A Buprenorphine depot formulation provides effective sustained post-surgical analgesia for 72h in mouse femoral fracture models

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Summary

Adequate pain management is essential for ethical and scientific reasons in animal experiments and should completely cover the period of expected pain without the need for frequent re-application. However, current depot formulations of Buprenorphine are only available in the USA and have limited duration of action. Recently, a new microparticulate Buprenorphine formulation for sustained release has been developed and safely used in first studies serving as an alternative product within Europe. Pharmacokinetics indicate a potential effectiveness for about 72h; the period that must be covered with analgesia in mouse fracture models. Here, we investigated whether the administration of the newly developed sustained-release Buprenorphine formulation ensures a continuous and sufficient analgesia in mouse femoral fracture models as a potent alternative to the application of Tramadol via drinking water. Both protocols were examined for analgesic effectiveness and side effects on experimental readout in two femoral fracture models using rigid and flexible external fixators in male and female C57BL/6N mice. We monitored general parameters of wellbeing, and model-specific pain parameters. Both, Tramadol in the drinking water and the sustained-release Buprenorphine provided effective analgesia. However, in male mice with flexible fixation, composite pain scores were significantly higher with Tramadol than in the other groups, and locomotion during gait analysis was more profoundly altered. Fracture healing outcome was not different between analgesic regimes. The availability of a sustained-release formulation of Buprenorphine in Europe would be beneficial for pain management in preclinical studies to increase animal welfare and actively support the refinement of painful animal experiments.

Keywords: pain management, refinement, bone repair, animal model, tramadol

1 Introduction

Despite the increasing progress in alternative methods, animals – especially mice – are still widely used and required in fundamental and translational research to study the complexity of biological and pathophysiological processes. Therefore, the active implementation of the 3R principle (Replace – Reduce – Refine), with a particular importance of *Refinement* forms the indispensable basis for a humane approach to conduct animal experiments. Thus, adequate pain assessment and medication in animals before, during and after the experimental procedure is crucial to decrease suffering and support data quality. Furthermore, handling-induced stress and insufficiently treated pain can affect animal behavior and physiological responses, especially in an immunological context, leading to potential falsification of the scientific outcomes and reduced reproducibility (Balcombe et al., 2004; Carstens and Moberg, 2000; Guo and Dipietro, 2010; Page, 2003; Peterson et al., 2017; Jirkof, 2017). However, evidence-based data on individual pain management efficiencies in surgical mouse models is still rare (Flecknell, 2018; Jirkof, 2017) and the reporting quality of the used analgesic protocols is often insufficient (Carbone and Austin, 2016; Wolter et al., 2021).

The potent opioid Buprenorphine is very often used in rodents after surgical intervention (Guarnieri et al., 2012; Roughan and Flecknell, 2002). Due to a short half-life of approximately 3 hours (Bullingham et al., 1980; Gades et al., 2000; Yu et al., 2006), frequent application intervals are required resulting in repeated handling of the animals. The commonly reported application intervals of subcutaneous or intraperitoneal injection every 8 – 12h can lead to pain peaks and insufficient analgesic coverage (Jirkof et al., 2015; Wolter et al., 2021). The parenteral application of other opioids such as Morphine, Tramadol and Fentanyl is most often not suitable for pain alleviation in rodents, as their half-life is even shorter (Gades et al., 2000; Evangelista-Vaz et al., 2018; Girón et al., 2002). It has also been suggested that the experience of pain and sensitivity to opioid analgesia can be strain and sex-dependent (Fillingim and Gear, 2004; Kest et al., 2000; Smith, 2019). Thus, the design of adequate pain management regimes needs to consider the duration and nature of the expected pain, animal species-specific sensitivity to opioid analgesia (including strain and sex) and the evaluation of options to reduce re-injection intervals and therefore, handling time.

To reduce handling-associated stress and to ensure continuous analgesic coverage, an alternative application route of Buprenorphine or Tramadol in the drinking water has been routinely used e.g., in orthopedics-related mouse models (Bucher et al., 2019; Jirkof et al., 2019; Rapp et al., 2018; Histing et al., 2016). As the uptake of analgesics with the drinking water is dependent on the drinking frequency and amount, the overall effectiveness of this treatment strategy might be highly influenced by e.g., reduced activity and water intake after anesthesia and surgery and circadian activity (Sauer et al., 2016). A drug formulation that extends the analgesic effects due to sustained parenteral drug release can overcome such challenges and serve as a powerful tool to refine today's analgesic regimens after surgical interventions in animal experiments. Current depot/sustained-release formulations of Buprenorphine for mice and rats are either not commercially available or only available in the United States of America (USA). Examples include Buprenorphine SR-LAB (ZooPharm) or Ethiqa XR (Fidelis Animal Health). Attempts to import these products to Europe have failed due to missing approval through the European Medical Evaluation Agency (EMEA).

To fill the gap, Schreiner et al. have successfully developed a poly-lactic-co-glycolic acid (PLGA) based microparticulate drug formulation for sustained drug release of Buprenorphine (Schreiner et al., 2020; Schreiner et al., 2021). In a proof-of-concept study in female C57BL/6J mice, they observed therapeutic-relevant drug levels of the sustained-release Buprenorphine (BUP-Depot) in the brain for more than 24h, an antinociceptive effect in the hot plate test and pain relief after a minor abdominal surgery for at least 72h. To test the BUP-Depot for the effectiveness in a wider range of surgical interventions, we here performed a pain management study in two mouse femoral fracture models. The selection of appropriate analgesics in experimental fracture healing studies is limited by potential adverse effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on inflammatory events during the initial and proinflammatory phase of fracture healing (Bove et al., 2009; Cottrell and O'Connor, 2010; Gerstenfeld et al., 2003). Moreover, the nature of pain divides into the pain sensitization during wound healing and the bone-related pain due to the cutting of the periosteum during osteotomy and is therefore a useful representation of potential pain conditions (Tappe-Theodor et al., 2022).

To this end, we tested the newly developed BUP-Depot as a potential alternative to the established application of Tramadol via the drinking water. Both pain management protocols were examined for their analgesic efficacy and adverse effects on experimental readouts in two femoral fracture models using rigid and flexible external fixators. We monitored (i) general parameters of wellbeing e.g., body weight, food and water intake, nestbuilding and explorative behavior and (ii) model-specific pain parameters including walking behavior (limp score) and CatWalk analysis. In addition, fracture healing outcomes were examined at the end of the study to exclude negative influences on the regeneration process. To overcome potential sex-dependent differences in response to the analgesic protocol male and female mice were included. A refined analgesic regime using a sustained-release Buprenorphine was implemented for post-surgical pain management in a mouse bone fracture models to explore its potential to improve animal welfare in a wider range of pre-clinical animal models.

2 Animals, Material and Methods

Animals and husbandry

The study was conducted according to the guidelines of the German Animal Welfare Act, National Animal Welfare Guidelines, and was approved by the local Berlin state authority (Landesamt für Gesundheit und Soziales – LAGeSo; permit number: G0044/20). Health monitoring in the animal facility was performed according to the FELASA guidelines (Supplementary Information). A total of 40 male and 40 female C57BL/6N mice aged 8 weeks were either provided by the Experimental Medicine Research Facilities (Charité-Universitätsmedizin Berlin, Berlin, Germany) or purchased from Charles River Laboratories (Sulzfeld, Germany). Mice underwent the first intervention (anesthesia/analgesia) at 10 weeks (body weight – males: 25.96 ± 2.1 g; females: 21.41 ± 1.3 g) and osteotomy with 12 weeks (body weight – males: 27.61 ± 1.9 g; females: 22.75 ± 1.3 g). Mice were housed in a semi-barrier facility with limited access, protective clothing, and individually ventilated cages (IVC). Housing conditions included a 12/12–h light/dark cycle (lights on from 6:00 a.m. to 6:00 p.m.), room temperature between 22 ± 2°C and a humidity of 55 ± 10%. Mice had access to food (Standard mouse diet, Ssniff Spezialdiäten, Soest, Germany) and tap water *ad libitum*.

