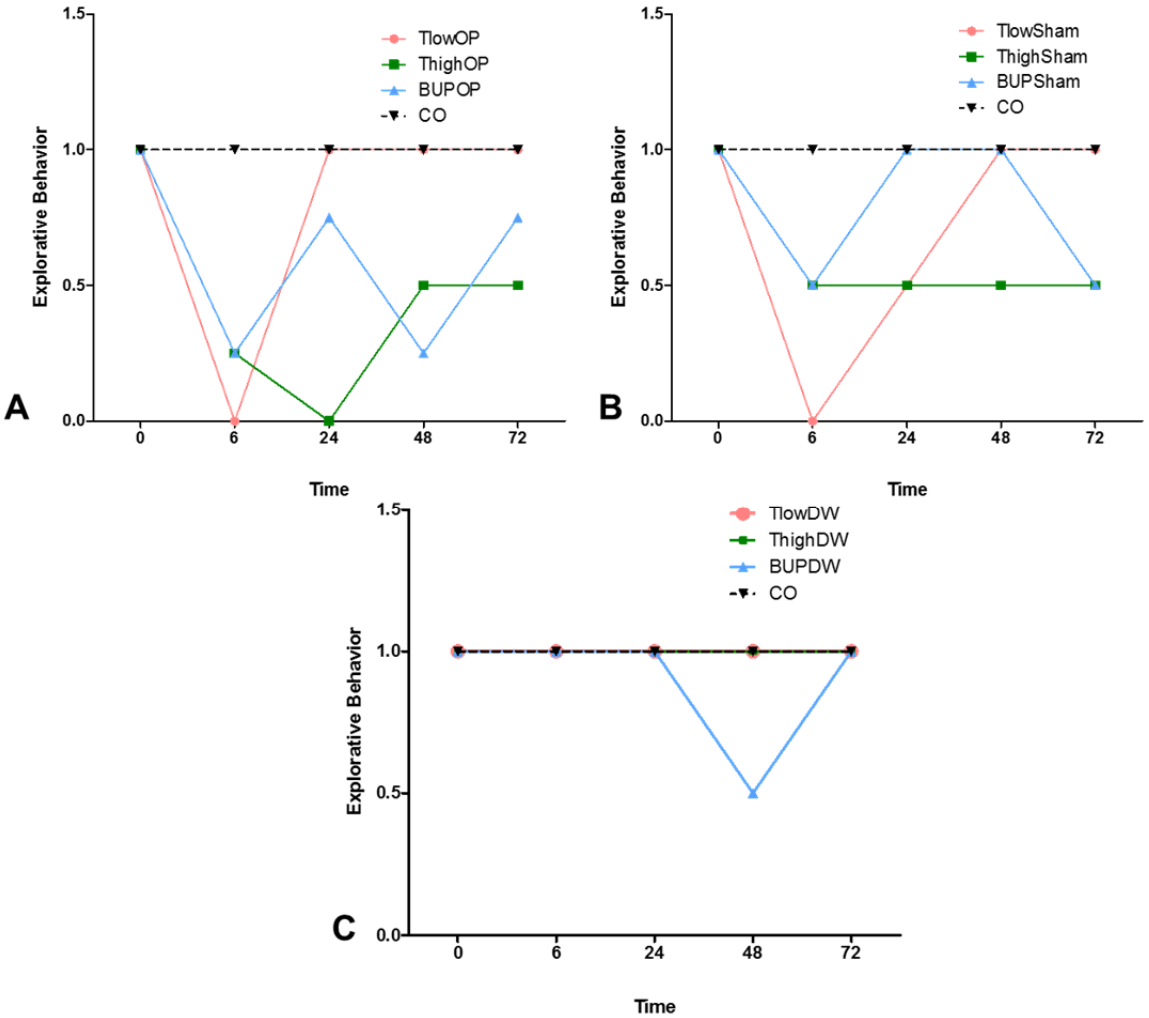

Figure 25: Explorative behavior. A) Operated, B) sham operated and C) animals with analgesia only. Explorative behavior was tested per cage with two animals each. 1=positive, 0=negative. (Graphs show Mean; OP n=4, rest n=2)

5.9 Activity Analysis

- **Resting time**

With the help of video recording the RT was assessed manually in seconds per hour to grade animals' activity. The RT in operated animals from 18:00 to 4:00 was similar between groups but had a different temporal distribution. TlowOP and BUPOP got continuously calmer while ThighOP stayed active for a longer time. At 3:00 RT of the control group started to increase until 13:00 with stable levels of long RT until the end. TlowOP

and ThighOP stayed at long RT until 8:00, had lower levels for five respectively four hours and then came back to high levels. BUPOP stayed at long RT for longer with an active hour at 12:00. Towards the end, OP groups stayed at high RT with increased activity from the tramadol groups. (Figure 26).

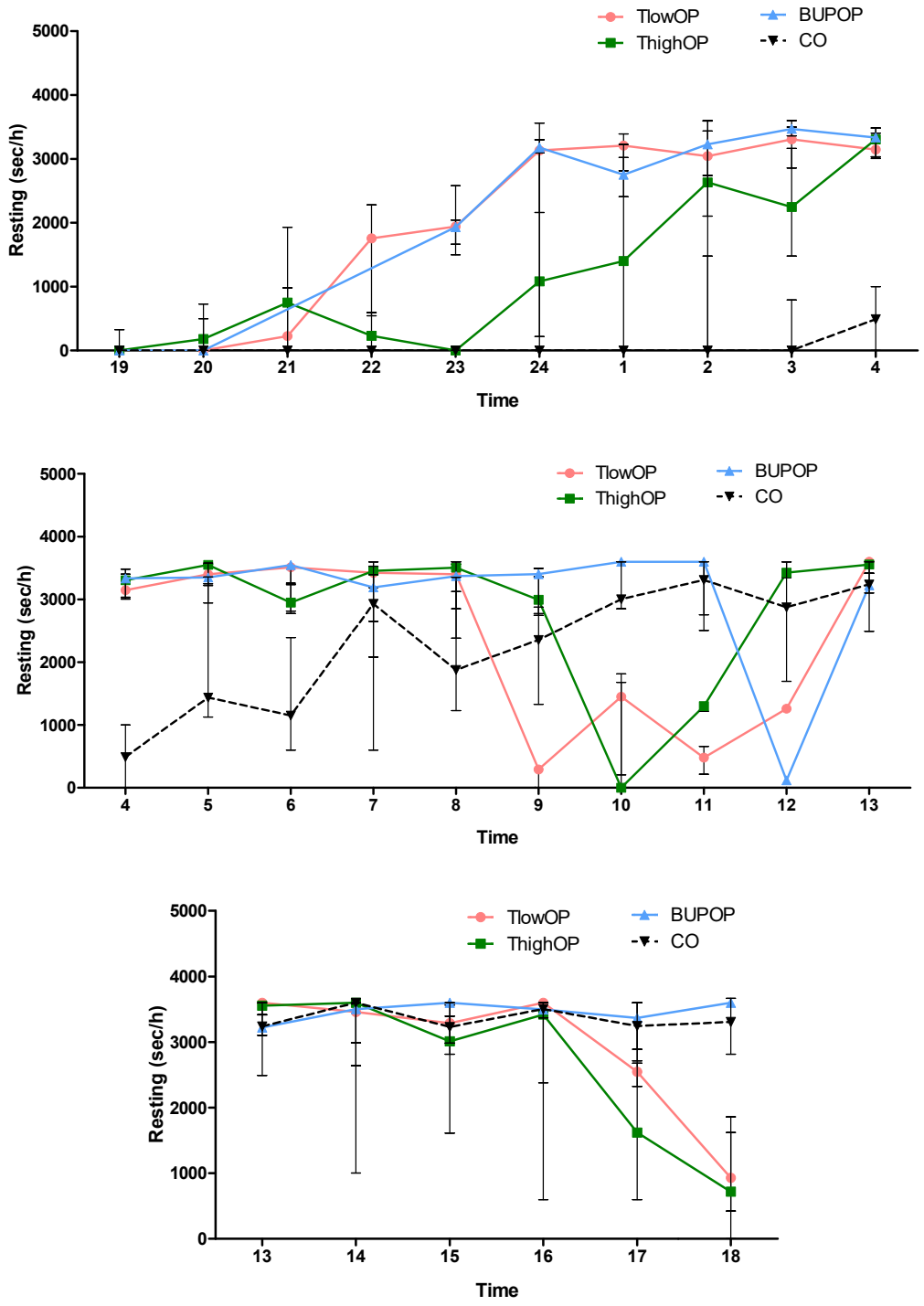

Figure 26: Resting time in operated animals. Starting from 18:00 for 24 hours on the day of surgery. Assessed in seconds of resting per hour. (Graphs show Median with Range; OP n=4, Control n=8)

Results of TlowSham from 21:00 to 9:00 could not be obtained due to technical problems. ThighSham were constantly active from 18:00 to 4:00. RT of BUPSham increased in this time. Values of TlowSham increased from 9:00 towards 12:00, animals than stayed calm. ThighSham increased from 4:00 to 8:00, followed by a slight decrease of RT. ThighSham then stayed calm. BUPSham showed constant levels of high RT with one active hour. At the end all groups showed a short active phase (Figure 27).

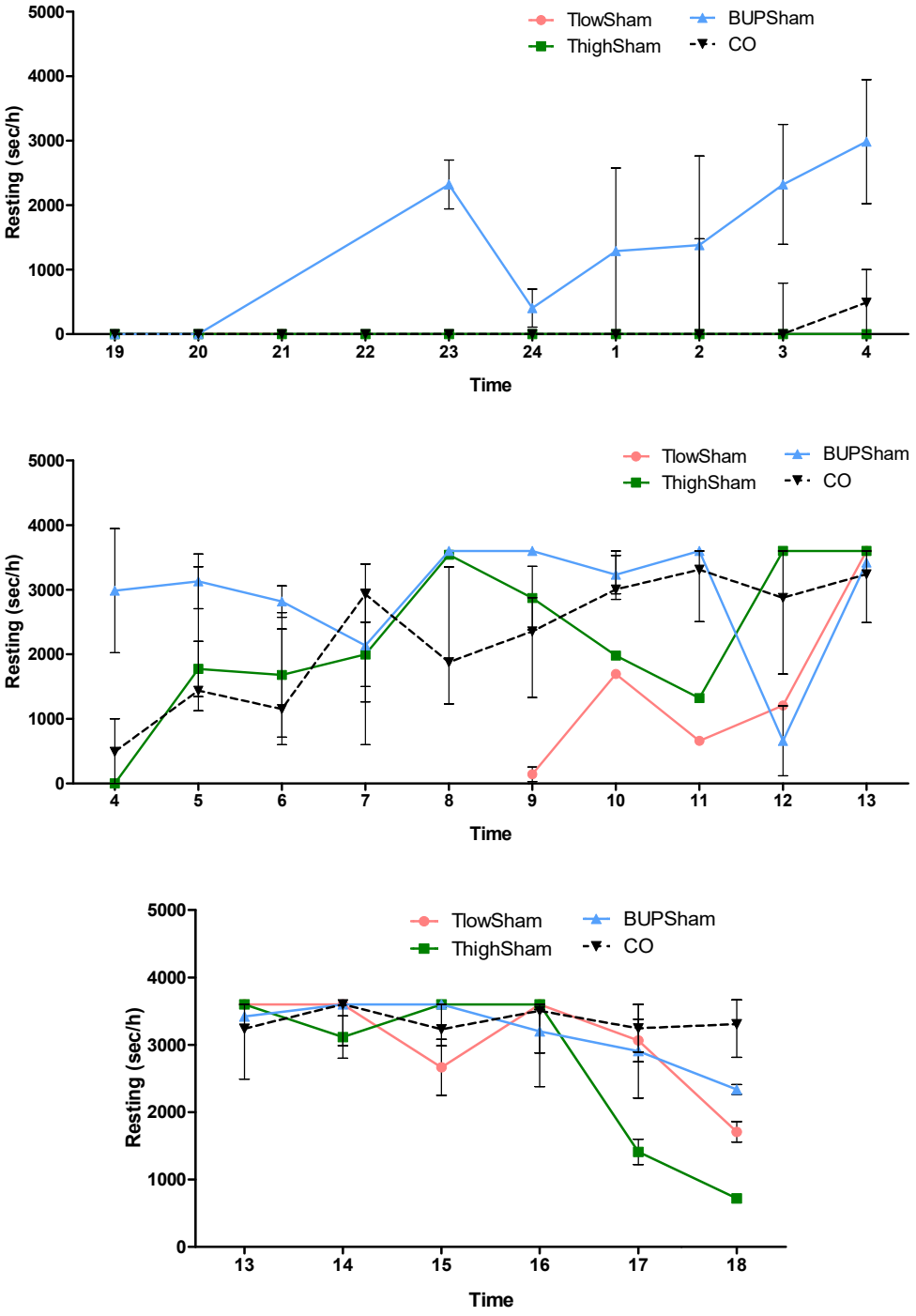

Figure 27: Resting time in sham operated animals. Starting from 18:00 for 24h on the day of surgery. Assessed in seconds of resting per hour. (Graphs show Median with Range; n=2, Control n=8)

Results of the TlowDW animals from 21:00 to 9:00 could not be obtained due to technical problems. ThighDW stayed active from until 2:00, then had increasing levels of RT. BUPDW animals were active from 18:00 to 4:00. RT of TlowDW stayed low until 12:00 and raised towards 13:00. The values of ThighDW were high until 13:00 with one more active period. BUPDW were active until 6:00 and were calmer with one active hour until 13:00. All DW groups had a resting phase until close to the end (Figure 28).