Upon arrival, the mice were randomly divided into groups of two per cage. All mice were housed in Eurostandard Type II clear-transparent plastic cages. In some instances, two males had to be separated due to aggressive behavior. Those mice were housed in a separated pair housing system consisting of compact cages (Green Line IVC Sealsafe PLUS Rat GR 900, Tecniplast, Milan, Italy), which were divided in two equally sized compartments by a perforated transparent cage partition wall enabling olfactory, acoustic, and visual interaction (Hohlbaum et al., 2020). All cages contained wooden chips (SAFE FS 14, Safe Bedding, Rosenberg, Germany) as well as 20 g Envirodri (Shepherd Specialty Papers, USA) and one additional shredded paper towel as bedding and nesting material. Cages were also equipped with a clear handling tube (Datesand Group, Bredbury, UK) and a mouse double swing (Datesand Group, Bredbury, UK). No houses were provided to allow reliable scoring and to reduce the risk of injury after osteotomy. In addition, tunnels and double swings were removed from the cages to reduce the risk of injury due to the external fixator. Two single swings (Datesand Group, Bredbury, UK) per cage were reinstalled 5 days after osteotomy. Cages were changed weekly by the experimenters only. When nesting material was dry, it was transferred to the new cage and replenished to 20 g with new nesting material. In the week of anesthesia and of the osteotomy, the cages were renewed the day before the intervention, to provide a clean surrounding and still allow the animals to get accustomed to the new environment and to build proper nests beforehand. Animals were tunnel handled only and all experimenters performing behavioral analyses were female. Animal husbandry and care was in accordance with contemporary best practice and all individuals involved in the study had a high level of competency.

Humane endpoints were predefined and divided into clinical parameters (e.g., body weight reduction >20%, apathy) and model-specific parameters (e.g., open skin wound, no usage of operated limb). No humane endpoints were reached during the conduct of the study. Potential 3R applications were thoroughly researched and if possible, implemented within this study e.g. enrichment, positive reinforcement during training (white chocolate) and tunnel handling.

Study design and experimental timeline

The study included 8 groups with each n= 9–10 mice, comparing male and female mice, rigid and flexible external fixators, and two different pain management protocols (Tramadol via drinking water vs. sustained-release Buprenorphine (BUP-Depot) (**Fig. 1A**). Exclusion of mice and data are detailed in the Supplementary Information. Cages and mice received individual random numbers which did not allow any inferences for the analgesic regimen or fixation group. Experimenters performing the pre- and post-surgical training and investigations were blinded for the analgesic regimen. Reporting of this study was carried out in compliance with the ARRIVE 2.0 guidelines, including the “Arrive Essential 10” as well as a majority of the “Arrive Recommended Set”. The study was pre-registered in the Animal Study Registry (Bf3R, Germany; DOI: 10.17590/asr.0000221).

After arrival, mice were allowed to acclimate for 5 days. Training was then performed by one female experimenter, following a precise 2-week schedule to accustom the mice to the experimenter, tunnel handling, the Noldus CatWalk XT (Noldus, Wageningen, Netherlands) and observation boxes (Ugo Basile, Gemonio, Italy). Baseline measurements were also obtained during this period (**Fig. 1B**). To correct for individual behavioral changes induced by anesthesia and analgesia alone, mice were first anesthetized and received their assigned analgesic protocol without any further surgical procedure (first intervention). This was followed by closely scheduled monitoring and assessment at 12h, 24h, 48h, and 72h. At day 14 after the first intervention, mice were then subjected to anesthesia, analgesia, and osteotomy and again assessed at the same timepoints as well as at day 10 post-surgical. Mice were euthanized at day 14 post-osteotomy to retrieve the osteotomized left femur.

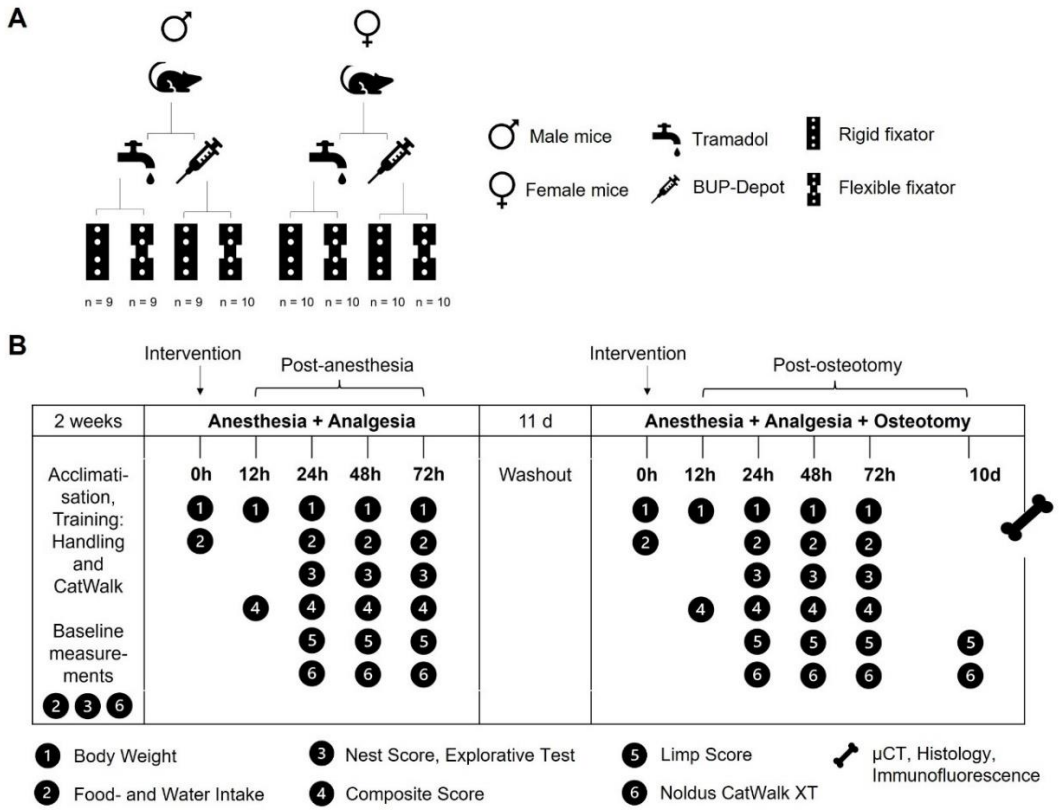

Figure 1. Group assignment and study design. Overview on (**A**) the group assignment, as well as (**B**) time points and measurements of the different parameters. To assure a rapid onset of the analgetic effect, each animal received a single s.c. dose of Temgesic (1 mg/kg) at the beginning of each intervention. Tramadol (0.1 mg/ml) in drinking water was provided one day before and for three consecutive days after interventions. A single dose of sustained-release BUP-Depot (1.2 mg/kg s.c) was administered after interventions.

Analgesic regimes

Each mouse received a single s.c. injection of fast acting standard Buprenorphine (1 mg/kg Temgesic, RB Pharmaceuticals, Heidelberg, Germany) at the beginning of each intervention (anesthesia/analgesia alone and osteotomy) (**Fig. 1**). Depending on the randomly assigned group, mice either additionally received Tramadol administered with the drinking water (0.1 mg/ml, Drops, Grünenthal, Stolberg, Germany) or a s.c. injection of the sustained-release Buprenorphine (BUP-Depot; 1.2 mg/kg). Tramadol via the drinking water was administered one day before and for three consecutive days after both interventions. The BUP-Depot was injected once at the end of both interventions. The PLGA based BUP-Depot (RG 502 H-Big) was prepared at the University of Basel, Switzerland, as described previously by Schreiner et al. (Schreiner et al., 2020; Schreiner et al., 2021). The BUP-Depot was imported in accordance with national regulations for controlled substances (BtM import authorization No. 4679477). Four different batches of BUP-Depot were used during the study. Each batch was analyzed before shipment with respect to drug content, reconstitution time, and drug release kinetics as described previously (Schreiner et al., 2020; Schreiner et al., 2021). The-BUP-Depot was stored as a lyophilizate in crimped glass vials at 4°C. Each vial was reconstituted with physiological saline (0.9% NaCl) immediately before administration.

Anesthesia and osteotomy

Independent of the intervention, all mice were anesthetized with isoflurane (~2–3%; provided in 100% oxygen) (CP-Pharma, Burgdorf, Germany) in an induction chamber before being weighed, and moved onto a heating pad (37°C). Anesthesia was maintained at ~2% via a nose cone. Eye ointment, physiological saline (0.5 ml, 0.9% NaCl (B. Braun Melsungen, Melsungen, Germany)), Clindamycin (45 mg/kg, Ratiopharm, Ulm, Germany) and a single s.c. injection of Buprenorphine (1 mg/kg; Temgesic) were administered. Anesthesia was then upheld for 15 minutes for the first intervention. For osteotomy, the left femur was shaved and disinfected with Braunoderm (B. Braun Melsungen, Melsungen, Germany), the osteotomy was conducted as described previously (Jirkof et al., 2019; Lang et al., 2019; Lang et al., 2022; Rapp et al., 2018). In brief, a longitudinal skin incision was made between knee and hip, and the musculus vastus lateralis and musculus biceps femoris were bluntly separated to expose the femur. Two standardized external fixators (rigid: 18.1 N/mm; flexible: 3.2 N/mm, both RISystem, Davos, Switzerland) were used for stabilization. The external bar of the fixator was positioned parallel to the femur and all pins were positioned accordingly. Afterwards, an approximately 0.5 mm osteotomy gap was created between the second and third pin using a Gigli wire saw (0.44 mm; RISystem, Davos, Switzerland) and the gap was flushed with saline (NaCl (B. Braun Melsungen, Melsungen, Germany)). Muscle and skin were closed with two layers of sutures (muscle: 5-0 Vicryl, skin: Ethilon 5-0, both Ethicon, Raritan, USA). For recovery, the mice were returned to their home cages under an infrared lamp and were closely monitored.