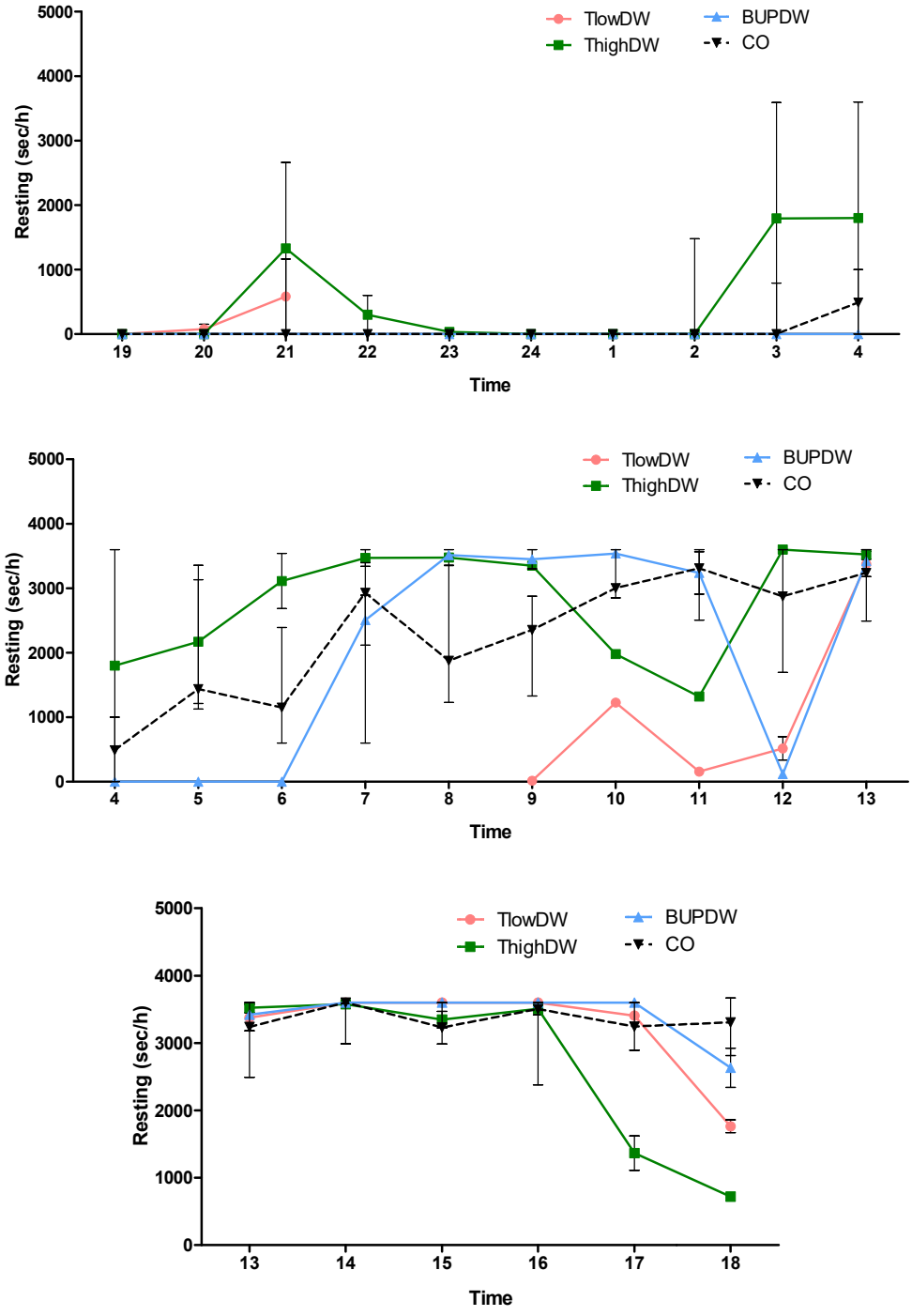

Figure 28: Resting time in animals with analgesia only. Starting from 18:00 for 24h on the day of surgery. Assessed in seconds of resting per hour. (Graphs show Median with Range; n=2, Control n=8)

RT of TlowOP alternated between high and low from 18:00 to 23:00 on the day after surgery. From there, levels stayed high for until the end of filming. ThighOP and BUPOP stayed at constant high RT on the day after surgery with one drop of BUPOP at 12:00. (Figure 29).

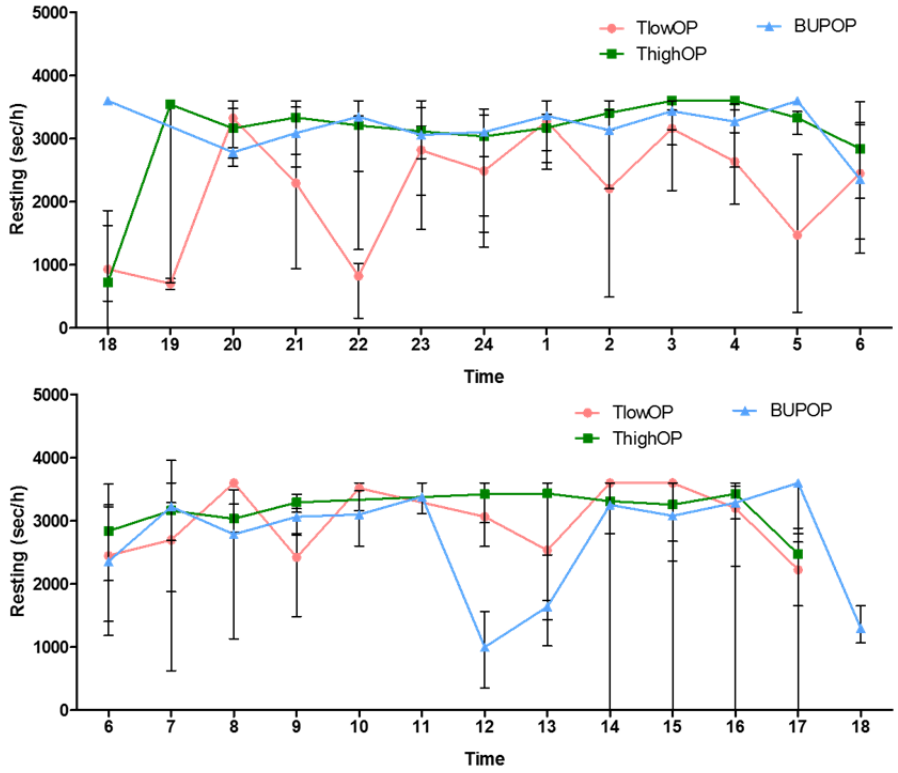

Figure 29: Resting time in operated animals. Starting from 18:00 on the first day after surgery. Assessed in seconds of resting per hour. (Graphs show Median with Range; n=4)

The results of Sham animals from 18:00 to 6:00 were variable with phases of high activity and resting. On the other half of the filming period Sham animals were more calm and stable. (Figure 30).

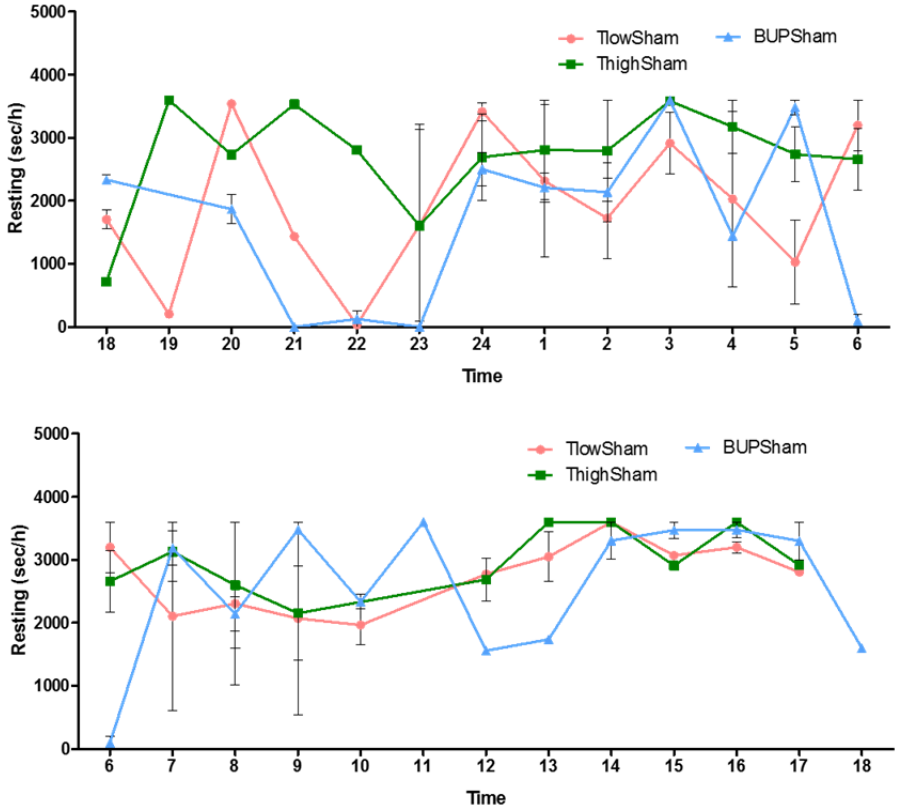

Figure 30: Resting time in sham operated animals. Starting from 18:00 on the first day after surgery. Assessed in seconds of resting per hour. (Graphs show Median with Range; n=2)

DW animals showed a lower total RT than the other groups from 18:00 to 6:00 on the first day after surgery varying between activity and resting periods. From 6:00 to 18:00 DW animals were more calm and stable than in the 12 hours before (Figure 31).

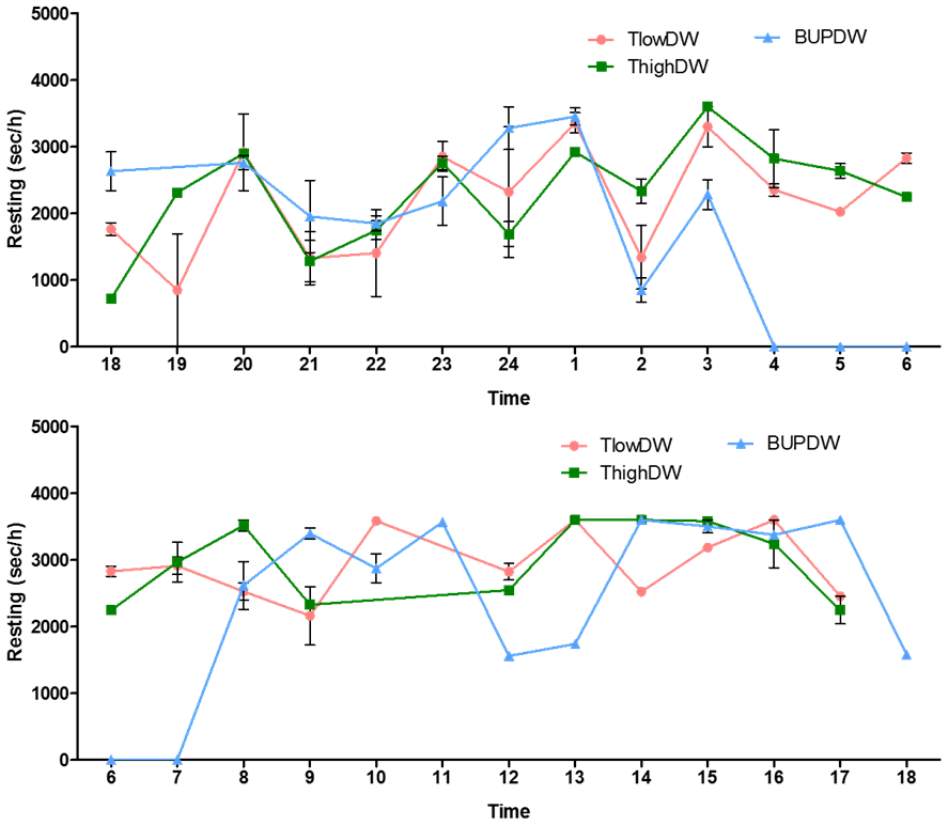

Figure 31: Resting time in animals with analgesia only. Starting from 18:00 on the first day after surgery. Assessed in seconds of resting per hour. (Graphs show Median with Range; n=2)

Taken together, DW had a higher activity than Sham, while OP had the lowest activity. In the first 24h in the OP and Sham group, Thigh had the highest activity. BUP had the highest activity in the DW group. In the second 24h TlowOP started with a higher activity, BUPOP was more active towards the end. ThighSham had a lower activity than the other Sham groups in the beginning. At the end Sham groups had a similar activity. DW groups were similar throughout the second 24 hours.

5.10 Model-specific Pain Parameters

5.10.1 Limp score

The limp score is a grade for limb use and function in the animals. A maximum score of 4 can be reached. It was only visible in the operated animals. TlowOP had an increased score at 1h (Median: 1, Range: 3). ThighOP had a raised score 1 and 6h after surgery (Median: 1 and 1, Range: 3 and 3). BUPOP had a low score throughout the measurement (Figure 32).

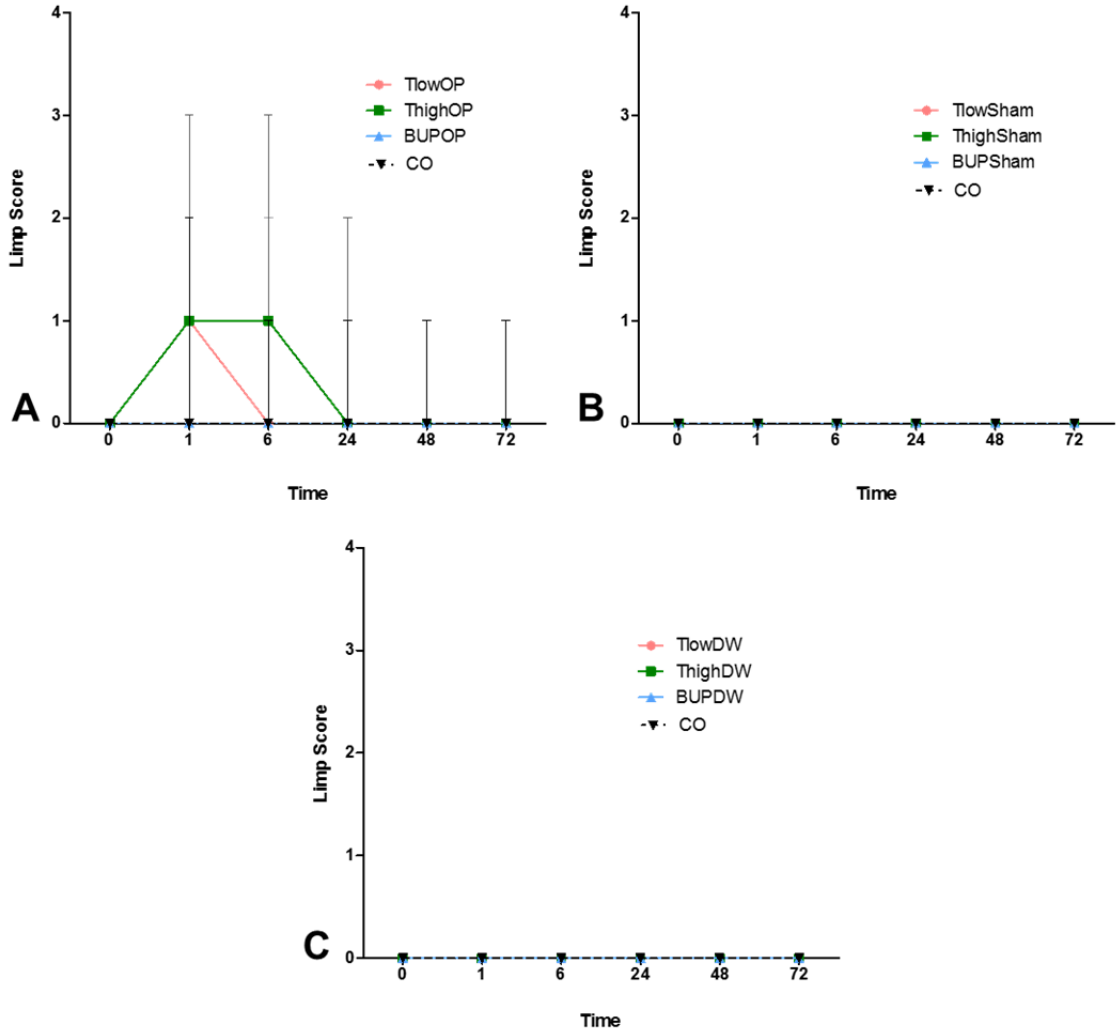

Figure 32: Limp Score of the left hind limb. A) Operated, B) Sham and C) animals with analgesia only. Assessed in the 3min video recording. (Graphs show Median with Range; Tlow/BUP n=8, Thigh n=7, rest n=4)

5.10.2 Dragging

Dragging of the left hind limb was assessed with a dragging score. A maximum score of 4 can be reached. Dragging was only observed in operated animals. The score was increased 1 and 6 hours after surgery only in the Thigh group (Median: 2 and 1, Range: 3 and 3), then decreased to the same low values as TlowOP and BUPOP (Figure 33).

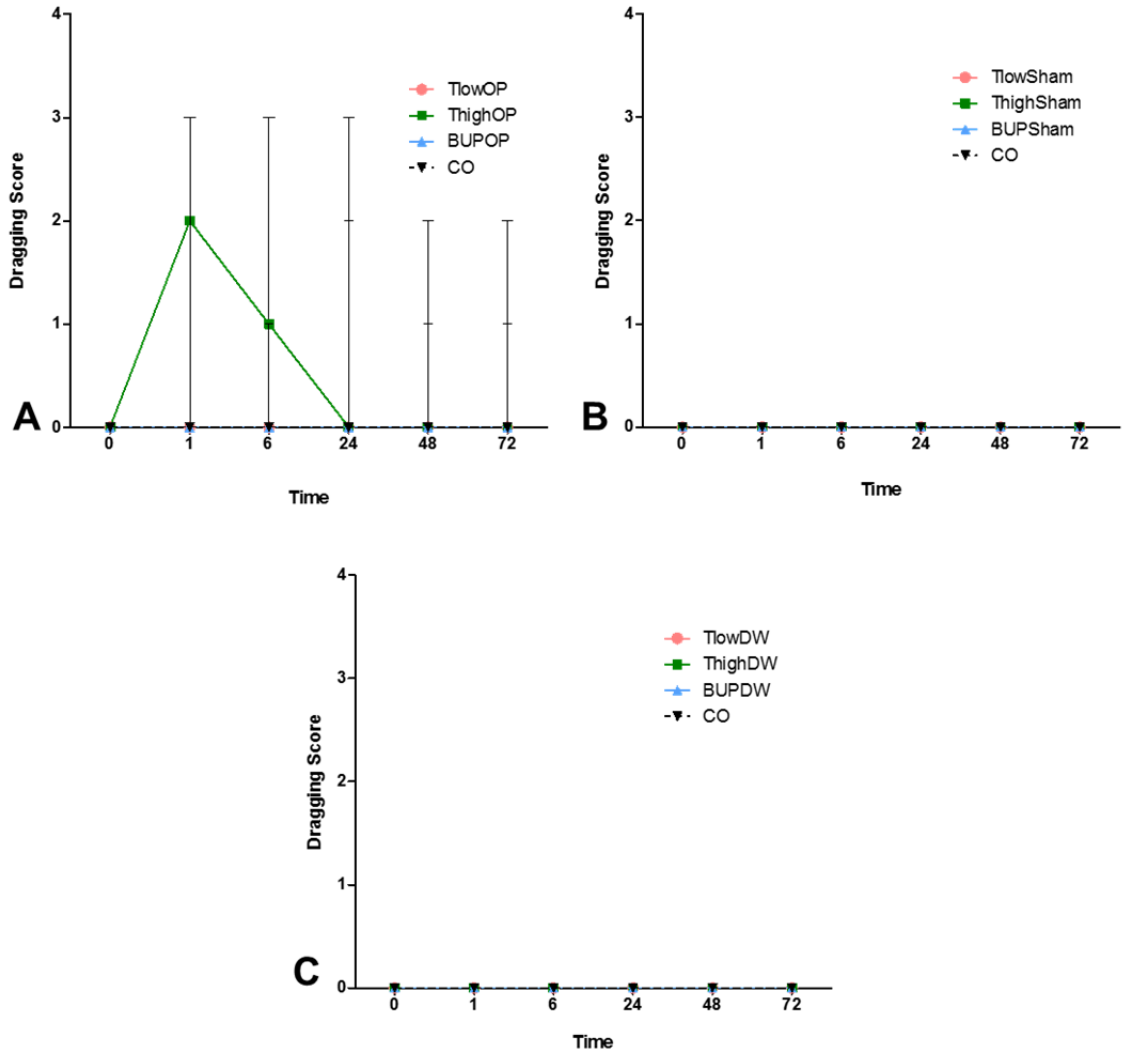

Figure 33: Dragging Score of the left hind limb. A) Operated, B) Sham and C) animals with analgesia only. Assessed in the 3min video recording. (Graphs show Median with Range; Tlow/BUP n=8, Thigh n=7, rest n=4)

5.10.3 Rear up

Time of rear up on both legs was measured within assessing the model-specific pain parameters. In all operated animals (Figure 34), it completely decreased from baseline values at 1 and 6h after surgery. Thigh recovered the slowest, never reaching control values. BUP was then similar to control values. Tlow continued to be slightly lower than BUP. Baseline values of all groups were lower than in control animals.

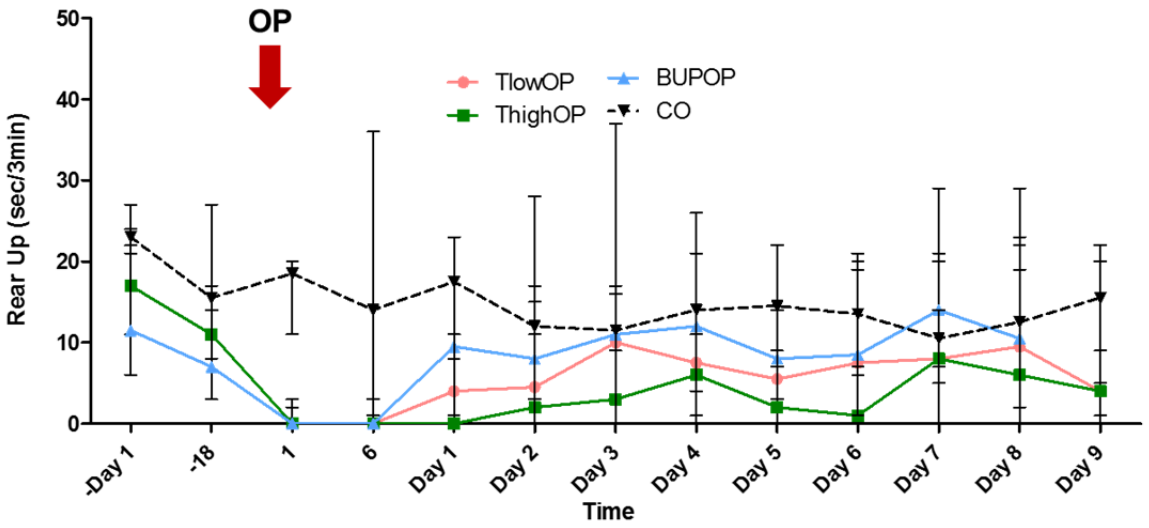

Figure 34: Rear up in operated and control animals. Assessed in seconds per 3 min video recording. The arrow marks the time of osteotomy surgery. (Graph shows Median with Range; Tlow/BUP n=8, Thigh n=7, Control n=4)

Sham showed the same drop as operated animals 1 and 6h after their anesthesia. The decrease in Tlow was prolonged, then values were similar to control animals. ThighSham levels were then similar to control animals, while BUP had increased results (Figure 35).

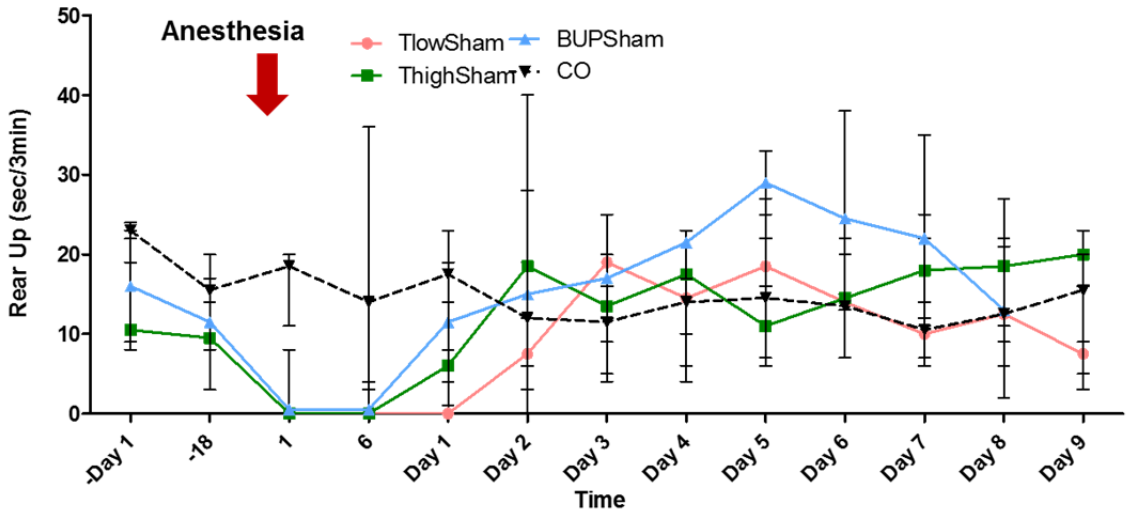

Figure 35: Rear up in Sham and control animals. Assessed in seconds per 3min video recording. The arrow shows the time point of anesthesia. (Graph shows Median with Range; n=4)

Rear up time in DW animals was decreased at 1 and 6h. Tlow and Thigh reached control levels on day 1, while BUP reached them on day 2. Rear up time of all DW groups varied around the control values until day 9 (Figure 36).

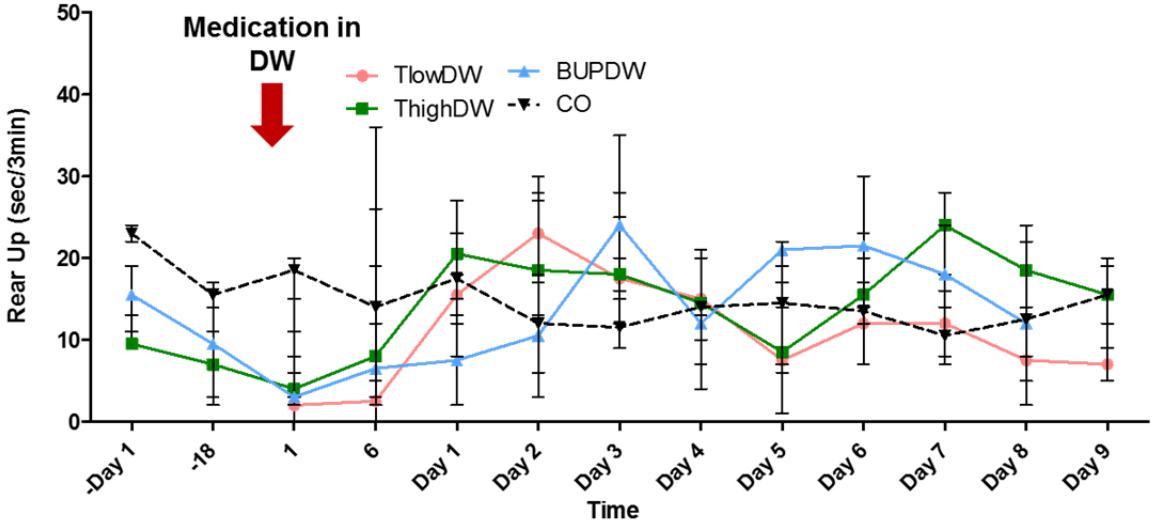

Figure 36: Rear up in animals with analgesia only and control animals. Assessed in seconds per 3min video recording. The arrow indicates the time point when analgesic pain medication in the drinking water was first given. (Graph shows Median with Range; n=4)

Taken together, the rear up time was reduced after the procedure in all animals. TlowOP and ThighOP animals did not recover to control values.