Body weight and food/water intake

Animals were weighed right before the intervention (defined as 0h), and at 12h, 24h, 48h and 72h after each intervention and then every other day until osteotomy/euthanasia. Food/water intake were measured per cage (i.e., two mice) by weighing the food and water bottles every 24h, beginning 1 day

prior to each intervention and ending 3 days after. The difference to the previous value was calculated. All measurements were normalized to the respective baseline values at time point 0h.

Explorative test and nest complexity score

Both scores were assessed before any other assessment or handling of the mice. To examine the motivation of the mice to explore and interact (sniffing, holding with forepaws, or carrying) with a foreign object in the home cage, we added a Nestlet (Ancare, Bellmore, USA) to the cages and observed the mice for one minute. The explorative test was scored 1 (interaction) or 0 (no interaction) per cage while an interaction of one animal of the cage was deemed as sufficient for a positive score. Nest complexity scoring was performed following Hess et al. (Hess et al., 2008) in the home cage assigning scores between 0 – 5.

Composite pain score

A composite pain score was used to combine the assessment of facial expression and clinical appearance (Jirkof et al., 2019; Langford et al., 2010) (**Table 1**). The maximal score was 9. Mice were transferred into a clear observation box (Ugo Basile, Gemonio, Italy) and were individually filmed for 3 min after an acclimatization period of 1 min (Basler Video Recording Software, Ahrensburg, Germany). Video analysis was performed by one blinded observer at timepoints 12h, 24h, 48h, and 72h post-intervention. As anesthesia and analgesia also impact the composite pain score, we calculated the delta composite pain score for each mouse by subtracting the scores from each individual mouse post-anesthesia from their respective scores post-osteotomy. This allows us to evaluate the effect of the surgical procedure on an individual base without the interference of behavioral or clinical changes induced by the anesthesia and analgesia alone (without surgical intervention) (Miller et al., 2015).

Table 1: Composite Pain Score

Parameter	**Specification**	**Score** (0 = not present)
Orbital tightening	Faint narrowing of the orbital area up to a tightly closed eyelid	Range: 0 – 2 0.5 = minimal 1 = moderately 1.5 = moderately severe 2 = severe
Ear position	Ears pulled back or rotated outwards and/or backwards	Range: 0 – 2 1 = moderately 2 = severe
Posture	Crouched posture, head and nose positioned towards the ground	Range: 0 – 1 0.5 = held for less than 10 s 1 = held for more than 10 s
Coat condition	Coat appeared disheveled or unkempt	Range: 0 – 1 0.5 = only certain body parts 1 = all over the body
	Erected fur, mice appear scruffy	Range: 0 – 1 0.5 = only on one body part 1 = on more than one body part, generalized
Movement	Apathetic, sedated, tipsy (0 – 1); Crawling (0 – 1)	Range: 0 – 1 0.5 = minimal 1 = moderately

Walking behavior – limp score

To assess the walking behavior of each individual mouse, limp scoring was performed as described by Jirkof et al. (Jirkof et al., 2019). The mice were individually transferred to conventional type III cages that contained the same type of wooden chips as the home cages. After an acclimation period of 5 min, a 3 min video was recorded. Walking behavior was examined at time points concurrent with the CatWalk analysis at 24h, 48h, and 72h post-anesthesia and post-osteotomy as well as 10d post-osteotomy. Video analysis was performed by two blinded observers and scores from 0 – 4 were assigned (**Table 2**). If walking seemed to be impaired due to a mechanical problem (e.g., displacement of the patella) one point was subtracted from the assigned score.

Table 2: Limp Score

Specification	Limp Score
Normal use, physiological gait	0
Complete ground contact and sporadic limping or alteration of the gait pattern	1
No complete ground contact or limping, constant alteration of the gait pattern	2
Partial non-use of the limb	3
Complete lack of use	4

CatWalk analysis

Specific gait analysis was performed using the CatWalk XT Gait Analysis system for rodents (Noldus, Wageningen, Netherlands). Prior to data acquisition, mice were trained to the setup during three training sessions. Data was then acquired at 24h, 48h, 72h and 10d post-anesthesia (not shown in results) and post-osteotomy. To acquire at least three evaluable runs for analysis per timepoint, multiple runs per animal were recorded. Post-acquisition, the runs were screened, and non-compliable runs as well as interrupted runs (e.g., by sniffing, rearing) were deleted. Runs that noticeably differed from the rest of the runs of these trials were also deleted, leaving an average of 4.4 runs per animal and timepoint for analyses. All runs were classified automatically by the Noldus CatWalk XT software (version XT10.6). Afterwards, every run was reviewed by one researcher and classification errors were corrected manually.

From the obtained data, mean speed (cm/s) (velocity) and the following parameters were analyzed for the osteotomized left hind leg: mean intensity, stand duration (s) and stride length (cm). The Noldus CatWalk automatically calculated an average speed (velocity) of all runs per animal and timepoint. The two mean velocity values from both baseline timepoints were used to create one baseline average velocity. The velocity values at all time points were then normalized to the baseline values (timepoint value divided by average baseline value). The same calculation was done for mean intensity (a measure of load bearing), stand duration and stride length, resulting in relative mean intensity, relative stand duration and relative stride length.

Euthanasia and sample collection

Euthanasia was carried out according to contemporary best practice. At 14 days post-osteotomy, mice euthanized by cervical dislocation in deep anesthesia. The osteotomized femora were retrieved and fixed in 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, USA) at 4°C for 6–8h. The femora were then transferred into PBS until ex-vivo µCT was completed.

Ex-vivo µCT

To determine bone formation three-dimensionally, femurs were scanned in a SkyScan 1172 high-resolution µCT (Bruker, Kontich, Belgium). To ensure sufficient stability, the bones were placed within a narrow PBS filled serological pipette and the pins and the external fixator bar were carefully removed. Voxel size was set to 8 µm and the bones were scanned with a source energy of 70 kV, 142 µA, a rotation step of 0.2 degree and an 0.5 mm aluminum filter. Subsequently, scan images were reconstructed using NRecon (Bruker, Kontich, Belgium), applying ring artefact reduction and beam hardening corrections. The CT Analyser software (version 1.20.3.0; both Bruker, Kontich, Belgium) was used for 2D and 3D analyses. By excluding the original cortical bone within the callus, the total volume (TV, mm^3), the total bone volume (BV, mm^3) and the bone volume fraction (BV/TV) of the newly formed bone were analyzed in a manually defined VOI (Bucher et al., 2019).

Histology and immunofluorescence

Following *ex-vivo* µCT, bones were placed in an ascending sugar solution as a cryoprotectant (10%, 20%, 30%) at 4°C for 24h each, then cryo-embedded in SCEM medium (Sectionlab, Japan) and stored at -80°C. Consecutive sections of 7 µm were prepared from the cryo-embedded bones using a cryotome (Leica, Wetzlar, Germany) and cryotapes (Cryofilm 2C(9), Sectionlab, Japan). Sections were fixed onto glass slides, air-dried, and stored at -80 °C until staining.

Movat's pentachrome staining comprised the following steps: briefly, sections were air dried for 15 min, fixed with 4% PFA (Electron Microscopy Sciences, Hatfield, USA) for 30 min at room temperature and washed in distilled water (H_2O_{dest}) for 5 min. Then, sections were pretreated for 3 min in 3% acetic acid (Carl Roth, Karlsruhe, Germany), stained 30 min in 1% alcian blue (Sigma Aldrich, St. Louis, USA) pH 2.5, followed by 10 min washing in 3% acetic acid under light microscopic control. Sections were rinsed in H_2O_{dest} and immersed in alkaline ethanol (Carl Roth, Karlsruhe, Germany) for 60 min, washed in tap water for 10 min, rinsed with H_2O_{dest} and incubated in Weigert's hematoxylin (Waldeck, Münster, Germany) for 15 min. Then, the sections were washed in tap water for 10 min, shortly rinsed with H_2O_{dest} and stained in crocein scarlet-acid fuchsin (Waldeck, Münster, Germany) for 15 min. Afterwards, the slices were treated with 0.5% acetic acid for 1 min, followed by 20 min incubation in 5% phosphotungstic acid (Waldeck, Münster, Germany), and again 1 min in 0.5% acetic acid. The sections were washed three times for 2 min in 100% ethanol, followed by alcoholic Saffron du Gâtinais (Waldeck, Münster, Germany) for 60 min. Finally, the slides were dehydrated in 100% ethanol three times for 2 min and cleared shortly in xylene. Sections were covered with Vitro-Clud (R. Langenbrinck, Emmendingen, Germany) and a cover slip. Imaging was performed on a Leica light microscope using AxioVision software (Carl Zeiss Microscopy, Jena, Germany) at 10x magnification. Quantitative analyses of the Movat's pentachrome staining were evaluated using an ImageJ macro. All analyses were performed blinded to sex, fixation, and pain management protocol.