5.10.4 Grooming

Grooming time was assessed to check if any pain would lead to an increased care of the affected leg (Figure 37). The results were highly variable. It was elevated in OP groups up to two days after surgery with no difference between the three groups. Grooming was also seen in ThighSham 6h, in TlowSham and in TlowDW on day 1 after surgery. In conclusion, increased grooming the affected leg was mainly seen in OP and Sham groups.

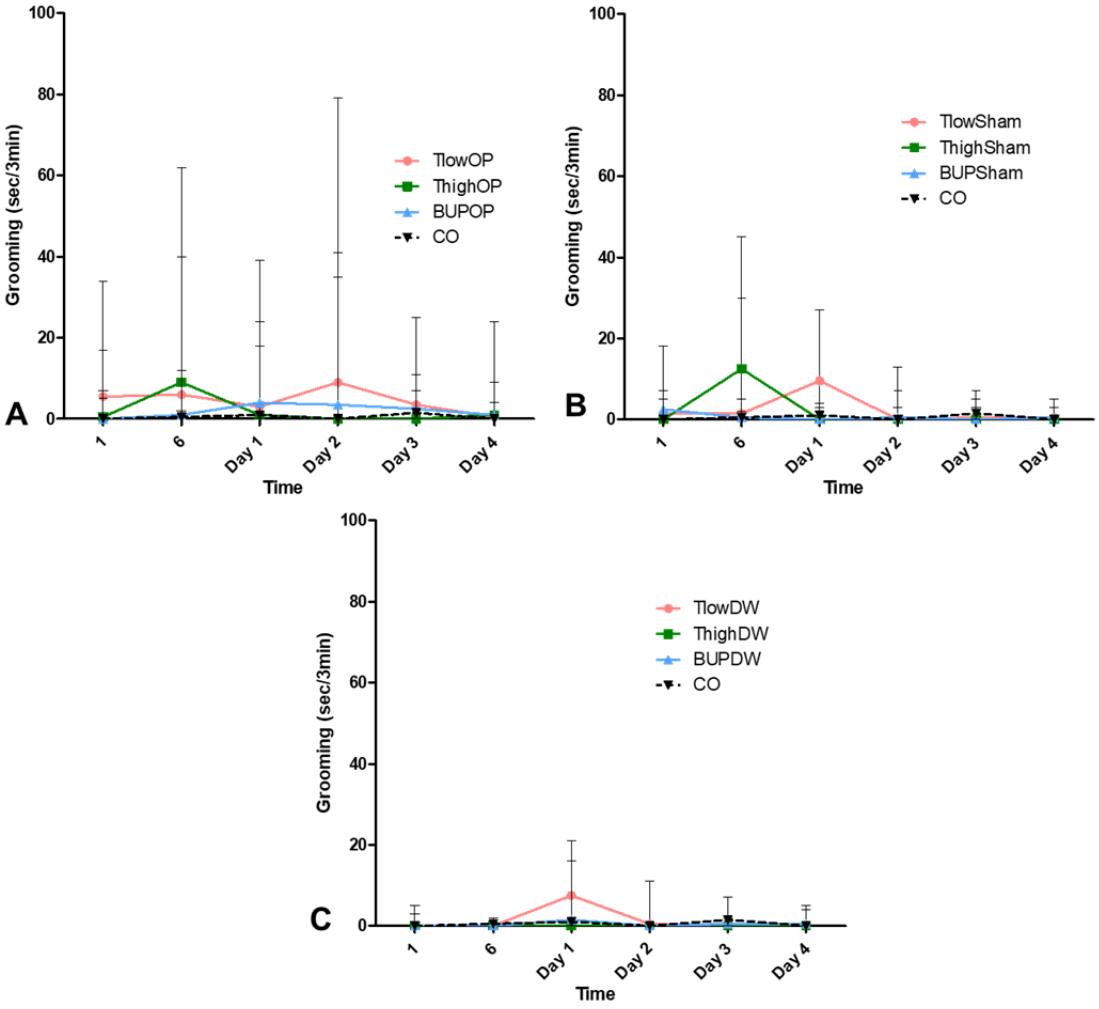

Figure 37: Grooming time of the left hind leg. A) Operated, B) Sham and C) animals with analgesia only. Measured in seconds per 3min video recording. (Graphs show Median with Range; Tlow/BUP n=8, Thigh n=7, rest n=4)

5.10.5 Flinching

Flinching was assessed as a specific pain parameter in the osteotomy model. Flinching only occurred in operated animals. The frequency was slightly increased in ThighOP 6h postoperative (Median: 4, Range: 10). TlowOP and BUPOP had low values throughout the experiment (Figure 38).

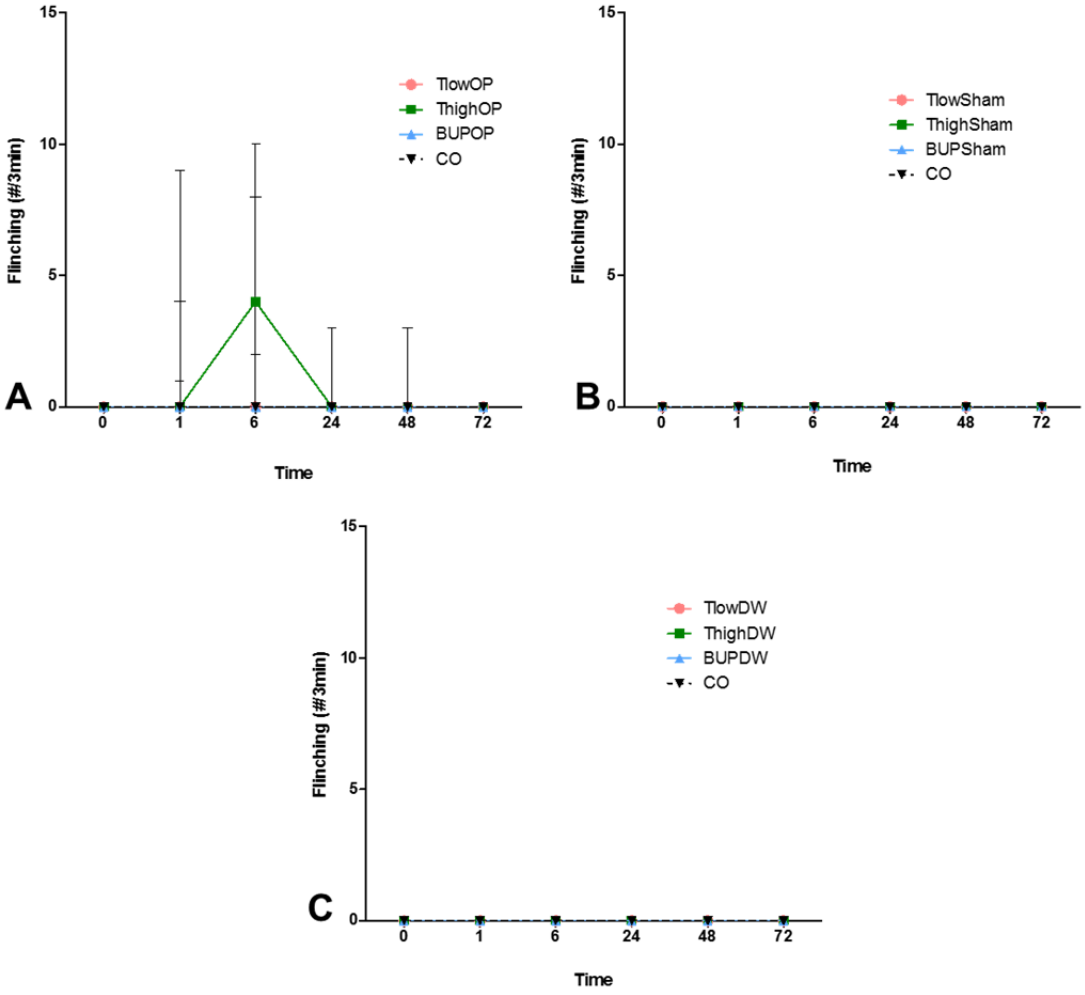

Figure 38: Flinching frequency of the left hind leg. A) Operated, B) Sham and C) animals with analgesia only. Measured in numbers of flinching per 3min video recording. (Graphs show Median with Range; Tlow/BUP n=8, Thigh n=7, rest n=4)

5.10.6 Guarding

Guarding did not occur.

5.11 Assessment of Fracture Healing

5.11.1 *In vitro* µCT measurements

In vitro µCT was done in osteotomized animals. Relative bone volume, bone volume and total volume were analyzed to grade bone healing after the two-week healing period (Figure 39). The fracture was either treated with a vehicle (Lyostypt) or left untreated (control) during surgery (see Figure 2). In all three measurements the control had higher results than the vehicle animals. With a significant difference in Tlow (p=0.0014) and BUP

(p=0.0286) in the Rel. BV. Vehicle Thigh differed from vehicle BUP (p=0.0571). In BV values showed a significant difference in Tlow (p=0.0128) and BUP (p=0.0286). The TV showed a difference in Tlow (p=0.0545) and BUP (p=0.0571) animals. Taken together, there was only a small difference in the results between the analgesia groups, but high differences between control and vehicle treated animals.

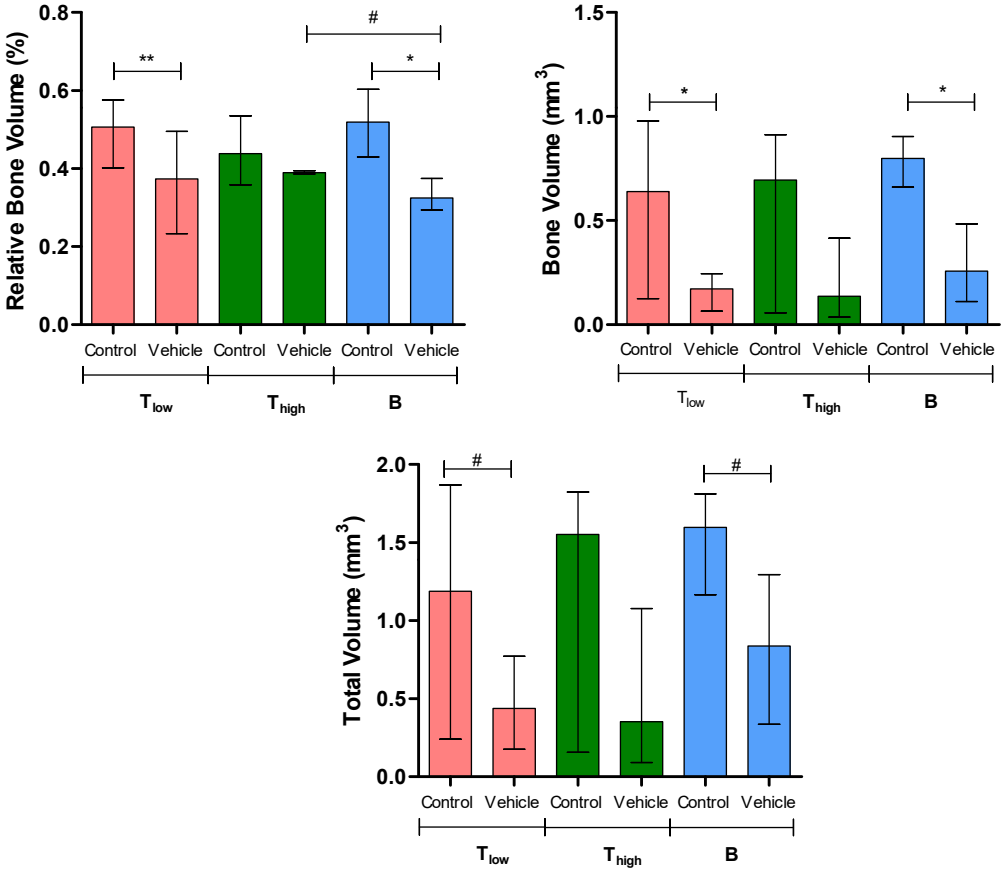

Figure 39: Parameters assessed in *in vitro* µCT. Relative bone volume (%), bone volume (mm3) and total volume (mm3). (Graphs show Median with Range; Control/Vehicle Tlow/BUP and Control Thigh n=4; Vehicle Thigh n=3; Mann Whitney test, *p<0.05)

5.11.2 Histology

A histological staining with Movat-Pentachrome was done to assess different parameters of bone healing. In all results besides the callus width, control values were higher than vehicle values (Figure 40). In the relative total cartilage, a significant difference in Tlow (p=0.0286) and a difference in BUP (p=0.0571) was found. The callus width had significant differences in Tlow (p=0.0286) and BUP (p=0.0286) and a difference in Thigh (p=0.0571). In the relative endosteal mineralized bone a significant difference was found in BUP (p=0.0286) and differences in control values between Tlow/BUP and Thigh/BUP (p=0.0571). In the relative intracortical cartilage a significant difference was seen in BUP (p=0.0294). The relative total mineralized bone showed significant differences in Tlow and

BUP (each p=0.0286), a difference between control Tlow and BUP (p=0.0571). Tlow showed a significant difference in the relative endosteal cartilage (p=0.0265), while there were no significant changes in the relative intracortical mineralized bone. To summarize, a difference in the bone healing parameters between control and vehicle groups was seen with small differences in the analgesia groups.

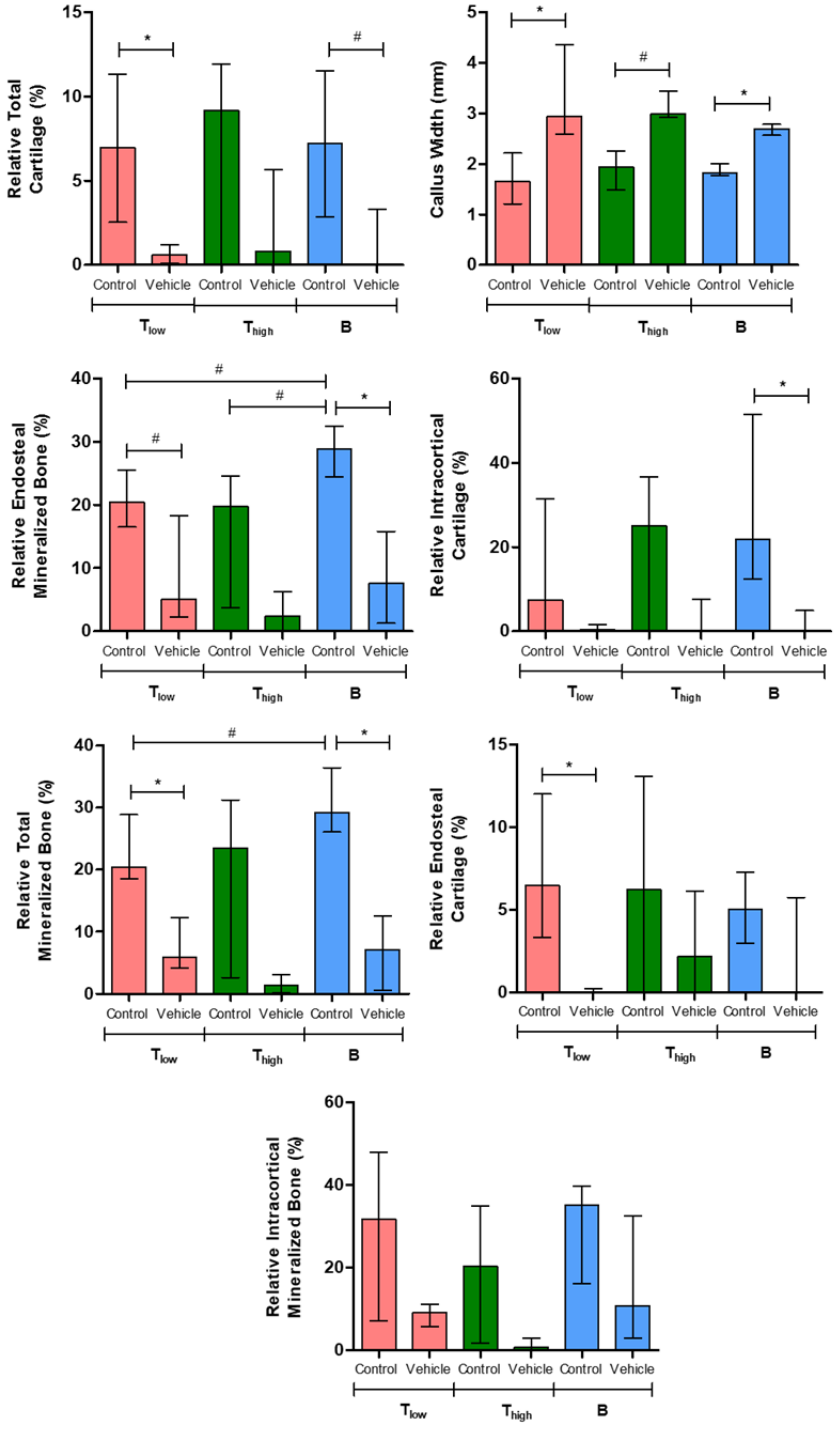

Figure 40: Parameters assessed with movat-pentachrome staining. Relative total cartilage (%), callus width (mm), relative endosteal mineralized bone (%), relative intracortical cartilage (%), relative total mineralized bone (%), relative endosteal cartilage (%), relative intracortical mineralized bone (%). (Graphs show Median with Range; Control/Vehicle Tlow/BUP and Control Thigh n=4; Vehicle Thigh n=3; Mann Whitney Test, *p<0.05)

5.12 Side effects

5.12.1 Liver histology

The livers of mice subjected to osteotomy were evaluated histologically. The livers of one animal each of the Tlow and the BUP group were found with no abnormalities (Figure 41 – A). The animal of the Thigh group that reached the humane endpoint and was euthanized two days after the surgery was diagnosed with a severe hepatic lipidosis (Figure 41 – C). The remaining seven animals of each analgesia group showed a mild hepatitis (Figure 41 - B).

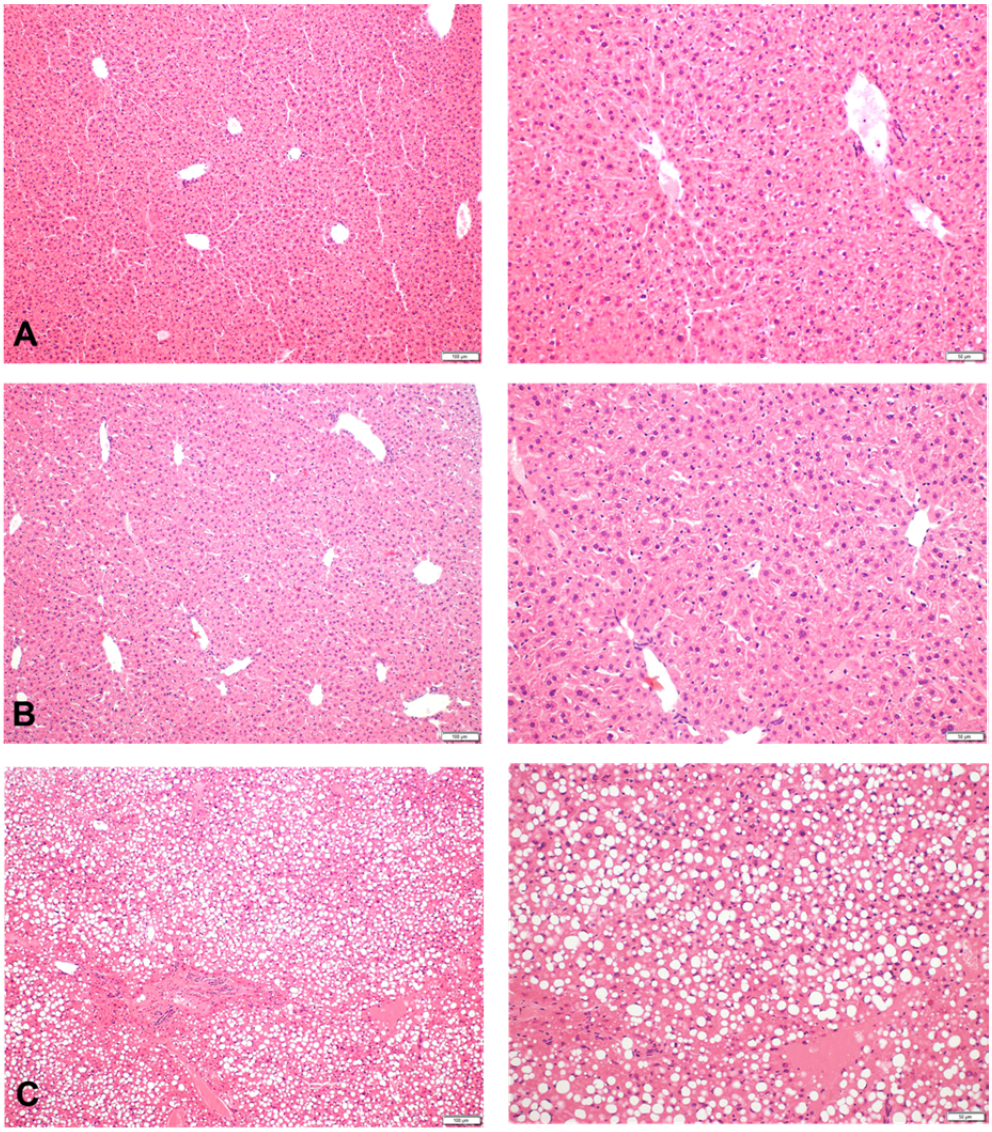

Figure 41: Liver histology. (HE stained, 100x, 200x). A) No abnormality detected in one Tlow and one BUP animal. B) Mild hepatitis in seven Tlow, Thigh and BUP animals. C) Severe hepatic lipidosis in the euthanized Thigh animal.

5.12.2 Unexpected occurrences

During the study, different abnormalities occurred and were further examined. The results are described in this chapter.

One animal of the ThighOP group had to be euthanized prior to the set date of two weeks after surgery. This mouse stood out with a poor clinical condition on the evening of the osteotomy. It was very calm with reduced movement and breathing heavily. The mouse was treated with 0.1 ml buprenorphine s.c. as a rescue pain treatment on the day of surgery at 22:00. One hour after the injection the animal's condition was reevaluated. No increase of the condition was seen. On the morning after the surgery the mouse was again treated with 0.1 ml NaCl s.c. for hydration and 0.1 ml buprenorphine s.c. at 9:00 am. In the evening at 18:00 again 0.1 ml NaCl s.c. was given. At the morning of day two after surgery the mouse was euthanized as it then reached the humane endpoints of the trial by a weight loss of over 20%. A dissection took place, no abnormalities beside an altered liver were found (Figure 42).

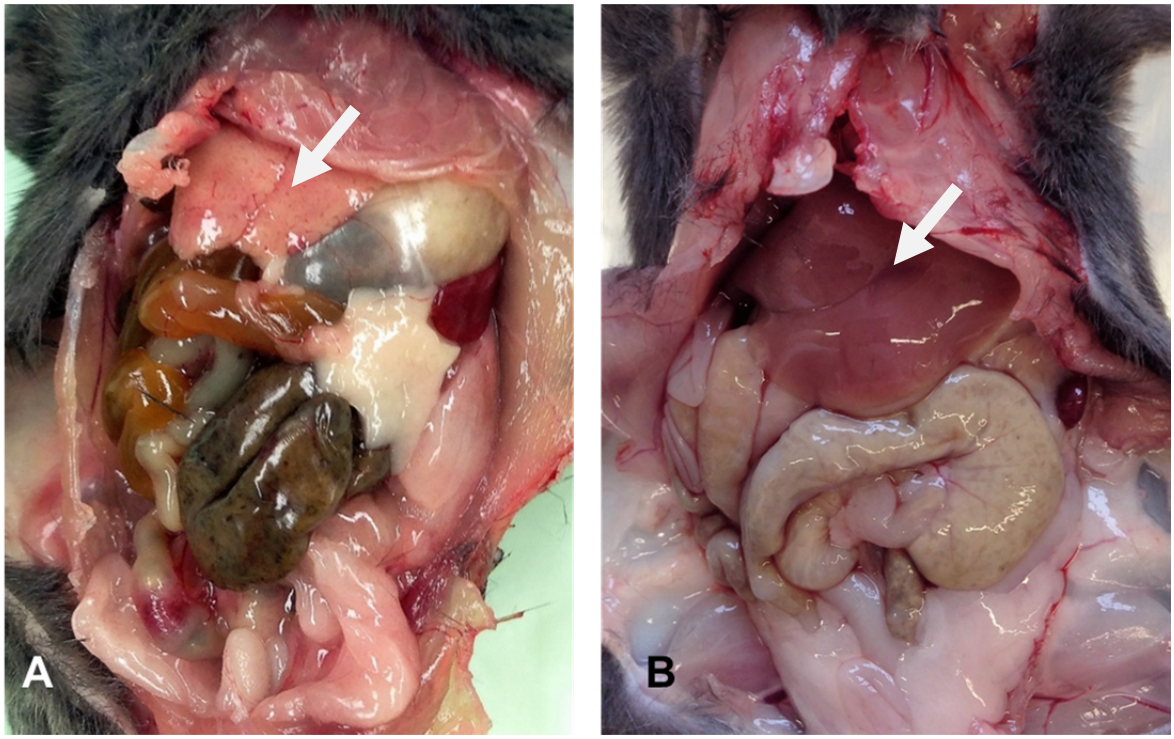

Figure 42: Dissection of abdominal region. A) In the Thigh animal euthanized prior to the euthanasia date and B) in a euthanized healthy animal. Arrows indicating A) the altered liver and B) a physiological liver.

One animal from the BUPOP group was conspicuous two days post-surgery with a reddened area around the wound and a minimal dry scab. This scab spread the following day but healed four days after surgery (Figure 43– A). Three days postoperative a crusted dry

area distal of the wound was noted in the cage mate. In the following days the wound decreased in size but was open and moist on a small streak towards the paw. Seven days postoperative the skin was then open around the external fixator. At day eight after surgery the distal part of the wound was slightly bigger (Figure 43– B). A second wound on the right shoulder was obvious with 0.5 cm in size. The two animals in the cage were then separated to exclude licking by the cage mate as a reason for the wounds. Nine days after the surgery the wound size was increasing and highly moist. The margin of the main wound was dry and slightly smaller at the distal part. The animal was treated with 0.05 ml clindamycin s.c. On the next day the wound was additionally cleaned with Octenisept spray, chlorhexidine wipes, Fucicort cream and one hour later with Bepanthen cream. On the evening, a third wound appeared on the left body side. Wounds were treated as mentioned above. On the following days the wounds were dry and decreased in size. Treatment was applied daily. On the 14th day postoperative, the animal was euthanized according to the study plan. The animal was observed daily, sometimes twice. The mouse was in a good condition and did not show signs of pain or reduced wellbeing due to the wounds.