Immunofluorescence staining was performed as described previously (Lang et al., 2019; Lang et al., 2022) using the following antibody: Endomucin (Emcn) (V.7C7 unconjugated, rat monoclonal, sc-65495, 1:100; Santa Cruz Biotechnology, Dallas, USA), goat anti-rat A647 (1:500; A-21247, polyclonal, Invitrogen, Thermo Fisher Scientific, Waltham, USA) and DAPI (1:1,000; Thermo Fisher Scientific, Waltham, USA). Blocking was performed with 10% FCS/PBS and staining solution contained 5% FCS

with 0.1% Tween20 (Sigma Aldrich, St. Louis, USA). Images were acquired using a Keyence BZ9000 microscope (Keyence, Osaka, Japan). The images were processed and analyzed with ImageJ (Rasband, 1997-2018; Schneider et al., 2012). An area of interest was established and managed via the built-in ROI-Manager, while cell number and signal distribution within the area were determined using the plug-ins Cell-counter and Calculator Plus. Data was processed with the ImageJ plugin OriginPro.

Statistical analysis

Sample size calculation was performed based on own preliminary data (Jirkof et al., 2019) using a nonparametric ranking procedure to analyze longitudinal data. In detail, the required number of animals was modeled in R using the package nparLD (Noguchi et al., 2012). The test revealed a 20% difference with a power of ~80% when the number of animals per group is n=10.

Statistical analysis was performed using RStudio and graphs were created in GraphPad Prism (V8). The package “nparLD” was used in RStudio (Noguchi et al., 2012). To test whether the data from female and male mice are homogenous and would allow for an integrated data analysis, we first exemplarily compared the body weight data of both sexes using the F2-LD-F1 design. Since we found significant interaction with regard to sex, we therefore analyzed both sexes separately for all statistical analyses. Thus, nonparametric analysis of longitudinal data (F1-LD-F1 design; further on named as non-parametric ANOVA-type tests), was further used to test for significant differences in the main effect of time and main effect of group, separated by sex. When mean group differences $p \leq 0.05$ were detected, group comparison for each timepoint was performed using the Kruskal-Wallis test (Kruskal and Wallis, 1952). To determine group differences, Dunn’s posthoc test with Bonferroni correction was performed for each timepoint, specifying which means were significant from the others (Dinno, 2015; Dunn, 1961). Non-parametric ANOVA-type tests as well as *p-values*, chi-squared and df for each analysis of the following parameters are provided in the Supplementary Information: body weight, food and water intake, composite pain score, delta composite pain score, limp score, CatWalk (relative velocity, relative mean intensity, relative stand duration, relative stride length), *ex-vivo* µCT (BV/TV, BV, TV), histology (relative bone fraction, relative cartilage fraction) and immunofluorescence (Endomucin, DAPI). Mice or data that were excluded during the study and following analyses are explained in detail in the Supplementary Information.

3 Results

Body weight, food and water intake are affected post-anesthesia and post-osteotomy independent of analgesic regime and fixation

An integrative study design was chosen to i) generate intra-individual controls for the behavioral assessments and ii) reduce animal numbers used in this study (**Fig. 1**). In brief, animals underwent a first intervention consisting of isoflurane anesthesia and administration of the assigned analgesics. Assessments were performed at 12h, 24h, 48h and 72h (referred to in the following as "post-anesthesia"). 14 days after the first intervention, the same animals underwent a second intervention including isoflurane anesthesia, administration of the respective analgesics and osteotomy on the left femur. Same assessments as post-anesthesia were performed at 12h, 24h, 48h and 72h (in the following referred to as "post-osteotomy") (**Fig 1B**).

To assess general indications for wellbeing, the body weight was monitored at 12h, 24h, 48h and 72h post-anesthesia (initial body weight at 0h – males: 25.96 ± 2.1 g; females: 21.41 ± 1.3 g) and post-osteotomy (initial body weight at 0h – males: 27.61 ± 1.9 g; females: 22.75 ± 1.3 g), showing a statistically significant reduction in the range of 5% in all groups at 12h and 24h regardless of sex, analgesic regime, and intervention (**Fig. 2A**). At 48h post-anesthesia, body weight normalized in all groups and even exceeded the pre-intervention weight (**Fig. 2A, B**). Of note, mice did not undergo osteotomy during the first intervention (= post-anesthesia). However, they were already assigned to the respective groups post-osteotomy. After osteotomy, we found that male mice showed prolonged body weight loss over 48h, when compared to post-anesthesia (**Fig. 2A**)**.** In contrast, female mice had less body weight loss at 24h compared to post-anesthesia, but a similar recovery at 48h (**Fig. 2B**). The body weight increase compared to the initial value was higher post-anesthesia than post-osteotomy at 72h in all groups independent of sex, analgesia, and fixation. After both periods of close monitoring, body weight was measured every other day until osteotomy/euthanasia with comparable weight development at any time point (**Fig. S1**).

Food and water intake per cage were assessed at 24h, 48h and 72h post-anesthesia and post-osteotomy. Initial values at 0h covering the previous 24h per cage were as follows: post-anesthesia - males 8.1 ± 0.7 g (food) and 9.2 ± 1.5 ml (water); females 7.8 ± 1.2 g (food) and 9.2 ± 1.2 ml (water); post-osteotomy – males 8.7 ± 1.0 g (food) and 9.9 ± 1.5 ml (water); females 8.4 ± 0.9 g (food) and 9.5 ± 0.9 ml (water). The lowest food and water intake (approximately 50% reduction to initial values) across all groups and sexes was measured 24h after each intervention and reached the level of the initial values at 48h (post-anesthesia) or 72h (post-osteotomy) (**Fig. 2C–F**). No significant main effects were detected between treatment groups (**Table S2, S3**). To rule out any constipating adverse effects of the BUP-Depot, all groups were closely monitored for defecation during the assessments, as constipation is a known-side effect of chronic-opioid usage (Jirkof et al., 2015; Webster et al., 2016). However, a reduction in defecation was only noticeable at 12h after both interventions (**Fig. S2**). The reduction in defecation was considerably more pronounced in female than in male mice but showed no differences between the Tramadol and BUP-Depot groups (**Fig. S2**).

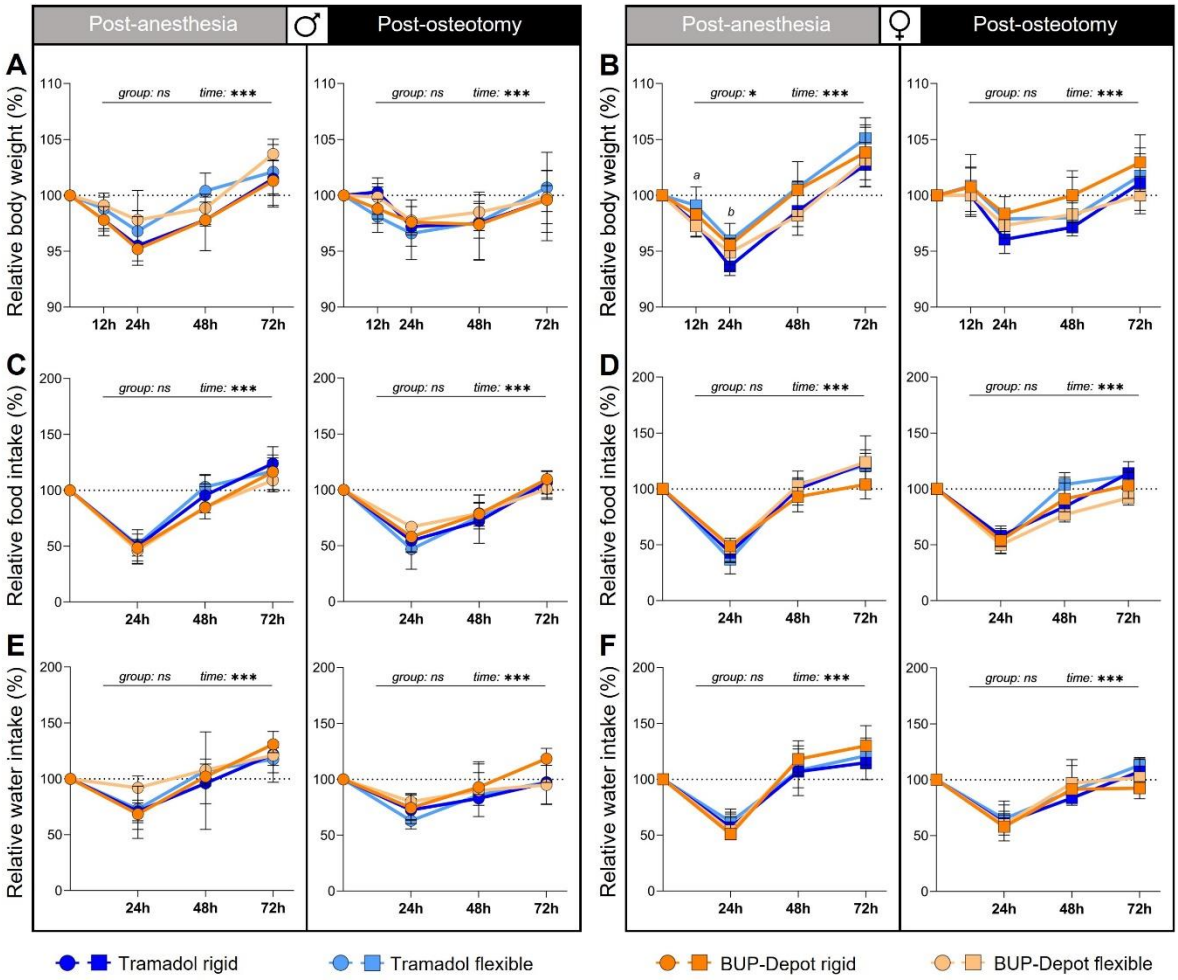

Figure 2. Reduction of body weight as well as food and water intake can be observed at 24h and 48h post-anesthesia and post-osteotomy. (**A, B**) Body weight was measured at 12h, 24h, 48h and 72h; (**C-F**) and food/water intake was measured at 12h, 24h, 48h and 72h post-anesthesia and post-osteotomy and normalized to the initial value (0h = 100%). Of note, mice did not undergo osteotomy during the first intervention (= post-anesthesia). However, they were already assigned to the respective groups post-osteotomy. All graphs show median with interquartile range for n= 9–10 (body weight) and n=4–5 (food/water intake). Non-parametric ANOVA-type test - main effects of time and of group are represented in the graphs; exact *p-values* are listed in Table S1–S3; $*p < 0.05$, $***p < 0.001$. To determine group differences Kruskal-Wallis test and Dunn's posthoc test with Bonferroni correction was performed. *a* – significant difference Tramadol flexible vs. BUP-Depot flexible; *b* – significant difference Tramadol rigid vs. Tramadol flexible.

Nest building and explorative behaviors are not influenced by the analgesic regime

To detect model independent changes in spontaneous behavior, monitoring of nest complexity scores and explorative behaviors was performed at 24h, 48h and 72h post-anesthesia and post-osteotomy. Analyses showed no differences in the nest building performance between the Tramadol and BUP-Depot groups post-anesthesia and post-osteotomy as medians ranged between scores of 4.5 to 5 (**Fig. 3A**). Explorative behavior was present in all cages with male mice post-anesthesia while reduced in some cages at 24h post-osteotomy (8/20) independent of analgesic regime or fixation. In the female mice, 3 out of 20 cages (Tramadol flexible and BUP-Depot flexible) showed no exploration 24h after both interventions (**Fig. 3B**).

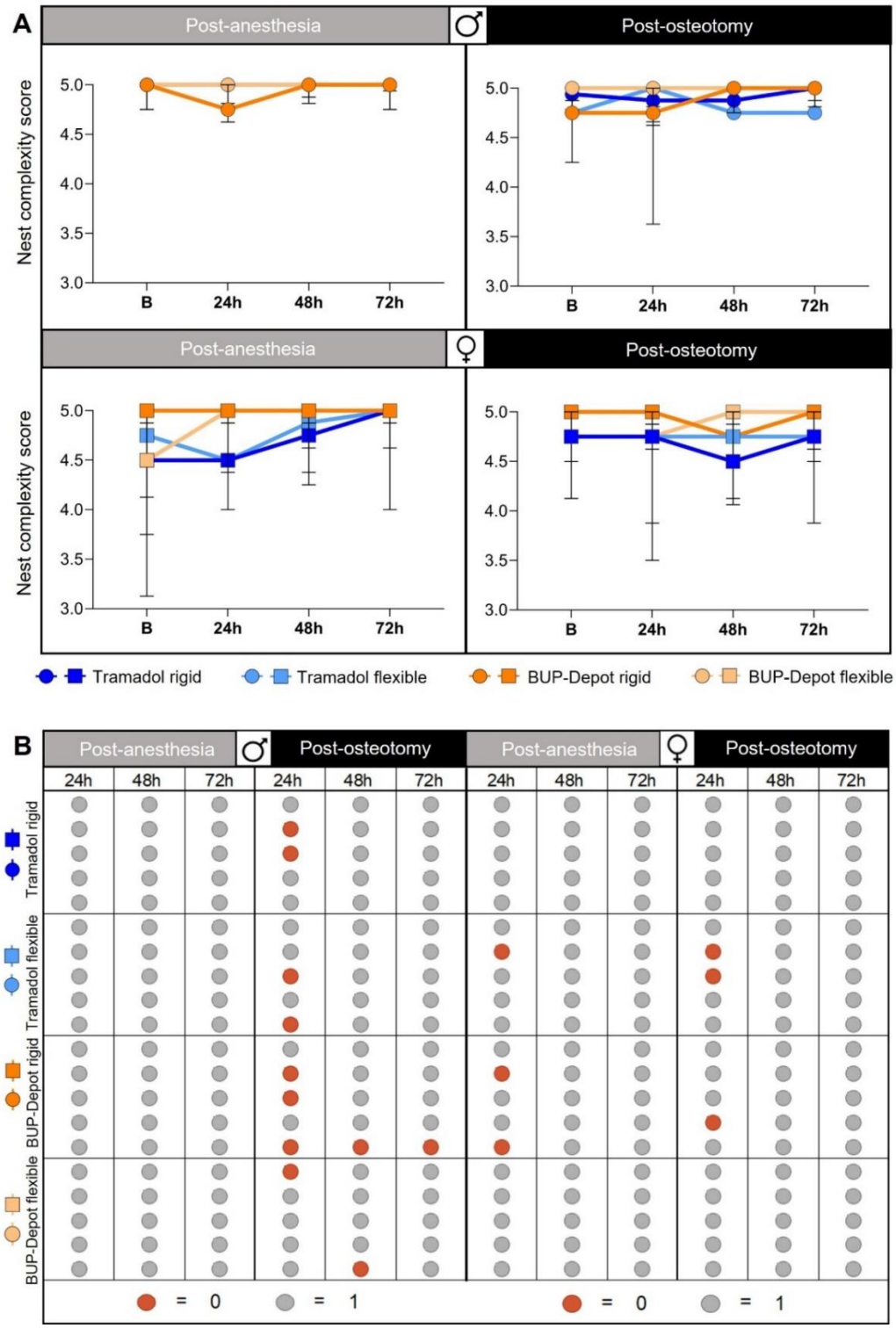

Figure 3. Nest building behavior remains unaffected post-anesthesia and post-osteotomy while exploration was negatively influenced post-osteotomy in male mice at 24h. (**A**) Nest building and (**B**) explorative behavior were monitored per cage at 24h, 48h and 72h post-anesthesia and post-osteotomy. (**A**) Shows median with interquartile range for n=4–5 (nest complexity score) based on cages (pair housing), (**B**) shows n = 5 (explorative behavior) based on cages (pair housing).

Delta composite pain score indicates limited analgesic capacity of Tramadol in male mice with flexible fixation

The composite pain score includes parameters of facial expression and overall appearance was assessed at 24h, 48h and 72h post-anesthesia and post-osteotomy. Since we observed a pronounced influence of the anesthesia and analgesic regime alone (**Fig. S3**; **Table S4–S4.3**), we corrected the

individual score post-osteotomy for the respective score post-anesthesia to obtain a delta composite pain score, that depicts the isolated effect of the osteotomy without the interfering effects of anesthesia and analgesia. The delta composite pain score was highest in all groups at 12h and 24h after osteotomy, and declined after 48h and 72h, reaching lowest scores in all groups after 72h (significant main time effect in both sexes, *p<0.001;* **Fig. 4A**; **Table S5**). We found a significant higher delta composite pain score in male mice from the flexible fixation group treated with Tramadol at 24h (median score = 2) and 48h (median score = 1.5) after osteotomy (Kruskal-Wallis test of all groups at 24h: *p= 0.031* and at 48h: *p= <0.001*; **Table S5.1**) when compared to the other groups (Dunn's posthoc test for Tramadol flexible vs. BUP-Depot flexible, BUP-Depot rigid and Tramadol rigid, respectively: 24h *p= 0.077, 0.042, 0.032*; 48h *p= 0.004, <0.001, 0.018* ; **Fig 4A**; **Table S5.2–S5.3**). Female mice showed comparable score developments between treatment and fixation groups with the highest median scores (1-1.5) at 12h.