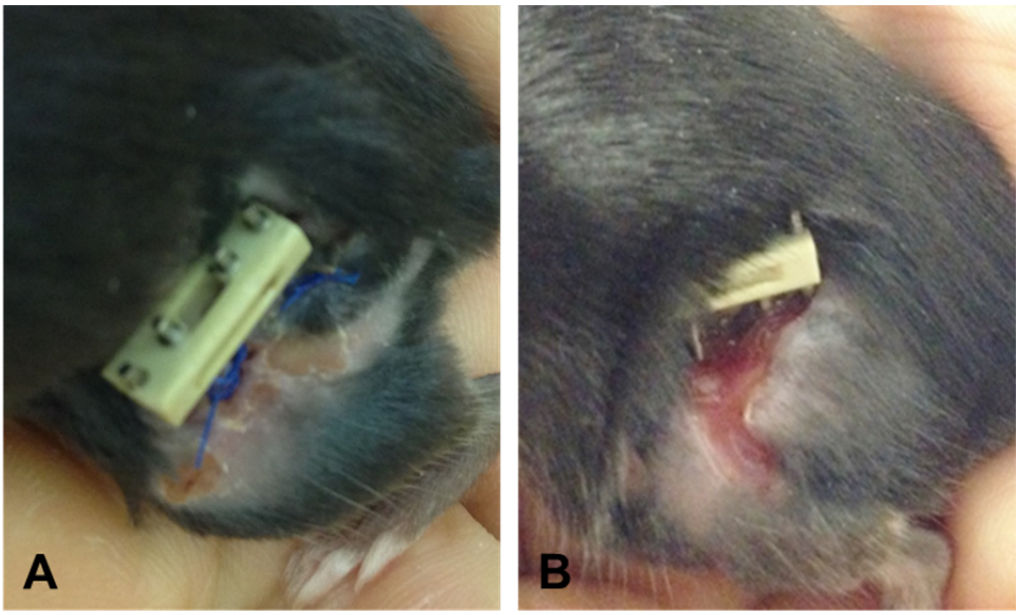

Figure 43: Wound development in two BUPOP animals. These animals were cagemates. A) Three days after surgery and B) at eight days after osteotomy.

5.13 Serum analysis of tramadol

A serum analysis was done to measure the amount of tramadol and M1 after treatment with the low tramadol dosage in the drinking water for three days. The results were then compared to the human minimal analgesic concentration of tramadol and M1 (Figure 44).

The serum of operated mice treated with tramadol low in the drinking water for three days contained a mean tramadol concentration of 28.3 ng/ml and a mean M1 concentration of 119.3 ng/ml. Compared to the human minimal analgesic concentrations (tramadol Median: 287.7 ng/ml; M1 Median: 36.2 ng/ml) described by Lehmann et al. [88], reversed results were found for the tramadol and M1 concentration. The concentration of the analgesic effective M1 is higher in mice than the human minimal effective analgesic concentration.

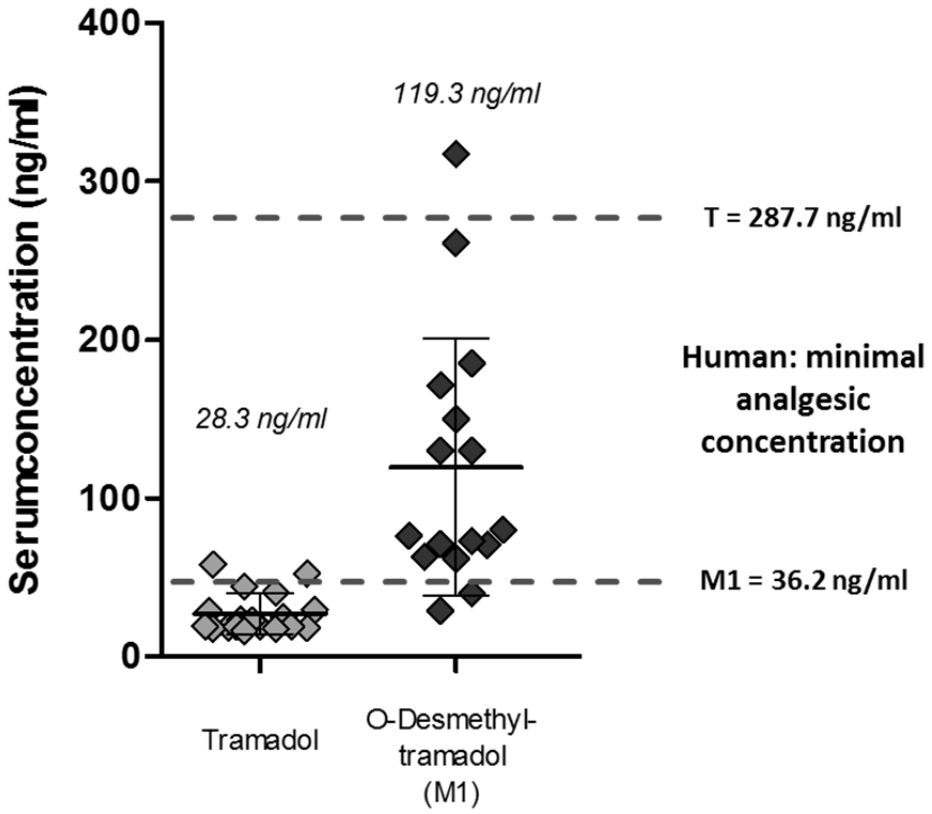

Figure 44: Serum concentration of tramadol and M1. Displayed in mice, compared to the human minimal analgesic concentration (dashed line, Median). T=Tramadol, M1=O-Desmethyltramadol. (Graph shows Mean with SD; mice n=18)

5.14 Impact of pre-emptive buprenorphine on bleeding during surgery

To grade the impact of pre-emptive buprenorphine on bleeding during surgery a bleeding score was calculated. The incision bleeding score showed similar results within both groups of animals injected 1h prior to the surgery. Animals injected to the right leg 1h prior to the surgery had a significantly higher incision bleeding score than animals injected during the preparation for surgery. In the pin bleeding score, animals of the left leg group injected 1h prior to surgery showed a significantly higher score than in OP mice. While there were no differences in prior injected animals in the total bleeding score, mice injected during surgical preparation had a significantly lower score than the mice injected prior to sur-

gery. Taken together, buprenorphine applied prior to surgery led to higher bleeding scores (Figure 45).

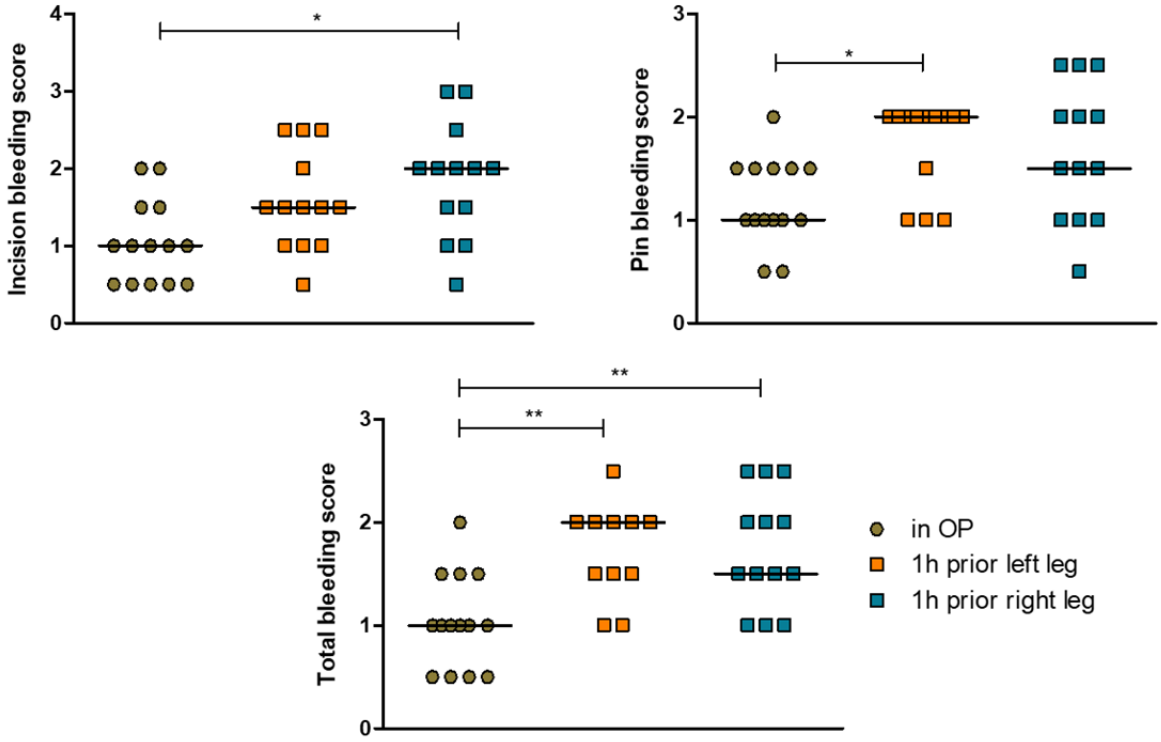

Figure 45: Scores on bleeding after buprenorphine injections. Incision, pin and total bleeding scores assessed during surgery with buprenorphine applied during surgical preparation or 1 hour prior to surgery. Kruskal-Wallis test with Dunn's correction. (Graphs show Median; n=13/14; *p<0.05, **p<0.005)

6 Discussion

Pain treatment with different dosages of tramadol and buprenorphine applied via the drinking water is evaluated in the mouse osteotomy model. On that account, three groups of animals are tested in every treatment protocol. A group with anesthesia, osteotomy and analgesia is compared to a group with anesthesia and analgesia. The third group receives analgesia only in the drinking water. Additionally, a control group with handling only is used. Parameters to assess the practicability, analgesic efficiency and possible side effects of the analgesia are used. Additionally, the bone healing with different treatments of the bone (vehicle or untreated), a main read-out of the basic research study using the mouse osteotomy model is assessed to test the influence of the analgesia on the fracture healing.

6.1 Feasibility of Pain Relief via the Drinking Water

Daily water intake in the used strain of mice is about 3 - 6 ml per mouse [93]. Lower baseline values are shown in the current study. Compared to these values the postoperative water intake is quite satisfying. Mice drink the analgesia enriched drinking water after the possibly painful surgery and after anesthesia. Animals treated with tramadol of the high dosage, osteotomized or with anesthesia only have lower values on day 1 and 2. An aversive reaction to the taste of the high tramadol dose could be suspected, as well as a reduced intake due to a reduced activity. The stable values in the group of analgesia only animals with no anesthesia and surgery show that the taste of the tramadol and buprenorphine itself probably does not interfere with the water intake. Evangelista et al. showed that mice treated with 0.2 mg/ml of tramadol in the drinking water achieved high and stable plasma levels [94]. A sufficient intake of the tramadol solution also during the inactive phase during the day is implied. Water intake could only be assessed per cage as single housing was not allowed by the legislation authorities, therefore differences in the intake of single animals could not be assessed.

In the drinking frequency, a day and night difference is seen in all animals where animals have more drinking events in the night, previously shown in another study with buprenorphine in the drinking water [95]. Less events take place especially in the beginning of the night in all groups compared to baseline. The low number of drinking events in analgesia only animals compared to Sham and operated animals and the lower values in all groups compared to tap water drinking baseline animals can be a sign of the aversive taste of medication. Animals treated with tramadol of the high dosage often show reduced intake events indicating an aversive taste of the high dosage tramadol. Additionally, the results show that number of drinking events does not always display the total water intake

amount. A critical point here is that drinking duration at the single drinking events is not assessed, so no differentiation of high intake versus low intake duration in one event was possible. In addition, drinking frequency was only assessed in half of the animals of each group due to structural reasons during video recording. Assessing drinking frequency in all animals with a second recording system would have been reasonable.

A serum analysis is done to test the tramadol concentration in operated animals after 3 days of low tramadol treatment. The analgesic efficient M1 value is nearly three times as high in the tested mice than the human minimal effective serum level of M1 [88]. Taken together, animals with an osteotomy treated with the low tramadol dosage in the drinking water can be considered to have a serum level effectively for pain relief.

6.2 Analgesia in the Drinking Water

All animals, especially the ones operated and anesthetized, lost body weight. Reasons for that can be the loss of fluid during surgery and the temporary drop in food and water intake after surgery and anesthesia. A result that was expected as previously seen in other studies [12]. Animals treated with tramadol in the high dosage, regardless of the group, lost the most body weight. They also recovered more slowly than the other animals. This high loss is in line with the reduced food intake in these groups. In a long administering study, tramadol in dogs leads to a reduced weight gain [96]. In Koutroli et al. tramadol treated mice show no differences in body weight loss compared to untreated mice after embryo transfer surgery [97].

Daily food intake in the used strain of mice is about 4 g per mouse [93]. These findings are in line with the baseline results in the current study. Sham and operated animals treated with tramadol in the high dosage showed reduced food intake after surgery and anesthesia. As a side effect tramadol in the high dosage may reduce hunger or wellbeing so animals eat less. In a study by Evangelista et al., mice treated with 0.2 mg/ml tramadol in the drinking water showed a stable food intake [25]. In Ratsep et al. food intake was also reduced after surgery in the tramadol and buprenorphine treated group but not in those treated with meloxicam [98]. Buprenorphine treatment led to no change in the food intake, while in other studies a reduced food intake was observed after buprenorphine treatment [14, 15]. In the study, food intake could only be determined per cage as single housing was not allowed by the legislation authorities, therefore differences in the intake of single animals could not be assessed.

Fecal samples were collected for measuring fecal corticosterone metabolites (FCM) in order to evaluate potential short-term stress. The fecal samples were quite variable in numbers between different treatment groups especially on the first and second day. In the

current study, fecal samples were collected during the observation process whenever the animals defecated voluntarily. A forced removal technique by manual manipulation described by Rakowski et al. may have been more successful [99]. A manual manipulation was not chosen because additional stress from needed handling should be prevented [100]. The distribution of fecal corticosterone metabolites was the same in all groups throughout the testing days with high values on day 1 and low values from day 2 until the end, even under the ones from animals that were handled only. This points to acute stress in all animals on the first day of treatment. The reversal to low values could be either a sign of no stress in the animals from day 2 or may be indicating a negative feedback inhibition of corticosterone release due to chronic stress [101]. The fact that animals with analgesia in their drinking water already show high values of FCM on day 1, shows that analgesia alone seems to have a great influence on the stress reaction of the animals. On the contrary, Goldschlager et al. see no effect of buprenorphine, meloxicam or bupivacaine on FCM in rabbits [102]. Also unlike our result, in two studies by Wright-Williams et al. mice receiving only analgesia show lower FCM values than mice undergoing a vasectomy [103, 104].