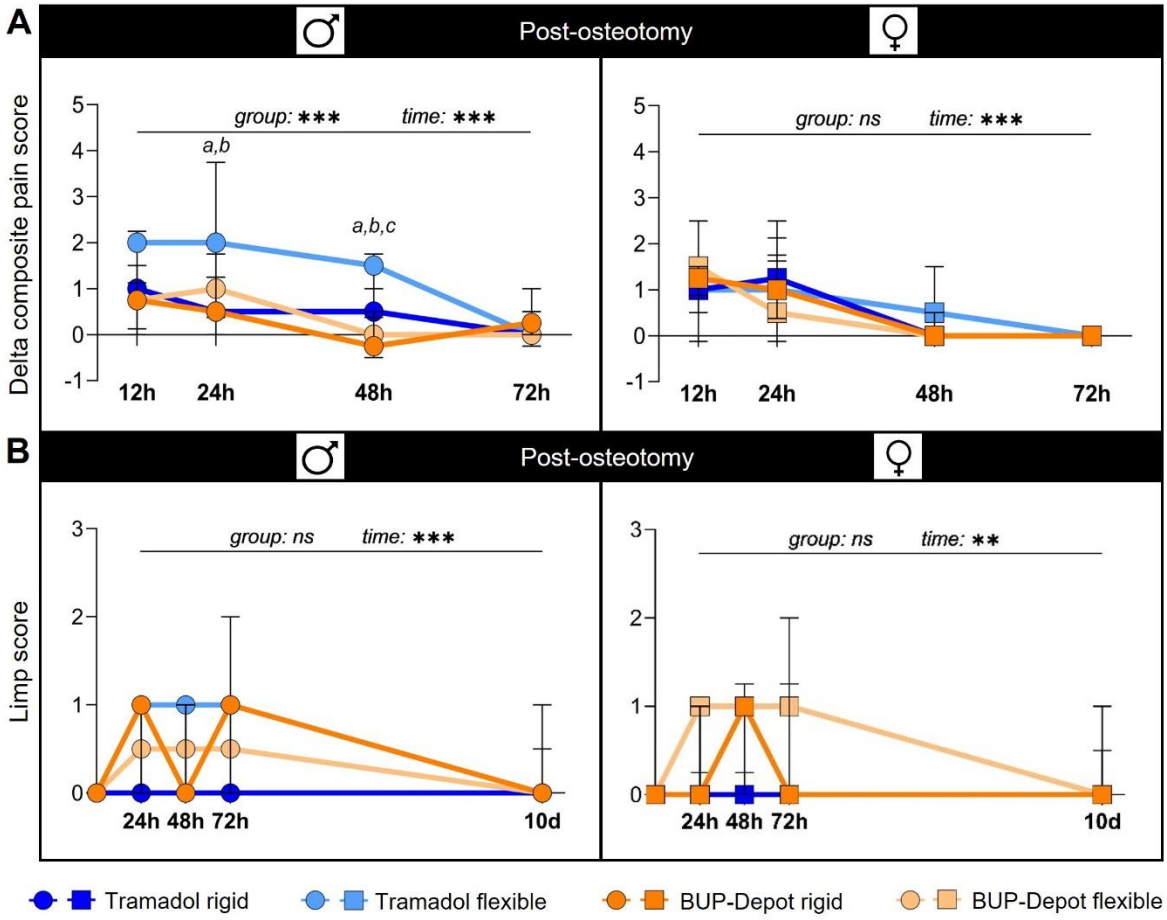

Figure 4. Delta composite pain score suggests adequate pain alleviation in almost all groups while limping behavior shows slight differences between groups post-osteotomy. (**A**) The delta composite pain score was evaluated at 12h, 24h, 48h and 72h post-anesthesia and post-osteotomy; to achieve the delta composite score, scores from each individual mouse post-anesthesia were subtracted from their respective scores post-osteotomy. (**B**) Limp score was assessed at 24h, 48h, 72h and 10d post-osteotomy. All graphs show median with interquartile range for n = 8–10 (delta composite pain score) and n= 9–10 (limp score). Non-parametric ANOVA-type test - main effects of time and of group are represented in the graphs; exact *p-values* are listed in Table S5-S5.3 and Table S6; **p < 0.05, ***p < 0.001*. To determine group differences Kruskal-Wallis test and Dunn's posthoc test with Bonferroni correction were performed. *a* – significant difference Tramadol rigid vs. Tramadol flexible; *b* – significant difference Tramadol flexible vs. BUP-Depot rigid; c – significant difference Tramadol flexible vs. BUP-Depot flexible.

Limping and gait analysis indicate model-related alterations in walking behavior with only slight differences between groups

Walking behavior was assessed by i) using a metric limping score applied to individual 3 min videos and ii) analyses of gait and locomotion using the Noldus CatWalk XT at 24h, 48h, 72h, and 10d post-osteotomy. The limping score was only considered post-osteotomy as mice did not show any alterations in walking post-anesthesia during routine monitoring. Individual baseline measurements for the CatWalk were taken before both interventions to correct the data post-osteotomy for variances in velocity.

As expected, limping was observed at all timepoints post-osteotomy until 10d with a general improving trend (significant main time effect in males $p<0.001$ and females $p= 0.018$; **Fig. 4B**; **Table S6**). In general, median scores ranged between 0 – 1 at 24h and 48h independent of sex and analgesics, while higher variations were seen at 72h (male BUP-Depot rigid; female BUP-Depot flexible; **Fig. 4B**). Slight alterations in limping behavior were still visible after 10d in some individual mice in the female BUP-Depot groups or male Tramadol groups (all = score 1; sporadic limping; up to two animals, respectively). Further analyses of gait and locomotion (CatWalk XT) exhibited a clear reduction in velocity after osteotomy in all groups (significant main time effect post-osteotomy for both sexes $p<0.001$; **Fig. 5A, B**; **Table S7**) with an upward trend surpassing baseline values in nearly all groups at 10d (exception: male, Tramadol, flexible fixation). Significant differences between the male groups were found at 24h and 48h with male mice (flexible fixation group treated with Tramadol) showing a lower relative velocity (non-parametric ANOVA-type test for group $p= 0.034$; Kruskal-Wallis test 24h $p= 0.052$ and 48h $p= 0.038$; Dunn's posthoc test – $p<0.05$ when compared to BUP-Depot rigid; **Fig. 5A**; **Table S7.1–S7.3**). Moreover, female mice showed significant group differences at 24h (non-parametric ANOVA-type test for group $p= 0.035$; Kruskal-Wallis test 24h $p= 0.009$; **Fig. 5B**; **Table S7.4–S7.5**) as mice with rigid fixation treated with Tramadol exhibited elevated velocity compared to the other groups which was comparable to their baseline values (median 1.015). With respect to the relative mean intensity (a measure for load bearing) and relative stand duration, we did not observe significant group effects independent of sex and analgesic (**Fig. 5C–F**), while time-dependent reductions at 24h, 48h and 72h improved over time (relative mean intensity: non-parametric ANOVA-type test $p< 0.001$ in both sexes; relative stand duration $p< 0.001$ in males and $p= 0.005$ in females; **Table S8–S9**). As observed before, the relative mean intensity in male mice (flexible fixation group treated with Tramadol) was also numerically reduced (**Fig. 5C**) with no differences in the relative stand duration (**Fig. 5E**). Interestingly, the relative stand duration was only slightly increased in all female mice over 10 days (median range 10d: 0.68 – 0.75; **Fig. 5F**). In addition, the stride length was only markedly modulated over the first 72h and normalized to baseline values at 10d (**Fig. 5G, H**; **Table S10**). Only male mice with flexible fixation and Tramadol treatment showed shortened stride length which was specifically significant at 24h compared to BUP-Depot rigid, but not to Tramadol rigid (non-parametric ANOVA-type test $p= 0.024$; Kruskal-Wallis test 24h $p= 0.044$; Dunn's posthoc test – $p<0.05$ when compared to BUP-Depot rigid; **Fig. 5G**; **Table S10–S10.2**). To evaluate the influence of the velocity on mean intensity, stand duration and stride length, we performed additional correlation analyses (spearman correlation) (**Fig. S4A–C**) which indicated that mean intensity (males r= 0.6, females r= 0.34; **Fig. S4A**) and stride length (males r= 0.8, females r= 0.52; **Fig. S4C**) correlate with the velocity (all $p< 0.001$) after osteotomy. Therefore, observations of those parameters need to be discussed in context of the overall velocity.

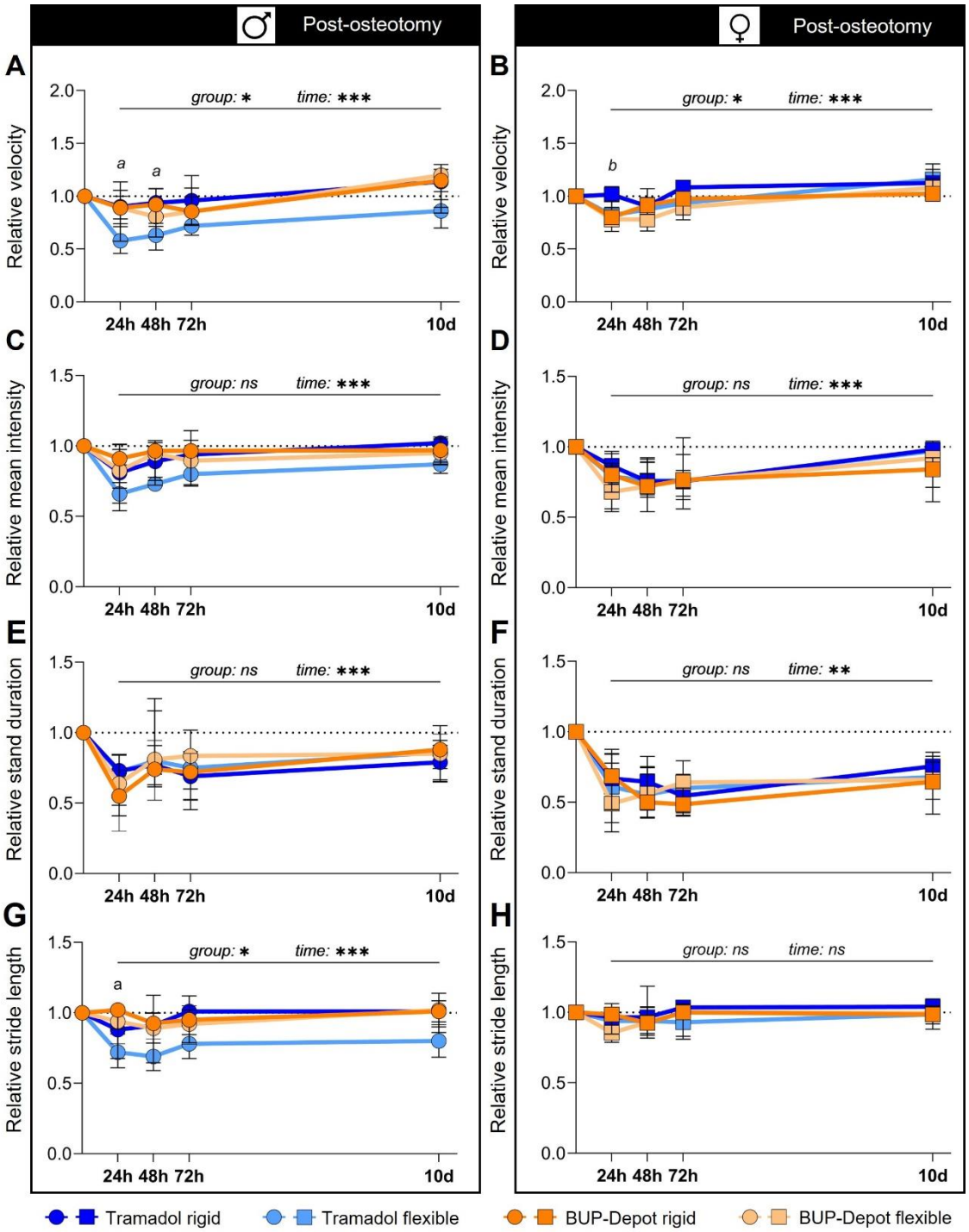

Figure 5. Results of limping and gait analyses yield model-related alterations in walking behavior which was specifically pronounced in male mice with flexible fixation and Tramadol treatment. CatWalk analysis was conducted at 24h, 48h, 72h and 10d post-osteotomy and normalized to the initial mean baseline value specifically focusing on (**A**) relative velocity, (**B**) relative mean intensity, (**C**) relative stand duration and (**D**) relative stride length. All graphs show median with interquartile range for n= 8–10. Non-parametric ANOVA-type test - main effects of time and of group are represented in the graphs; exact *p-values* are listed in Table S7–S10.2; *$p < 0.05$, ***$p < 0.001$. To determine group differences Kruskal-Wallis test and Dunn's posthoc test with Bonferroni correction were performed. *a* – significant difference Tramadol flexible vs. BUP-Depot rigid; *b* – significant difference Tramadol rigid vs. BUP-Depot flexible.

Fracture healing outcome is affected by fixation stability but not analgesic regime

To evaluate potential effects of the BUP-Depot on fracture healing outcomes, we performed *ex-vivo* µCT, histomorphometric analysis and vessel staining at day 14 post-osteotomy (**Fig. 6**). We observed a numerically lower BV/TV when comparing flexible fixation compared to rigid - except for males treated with Tramadol, however the differences did not reach statistical significance (BV/TV; **Fig. 6A, B**; **Fig. S5**; **Table S11**). The differences were slightly more pronounced in the BUP-Depot groups (male: median rigid = 25.87% vs. median flexible = 17.80%; female: median rigid = 26.12% vs. median flexible = 20.58%) than the Tramadol group (male: median rigid = 21.89% vs. median flexible = 21.52%; female: median rigid = 30.32% vs. median flexible = 23.31%).

Histomorphometric analysis revealed comparable differences between the rigid and flexible fixation in relative bone and cartilage fraction; as expected, bone was reduced while cartilage formation was elevated in the flexible groups compared to rigid fixation in both sexes and treatment groups (**Fig 6C–H**; **Table S12**). Analysis of the vessel formation (Endomucin/Emcn staining) within the callus area did not show significant or obvious differences in the male groups (**Fig. 6I**; **Fig. S5**; **Table S13**). Differences in vessel formation (Emcn) were conditionally statistically significant between fixation in female mice (Kruskal-Wallis test $p= 0.045$; Dunn's posthoc test $p> 0.05$ between all groups; **Fig. 6J**; **Table S13.1**) with flexible fixation groups showing less relative $Emcn^+$ areas when compared to the rigid fixation. Additionally, DAPI staining indicated lower cellularity in the female mice with flexible fixation independent of analgesic treatment (**Fig. S5**).