The adrenal glands showed no histological differences within the operated animals and compared to naïve animals. Analgesia or dosage does not seem to have an impact on the histological parameters in operated animals. There is no difference compared to the naïve animals so there may be no long-term stress in the mouse osteotomy model or the experienced stress may not be long or not severe enough. Additionally, this method may also not be sensitive enough to detect stress in the two-week experiment period. However, in the study on a colitis mouse model by Hausmann et al. where colitis was induced over a period of over nine days applying this method to detect long-term stress, the adrenal glands showed signs of chronic stress compared to healthy animals on the day of euthanasia [81]. In another study by Ulrich-Lai et al. researchers investigate histological changes of the adrenal gland in rats due to chronic variable stress [105]. Ulrich-Lai et al. work with specific cell markers to distinguish between cells of the outer and inner zona fasciculata. As a result, they observed hypertrophy in the inner zona fasciculata and medulla and hyperplasia in the outer zona fasciculata. With the method used in the refinement project a differentiation between the two parts of the zona fasciculata was not possible and therefore different cell changes could not be identified. In conclusion, the used method in the refinement project could not be appropriate to detect stress-induced histological changes in the adrenal gland.

6.3 Analgesic Efficiency of Tramadol and Buprenorphine in the Drinking Water

To investigate changes in behavior that might hint on pain, reduced wellbeing or impairment of movement, a clinical score was assessed. In the clinical scoring, operated animals treated with tramadol in the high dosage reached the highest scores. Thus, tramadol in the high dosage does not seem to have a better pain-relieving activity in the operated animals. Additionally, the clinical score of Sham animals in all three treatment groups was also elevated, indicating a reduced wellbeing or side effects from anesthesia and analgesia. The influence of a reduced wellbeing or potential side effects of anesthesia and an analgesic drug on the clinical score is in line with previous findings that the score is not solely pain-specific. An increase in the MGS, which was a part of the clinical scoring, after isoflurane anesthesia is also shown by Miller et al. [31]. Mice with Tlow had the lowest scores in the Sham group and slightly lower scores in the operated animals suggesting less side effects from the anesthesia and low tramadol dosage. Therefore, the animal's wellbeing may be better when treated with Tlow.

In the nest complexity, the high influence of anesthesia and even analgesia on this score was seen in all of the treatment groups, values decrease after treatment. This influence of anesthesia was also seen in the study by Jirkof et al. [20]. A decrease of nest complexity was also observed in fentanyl treated mice [106]. Operated animals of the Thigh group recovered slowest which may implicate a negative effect of tramadol on the nest building activity. The slow recovery of the nesting score in buprenorphine animals without anesthesia and surgery could be explained by the reduced resting time in these animals compared to the other groups (5.9). Mice may spend less time on nest building behavior or existing nests are accidently destroyed by the active animals between the observations. The nest complexity score showed a similar distribution in all groups with a reduction after analgesia or anesthesia. Implicating this test to be rather good in assessing analgesic side effects associated with a reduced wellbeing of the animal. In conclusion, wellbeing seems to be negatively influenced by the high tramadol dosage. Again, this test was cage based so no single animal based data could be assessed.

The explorative test was originally planned as a time-to-integrate-to-nest test. Rock et al. rate the TINT as positive when the new nesting material is integrated into the existing nest while Gaskill et al. describe it as positive once the given nesting material is moved from its original location [21, 24]. The animals in the current study failed to integrate or move the given nesting materiel. Rock et al. stated that single housed animals are more likely to have negative scores. The C57BL/6N perform good in the TINT compared to other strains, sex does not influence the test in this study. Gaskill et al. mentioned to better use

a nestlet than Envirodri crinkle paper as a new object because it is easier to score. Hager et al. also find group housing with four to five animals more prone to a better performance [26]. The reason for the failure of the TINT in the current study is therefore not clear. A longer habituation time to this test may lead to an integration but contradicts with the purpose of the TINT as a quick and easy way to assess pain and wellbeing. The obtained data was therefore used to grade explorative behavior. All mice scored positive in the baseline measurements but the scores were afterwards decreased by the surgery and the anesthesia. The test on explorative behavior seemed to display wellbeing more than just pain. In this explorative behavior Sham and operated animals treated with Thigh score lowest. In conclusion, tramadol in the high dosage seemed to negatively influence the wellbeing as previously seen in the nest complexity. This test was cage based and results were rated positive or negative with the reaction of only one animal so no single animal based data could be assessed.

Operated animals had the lowest activity in all groups over the whole period of 48h. This result is in line with many studies assessing activity after surgery or a painful inflammation with either locomotor analysis or voluntary wheel running [107, 108]. Roughan et al. described that mice undergoing vasectomy increase their inactive periods due to pain which is comparable with the activity levels in the osteotomized mice in the current experiment [109]. The higher resting time in operated animals indicated a pain induced reduction of the animals' activity. The operated and Sham animals treated with Thigh showed a lower decrease of activity compared to Tlow and BUP in the first 24h.This may display a positive influence of tramadol in the high dosage on pain relief but could also indicate a stimulatory effect of tramadol itself on the activity. Data from Evangelista et al. did not hint towards an increased activity with the use of tramadol in mice [25]. In the first 24h in the DW group BUP have the highest activity. Many studies showed the side effect of buprenorphine, increasing the locomotor activity and distance moved in mice and rats [14, 15, 17, 54, 110]. This means that the assessment of activity as a parameter of pain and pain relief has to be used carefully, as the used analgesia could stimulate activity and therefore the results can be interpreted as the analgesia actually relieving pain. Unfortunately, baseline video recordings were only done for the first 24h. Activity might have been changed in the second 24h because animals get more used to the cage and the environment. It should also be kept in mind that the resting times are variable between the animals.

An elevated limp and dragging score as well as flinching only occurred in operated animals. These parameters are therefore viewed as pain-specific in the current study. Though the differences were not significant, operated animals treated with the high tramadol dosage scored high results over a longer time in all three tests. This implicates, that

tramadol in the high dosage of 1 mg/ml does not lead to a better pain relief in the mouse osteotomy model. A classical approach to assess sensitivity, allodynia and therefore the analgesic efficacy of a pain medication is the testing of mechanical or thermal nociception. The time until withdrawal to a noxious stimulus is measured. Animals experiencing pain show decreased response times to the stimuli. Mechanical nociception can be tested with the von Frey assay while thermal nociception can be assessed with the hotplate test. In studies evaluating fracture pain these tests in combination with subjective pain scales and the assessment of weight bearing are commonly used [84, 111-113]. In the present work, a von Frey test was not scheduled as an increased severity due to the mechanical stimulation of the operated hind limb was expected.

Rear up seemed to be influenced by pain and anesthesia. Operated animals did not recover towards baseline values, so pain or the impact of the osteotomy with the external fixator influenced the rear up time over the complete measuring time. The mice seemed to avert putting load on the affected limb. In humans and animals weight bearing after a fracture or orthopedic surgery is reduced as well. In the operated animals, the ones treated with the high tramadol dosage recovered not significantly but more slowly than the other two groups. In conclusion, that implies that tramadol in the high dosage does not lead to a better pain relief after the osteotomy. A slight decline of the rear up time also occurred in analgesia only animals. As opioids are known to cause nausea and dizziness in humans as well as in animals, these mice may feel nauseous and lightheaded after receiving opioids and therefore loose balance and lift one leg when rearing to regain balance [114, 115].

A grooming directed to the left leg and an increased engagement with this extremity occurred in all animals, mainly in the operated and sham animals. The grooming time did not differ between the two groups. As both groups received a dressing spray to close the wound, it is assumed that grooming is not due to pain but rather caused by the sticking spray on the animal’s skin.

Guarding did not occur in any animals. A possible explanation for the absence of this pain-specific behavior in operated animals could either be that these animals are not able to show guarding behavior due to the restricted movement of the external fixator or that there was no strong pain as the animals were given an analgesic. In rats guarding behavior is used as a measure of ongoing pain in inflammatory, induced by complete Freund’s adjuvant, and bone cancer pain [85, 87]. But is also shown by rats in a fracture pain model [116]. In mice, guarding behavior is also observed in the course of bone fracture pain [86, 112, 117]. The fractures in these studies are fixated with a pin not with an external fixator.

It is possible that these animals are more free in their movements and are therefore able to perform the guarding behavior.

6.4 Impact of the Pain Management on Fracture Healing

To check for possible implications of the pain medication on the bone healing, *in vitro* µCT and a histological staining was applied. In all three *in vitro* µCT parameters, differences of the bone healing between control and vehicle animals were found. Animals treated with the vehicle had lower bone healing in all three pain management protocols, but only in Tlow and BUP this difference was significant. In the histological parameters, enabling a differentiation of all relevant tissues, a reduction of the bone healing was also visible in the vehicle group, underlining the *in vitro* µCT results. No significant differences were found between the analgesia groups. The reduced bone healing in animals treated with Lyostypt is just recently shown in a study conducted by Lang et al.[118].

In conclusion, these results are in line with previous findings of the basic proof-of-concept study. Namely, the difference of bone healing between control and vehicle (Lyostypt) groups in animals treated with the low tramadol dosage of 0.1 mg/ml. That implies, that the different pain management protocols, tramadol in the low or high dosage and buprenorphine do not impact the bone healing in this mouse osteotomy model. Nevertheless, the impact of an analgesia should be evaluated in every animal model and the pain management should not be changed in a running study.

6.5 Side effects and unexpected occurrences

One osteotomized animal of the tramadol high dosage group had to be euthanized prior to the set date of 14 days due to achieving a humane endpoint. In the histology, a high-grade hepatic lipidosis was confirmed in this animal. A pre-existing metabolic condition can be assumed leading to a potentially altered liver function. The impact of the anesthesia and surgery led to a reduced food and water intake which cannot be easily overcome with an already existing health condition. Food and water intake will further decrease and lead to more liver damage, a rapidly decreasing body weight loss and a highly reduced wellbeing. With a reduced wellbeing in combination with the high body weight loss this animal reached the humane endpoint of the study. It has to be noted that this mouse did not appear to have a reduced wellbeing or health status prior to the beginning of the experiment.

In the livers of one Tlow operated and one BUP operated animal no abnormalities were detected. The remaining livers showed a mild hepatitis with suspicion of a *Helicobacter*

spp. infection. A bacterium often found in laboratory rodent facilities and not assumed to confound orthopedic research [119].

The bleeding during surgery was influenced by the time point of the buprenorphine injection. There was more bleeding intraoperative when the buprenorphine was applied 1 hour before the surgery. No literature was found on the direct effect of buprenorphine on bleeding during a surgery. The bleeding might be caused by an elevated heart rate due to the injection as seen in the study by Ilback et al. [120]. In the osteotomy surgery, with its short duration and low blood loss, this result does probably not impact the mice wellbeing. In surgeries with longer duration and where blood loss could play an important role, this should be kept in mind. Buprenorphine was injected 1 hour prior to surgery rather than at the immediate preparation to ensure an analgesic action once the surgery starts. A long term subcutaneous buprenorphine for cats is recommended to be given 1 hour prior to an operative procedure [91]. Other data shows an onset of action of 15-30 mins and a peak in analgesic activity within 1 hour in intramuscularly injected buprenorphine [89, 90].

In one animal we observed a delayed wound healing, followed by increased licking of the own wound or licking by cage mate. The wound of this animal healed quite fast and was not treated. The second animal with delayed wound healing showed the same wound healing disorder but healing was slower but improved under treatment with adequate ointment. A delayed wound healing never occurred before in the Tlow treated animals of the proof-of-concept study. As both of these animals were treated with buprenorphine it may indicate an impact of the opioid on the wound healing. Several studies on rats show differences in wound healing on a histological basis within individual analgesics but no overall delay is seen in buprenorphine [121-123]. As mentioned before, self-manipulation is suspected in the mice with altered wound healing. Autotomy is increased by buprenorphine following brachial nerve section in rats [124], but there is no data on increased autotomy in mice with buprenorphine treatment. Additionally, a second and third wound appeared in the second mouse. As this mouse was treated with clindamycin three times to prevent infection, an irritation and therefore skin lesions by the injection could be suspected. There is no literature on clindamycin itself being the cause for the skin lesions, but Burns et al. report tissue necrosis after repeated subcutaneous enrofloxacin injections in the chinchilla [125]. The additional wounds could also be due to ulcerative dermatitis a common disease in the strain of C57BL/6 [126].

6.6 Integration of Refinement Study

Several advantages of integrating a refinement study were worked out. On the one hand gaining information with the use of a lower number of animals could be possible. Refine-

ment measures can be tested in a specific animal model and therefore recommendations, for examples on pain management, can be directly applied by the researcher. On the other hand, concerns were raised about possible disadvantages of the integration approach. The refinement measures, in this case different pain management protocols could impact the underlying study. This should be kept in mind and the implications on the underlying study should be properly checked during the trial. Attention must be payed because compromises and changes made for integrating the refinement study could decrease the validity of the original study. The reduction of the animal number mentioned previously would be void, if additional animals must be used in that case. Assessing the benefits of a refinement measure during a basic research study could put the animals through additional pain, suffering or stress. The severity could be increased and therefore approval by the authorities could be denied. Submitting an additional animal test proposal for the refinement study should be considered. These implications were taken into consideration while planning, executing and analyzing this project.

Prior to the study, the application for an independent animal test proposal for the refinement study was actually demanded by the local animal rights protection authorities as the integrated refinement tests could possibly increase overall severity. During the study, it was recognized that the mice are indeed influenced by the additional behavioral testing procedures. An increased severity compared to the underlying basic research study solely investigating bone healing can be assumed.

In the current study, useful results on the pain management in the mouse osteotomy model were gathered. With the integrational approach, the animal number was reduced compared to having two separate studies. The fear of losing outcome or value of results in the bone healing setup due to additional experiments done in the course of the refinement study could not be confirmed. All read-outs could be acquired and were somehow in line with previous results gained by the working group.

7 Conclusions and Recommendations

The aim of this study was the evaluation of three different analgesia protocols in a mouse osteotomy model. An approach of integrating this refinement study into a basic research proof-of-concept project on bone healing was used.

Overall, the concept of integrating a refinement study on pain management into a basic research project on bone healing was purposeful, especially in the light of reducing animal numbers and finding recommendations on the pain management for one specific animal model.

This refinement study shows a sufficient pain relief in animals treated with analgesia in their drinking water. A preemptive analgesic treatment, prior to the painful event such as a buprenorphine injection to ensure sufficient pain reduction immediately after surgery is important. In our study, animals were able and willing to drink the analgesia enriched water directly after surgery and took in sufficient amounts.

The clinical score, nest building and explorative behavior as well as the model-specific pain parameters proved the dosages used for tramadol and buprenorphine to provide a sufficient pain relief in the mouse osteotomy model when applied via the drinking water. The recommendation of the GV-SOLAS for tramadol in the drinking water is currently 1 mg/ml [6], the Thigh dosage that was tested in this study. One should keep in mind, possible side effects and reduced wellbeing resulting from the higher tramadol dosage. Taking the results of the current study into account, the GV-SOLAS should consider recommending the use of tramadol with a dosage of 0.1 ml tramadol per ml drinking water for pain relief in the mouse osteotomy and other animal models.

This study contributes to the idea of evidence-based recommendations on pain management specific to one animal model. Further studies providing information on side effects of analgesia and sufficient pain management protocols in different animal models should be conducted.

8 Summary

8.1 Summary

Evaluating the Pain Management in a Mouse Osteotomy Model - Integrating a Refinement Approach in a Basic Research Study

Tramadol applied via the drinking water is a commonly used analgesia in the mouse osteotomy model. Another opioid that can be used is buprenorphine. The recommendation for tramadol in the drinking water was increased 40-fold by the GV-SOLAS from 2010 to 2015. A recommendation on buprenorphine is given for injection but not for the application in the drinking water. Nevertheless, some standard operating procedures are found on buprenorphine applied via the drinking water. Model-specific recommendations on pain management in the mouse osteotomy model are not available. In the current study, three pain management protocols, two dosages of tramadol and buprenorphine applied via the drinking water in the mouse osteotomy model were tested. This refinement project was integrated into a basic research study. The aim of this project was to provide researchers with a specific recommendation on pain treatment in bone-linked mouse models.

The three pain management protocols (tramadol 0.1 mg/ml, tramadol 1 mg/ml and buprenorphine 0.009 mg/ml in the drinking water) were evaluated for feasibility, analgesic efficacy and impact on the bone healing in the mouse osteotomy model. The feasibility was tested with the assessment of body weight, food and water intake, serum level of tramadol and M1 as well as stress measurements. The stress measurements consisted of the analysis of fecal corticosterone metabolites as a parameter for short-term stress and the histological assessment of adrenal glands as a measurement for long-term stress. To check for possible side effects of the different medications, the livers were histologically assessed. With the help of parameters such as a clinical score, nest complexity, explorative behavior and an activity assessment the animals were screened for behavior indicating pain or reduced wellbeing. Model-specific pain parameters like the limp score, dragging score, flinching, guarding, grooming time of the operated limb and rear up time were applied. These behavioral and model-specific tests are used to evaluate the analgesic efficiency of tramadol and buprenorphine. To grade the impact of the different pain management protocols on the animal model parameters of bone healing were assessed. These included *in vitro* µCT and histology of the osteotomized bone after euthanasia.

During the refinement project, more questions came up regarding the pre-emptive injection of buprenorphine. Therefore, a study on the impact of pre-emptive injected buprenorphine on bleeding during the surgery was conducted.

The animals used in the study drank after the procedure with more drinking events in the night compared to the day. A reduced wellbeing in animals treated with the tramadol dosage of 1 mg/ml was found. Also seen in the body weight loss, reduced food intake and drinking events, the clinical scoring and nest complexity. Short stress was visible on day one in all animals, but there were no signs of histological changes in the adrenal glands. The used clinical scoring was not specific to pain, but rather displayed a reduced wellbeing in Thigh animals. In the explorative behavior Tlow animals showed the fastest recovery. Operated animals had lowest activity with no clear differences between the treatment groups. The pain-specific parameters showed no better pain relief in the high tramadol group. No impact of the changed pain management protocol on the bone healing and therefore on the main read-out of the mouse osteotomy model was seen. One animal was euthanized prior to the end of the study which was not related to the analgesic treatment but rather due to a preexisting potential metabolic disorder. Tramadol of the low dosage showed serum concentrations that can be assumed sufficient to relief pain compared to human values. The time point of pre-emptive buprenorphine injection influenced the bleeding during surgery, specifically an injection 1h before the surgery increased bleeding. Additionally, buprenorphine may alter wound healing.

After successfully conducting this integrated refinement study, more projects of this kind can be recommended to gain knowledge of possible refinement benefits in specific animal models. Tramadol and buprenorphine in the drinking water is an efficient route of applying analgesia in the MOMo. The concern of a reduced water intake was not confirmed in tramadol of the low dosage and in buprenorphine. The use of tramadol in the dosage of 1 mg/ml is not necessary in the MOMo. It offers no extra benefit in pain reduction but rather reduces wellbeing in the animals. Tramadol in the dosage of 0.1 mg/ml or buprenorphine in the drinking water are a sufficient method to relief pain in the MOMo.

8.2 Zusammenfassung

Evaluierung des Schmerzmanagements in einem Maus-Osteotomie-Modell – Integration eines Refinement Ansatzes in eine grundlagenwissenschaftliche Studie.

Tramadol wird im Maus-Osteotomie-Model häufig als Schmerzmittel im Trinkwasser verwendet. Ein weiteres häufig angewendetes Opioid in der Schmerzbehandlung in der Versuchstierkunde ist Buprenorphin. Die Empfehlung der GV-SOLAS für Tramadol im Trinkwasser wurde von 2010 bis 2015 um ein vierzigfaches erhöht. Eine Empfehlung für die Injektion von Buprenorphin ist vorhanden, die Empfehlung für eine Gabe über das Trinkwasser gibt es nicht. Standardarbeitsanweisungen zu Buprenorphin im Trinkwasser sind vorhanden während modell-spezifische Empfehlungen zum Schmerzmanagement im Maus-Osteotomie-Modell fehlen. In dieser Studie wurden drei Schmerzmanagement Protokolle, zwei Dosierungen von Tramadol und Buprenorphin im Trinkwasser im Maus-Osteotomie-Modell untersucht. Dieses Refinement Projekt war in eine grundlagenwissenschaftliche Studie eingebettet, die das Maus-Osteotomie-Modell benutzte. Ziel dieses Projekts war es, Wissenschaftlern spezifische Empfehlungen zum Schmerzmanagement im Maus-Osteotomie-Modell zur Verfügung zu stellen.

Drei Schmerzmanagement Protokolle (Tramadol 0.1 mg/ml, Tramadol 1 mg/ml und Buprenorphin im Trinkwasser) wurden unter dem Aspekt der Umsetzbarkeit, analgetischer Wirksamkeit und Einfluss auf die Knochenheilung im Maus-Osteotomie-Modell evaluiert. Die Realisierbarkeit wurde mit der Messung des Körpergewichts, Futter- und Wasseraufnahme und Messungen zur Stressbelastung bewertet. Diese bestanden aus einer Analyse der fäkalen Corticosteronmetaboliten als Parameter für Kurzzeitstress und der histologischen Untersuchung der Nebennieren als Parameter für Langzeitstress. Zusätzlich wurden die Serumkonzentration von Tramadol und M1 gemessen und die Lebern von operierten Mäusen nach der Euthanasie histologisch untersucht. Mit Hilfe von einem klinischen Score, der Nestkomplexität, des explorativen Verhaltens und einer Aktivitätsmessung wurden die Tiere auf Verhalten getestet, welches auf Schmerz oder reduziertes Wohlbefinden hinweist. Modell-spezifische Schmerzparameter wie der Limp Score und Dragging Score, Flinching, Guarding, Grooming und das Aufrichten auf beide Beine wurden angewandt. Diese Verhaltens- und Modell-spezifischen Tests dienten dazu die analgetische Wirksamkeit von Tramadol und Buprenorphin zu evaluieren. Um den Einfluss der verschiedenen Protokolle auf das Tiermodell zu untersuchen, werden Knochenheilungsparameter genutzt, die auch in der zugrundeliegenden Studie angewandt wurden. Diese beinhielten die Mikro-Computertomographie und die histologische Untersuchung der osteotomierten Beine nach der Euthanasie.

Während der Studie kamen neue Fragen auf, was zu zusätzlichen Untersuchungen führte. Eine Studie zum Einfluss von Buprenorphin, injiziert als präemptives Schmerzmittel, auf Blutungen während der Operation wurde durchgeführt.

Die Tiere in dieser Studie tranken zuverlässig nach der Operation, beziehungsweise Anästhesie und Gabe von Analgesie über das Trinkwasser. Während der Nacht war die Anzahl der Trinkevents höher als am Tag. Die Tiere mit Tramadol in der Dosis 1 mg/ml zeigten ein reduziertes Wohlbefinden, was sich im reduzierten Körpergewicht, der reduzierten Futteraufnahme, niedrigeren Wasseraufnahmefrequenzen, klinischen Scores und geringerer Nestkomplexität wiederspiegelte. Kurzzeitstress war sichtbar in allen Gruppen am Tag 1. Es gab keine Hinweise auf histologische Veränderungen in den Nebennieren. Der benutzte klinische Score war nicht schmerzspezifisch und wies vielmehr auf ein reduziertes Wohlbefinden in den Tieren mit einer hohen Tramadoldosis hin. Im explorativen Verhalten zeigten operierte Mäuse mit Tramadol in der niedrigen Dosis die schnellste Erholung. Operierte Tiere hatten die längsten Ruhezeiten, mit keinen deutlichen Unterschieden zwischen den einzelnen Behandlungsgruppen. Die schmerzspezifischen Parameter wiesen darauf hin, dass die hohe Tramadoldosis nicht zu einer verbesserten Schmerzausschaltung führt. Das geänderte Schmerzmanagement hatte keine Auswirkungen auf die Knochenheilung und damit auch nicht auf das hauptsächliche Auswertungskriterium des Maus-Osteotomie-Modells. Ein Tier wurde vorzeitig euthanasiert, was nicht in Verbindung mit der Behandlung stand, sondern vermutlich durch eine bereits bestehende Stoffwechselerkrankung hervorgerufen wurde. Verglichen mit der humanen effektiven Serumkonzentration, stellte die Serumkonzentration von Mäusen bei einer Behandlung mit der niedrigen Tramadoldosis vermutlich eine ausreichende Schmerzausschaltung sicher. Der Zeitpunkt einer präemptiven Buprenorphininjektion beeinflusste die Stärke und das Auftreten von Blutungen während der Operation. Zusätzlich veränderte Buprenorphin möglicherweise die Wundheilung.

Nachdem diese integrierte Refinement Studie im Maus-Osteotomie-Modell erfolgreich zu Ende gebracht wurde, sollten Studien dieser Art vermehrt durchgeführt werden, um mögliche Vorteile von Refinementmethoden in spezifischen Tiermodellen aufzuzeigen. Die Gabe von Tramadol und Buprenorphin über das Trinkwasser ist eine effiziente Art des Schmerzmanagements im Maus-Osteotomie-Modell. Die Befürchtung eines reduzierten Trinkverhaltens konnte für die niedrige Tramadoldosis und Buprenorphin nicht bestätigt werden. Die erhöhte Tramadoldosis von 1 mg/ml ist im Maus-Osteotomie-Modell nicht notwendig. Die erhöhte Dosis führt zu keiner erhöhten Schmerzreduktion, sondern eher zu einem reduzierten Wohlbefinden der Tiere. Die niedrigere Tramadoldosis von 0,1

mg/ml und Buprenorphin im Trinkwasser führen zu einer ausreichenden Schmerzausschaltung im Maus-Osteotomie-Modell.

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10 Publications

Durst M.*, Jirkof P.*, Klopfleisch R., Palme R., Thöne-Reineke C., Buttgereit F., Schmidt-Bleek K., Lang A. *Tramadol and Buprenorphine viadrinking water as analgesiain a mouse-osteotomy model – Why dosage matters!* Publication in revision

Lang A., Kirchner M., Stefanowski J., **Durst M**., Weber M., Pfeiffenberger M., Damerau A., Hauser A., Hoff P., Duda G. N., Buttgereit F., Schmidt-Bleek K., Gaber T. *Collagen I-based scaffolds negatively impact fracture healing in a mouse-osteotomy-model although used routinely in research and clinical application.* Acta Biomaterialia Volume 86, 1 March 2019, Pages 171-184

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Abstracts & Participation in Conferences

Durst M., Jirkof P., Arras M., Schmidt-Bleek K., Buttgereit F., Lang A. (2018). *Evaluating Tramadol and Buprenorphine applied via the drinking water as pain medication in a mouse osteotomy model.* EUSAAT Linz 2018. Oral Presentation

Durst M., Jirkof P., Arras M., Schmidt-Bleek K., Buttgereit F., Lang A. (2018). *Analgesia in mouse osteotomy: Tramadol and Buprenorphine applied via the drinking water*. 17th Day of Clinical Research, University Hospital Zurich. Poster Presentation

Durst M., Jirkof P., Arras M., Schmidt-Bleek K., Buttgereit F., Lang A. (2017). *Evaluierung des Schmerzmanagements im Maus-Osteotomie-Modell*. Annual Meeting of the Society for Animal Laboratory Science (GV-SOLAS). Oral Presentation

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12 Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die hier vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 18.12.2018

Mattea Sophie Durst

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