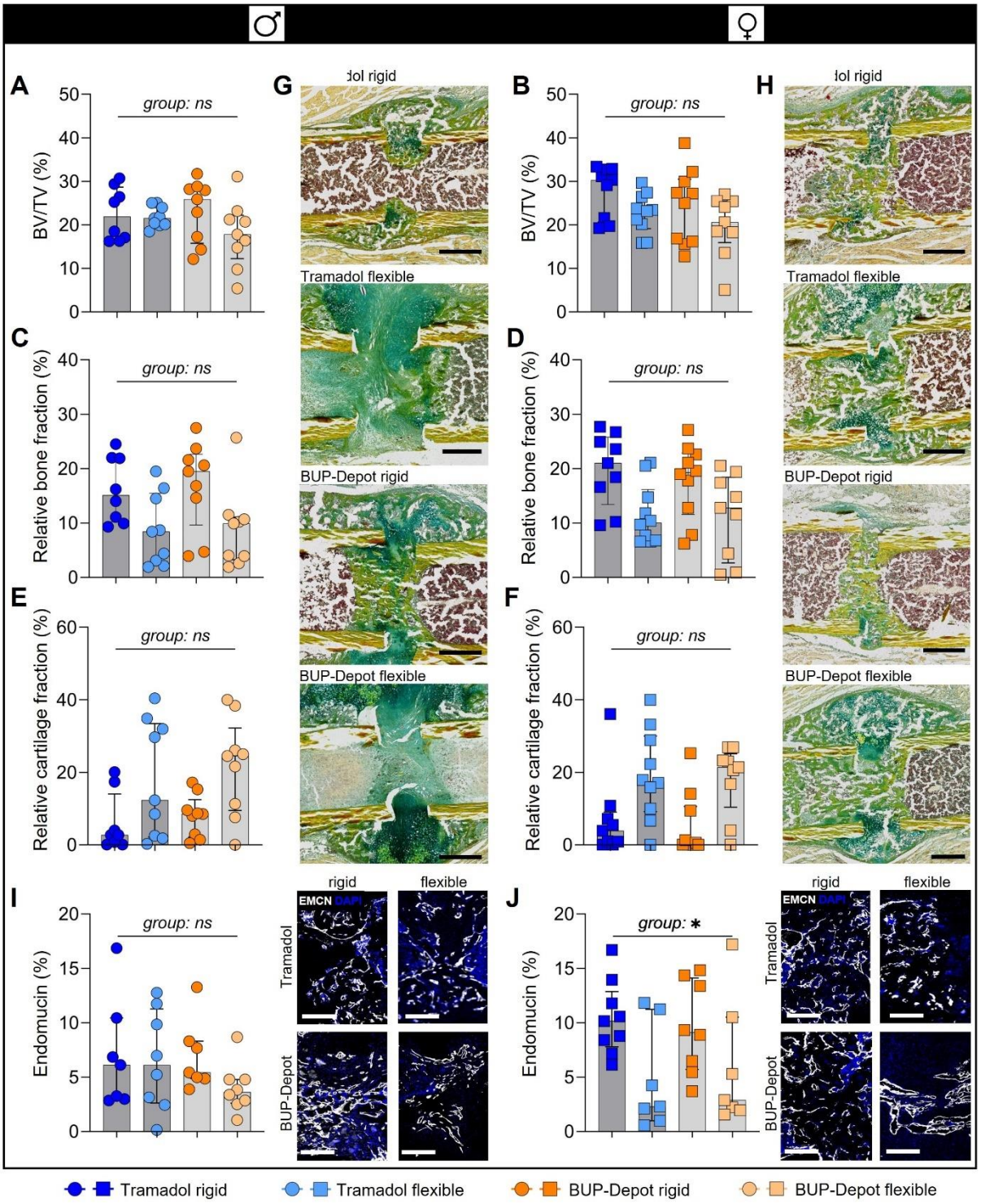

Figure 6. Analgesic regimes did not negatively affect fracture healing outcome at day 14, while the different fixations lead to differences in new bone formation. (**A, B**) Relative bone volume (BV/TV) (%), (**C, D**) relative bone fraction (%) and (**E, F**) relative cartilage fraction (%). (**G, H**) Exemplary images of the Movat's pentachrome staining: yellow = mineralized bone, green = cartilage, magenta = bone marrow; scale bar 500 µm. (**I, J**) Immunofluorescence staining of vessel formation (Endomucin) including quantification and exemplary images; scale bar 200 µm. All graphs show median with interquartile range for n = 8–10 (**A**–**H**) and n = 6–9 (**I**–**J**). To determine group differences, Kruskal-Wallis test and Dunn's posthoc test with Bonferroni correction were performed in RStudio; exact *p-values* are listed in Table S11–S13.1; *$p < 0.05$.

4 Discussion

We evaluated the analgesic efficacy and possible side-effects of a newly developed sustained-release Buprenorphine (BUP-Depot) in comparison to an already established protocol (Tramadol in the drinking water) after a pre-operative injection of Buprenorphine in two mouse-osteotomy models with different fixation stiffnesses (Schreiner et al., 2020; Schreiner et al., 2021; Jirkof et al., 2019; Rapp et al., 2015). The BUP-Depot delivered reliable pain relief over 72h post-surgical without side effects on fracture healing outcome, comparable to Tramadol applied with the drinking water. Unexpectedly, we found a possibly limited analgesic capacity of Tramadol in male mice with a more flexible fixation of the osteotomy based on an elevated composite pain score and more altered gait. Due to individual differences in behavioral changes and pain perception (Hohlbaum et al., 2018; Jirkof et al., 2019), we chose a consecutive study design to analyze the effect of anesthesia and analgesia alone and in combination with an osteotomy in the same animal.

General clinical parameters such as body weight, and food and water intake were noticeably reduced post-anesthesia and post-osteotomy. Reduction of food intake and therefore negatively influenced body weight development are known side-effects post-surgical but can also be related to anesthesia or Buprenorphine/Tramadol administration (Brennan et al., 2009; Clark et al., 2004; Goecke et al., 2005; Jirkof et al., 2015). Reduction of body size observed in the present study was very low (in the range of 5% and similar to the anesthesia only control), which is indicative of an excellent pain management and wellbeing in all animals. In none of the animals' values (i.e.20% weight loss) were reached, which would have necessitated immediate termination of the experiment. These findings are as well indicative of a high tolerability of the used drugs. Lowest values in body weight and food intake were reached after 24h in all groups, with an incline over 48h and 72h post-anesthesia and post-osteotomy. Mice were 10 weeks of age during the first intervention (anesthesia and analgesia alone) and 12 weeks at osteotomy. It is known that mice show a rapid body weight development until skeletal maturity between approx. 10-12 weeks which also includes the formation of more body fat (Ferguson et al., 2003; Yang et al., 2014). Since interventions entailing anesthesia results in short-term starvation during the recovery period, it can be speculated that mice at 10 weeks loose body weight more rapidly due to limited body fat reserves when compared to more mature mice (12 weeks) with most probably more body fat. The BUP-Depot showed similar effects on the body weight development as well as food and water intake as the established Tramadol treatment. We did not find differences in water intake between groups, excluding a negative effect of the Tramadol containing water on the overall drinking amount and ensuring a continuous uptake of medication as shown previously (Jirkof et al., 2019; Evangelista Vaz et al., 2018). However, the measurement of the overall 24h water intake does not allow to verify sufficient water intake during the first hours after surgery which might still be reduced as also reported by Evangelista et al. (Evangelista-Vaz et al., 2018). To reduce the chance of insufficient water intake due to altered water taste after surgery and to provide a pre-emptive level of analgesic, we administered Tramadol already one day before surgery (Ehrnthaller et al., 2013; Rapp et al., 2015). To improve the food intake and therefore body weight development, the food can be alternatively provided on the cage floor to prevent the animals from having to stand on their hind legs (Lang et al., 2016). However, this does not allow for precise monitoring of food intake as we did here, as food pellets might be hidden in the nest or be buried.

The use of high-caloric dietary gels could be a valid alternative ensuring the consumption of food and liquids and can be also used as route for oral analgesic administration (Nizar et al., 2018).

Changes in nest building and the willingness to explore foreign objects can indicate alterations in well-being in laboratory mice (Gaskill et al., 2013; Jirkof et al., 2013; Jirkof, 2014; Jirkof et al., 2019; Rock et al., 2014). In our study, nest building behavior was only scarcely influenced by anesthesia or osteotomy in the BUP-Depot and Tramadol groups independent of sex and fixation stiffness. This is in line with other studies suggesting that nest complexity scoring might not be perceptive enough to differentiate between minor pain and other potential stressors or reduced wellbeing after surgery (Jirkof et al., 2013). However, these findings are in contrary to our previous study where we have reported a reduction in the nest building performance after osteotomy (Jirkof et al., 2019). Technical differences (more or less nesting material) or individual differences in scoring might explain the variations in our findings and underline the necessity for more objective approaches. Moreover, the explorative behavior in male mice seemed to be negatively impacted by osteotomy, especially at 24h post-surgery, when compared to the post-anesthesia controls and female mice. This is in accordance with observations reported by Gallo et al. where they found that male CD1 mice more often failed to pass a Time to Incorporate into Nest Test (TINT) 3h after surgery (carotid artery catheterization) as compared to their baseline measurements after anesthesia alone (Gallo et al., 2020). In addition, Hohlbaum et al. showed that female C57BL/6JRj mice exhibited shorter latency to explore than male mice 1 day after last anesthesia in a repeated inhalation anesthesia trial indicating sex-specific difference that is in accordance with our findings with respect to explorative behavior (Hohlbaum et al., 2017).

We used a composite pain score to combine the assessment of facial expression, i.e., the mouse grimace scale, and clinical appearance post-anesthesia and post-osteotomy. Based on our consecutive study design, we were able to calculate a delta for each individual mouse representing numerically the “real” osteotomy effect. In line with other studies, the composite pain score was already slightly impacted by anesthesia and analgesia alone, indicating that some components of the composite pain score might not only be influenced by pain but rather also depict stress or discomfort (Jirkof et al., 2019; Miller et al., 2015). The highest delta composite scores were reached 12h and 24h after osteotomy suggesting the pain/discomfort peak due to the surgical procedure which constantly declined till 72h post-surgical. Median delta scores varied around 1 indicating only limited residual pain and/or discomfort over the first 48h. The BUP-Depot provided comparable and sufficient alleviation of pain signs as the Tramadol treatment and our data indicate that pain relief can even be achieved over 72h. This is an important advantage as compared to the commercially available sustained-release formulation Buprenorphine SR-LAB (ZooPharm – USA only), which yields effective serum concentrations over a maximum of 24h to 48h only with a need for re-administration (Clark et al., 2014; Kendall et al., 2014).

As model-specific parameters, we assessed limping behavior and locomotion using gait analysis. After osteotomy, limping behavior was observed over 72h post-osteotomy in all groups, irrespective of sex, fixation, and analgesic, with no significant differences between the groups. More detailed gait analysis using the CatWalk system revealed reduced velocity and altered gait patterns over 72h and up to 10

days. While velocity and mean intensity ameliorated over 10 days, stand duration and stride length remained reduced in all groups. Due to the functional impairment, limping as well as changes in gait parameters after osteotomy are expected as a mixture of residual pain due to local inflammatory processes and periosteal pain as well as the restricted functionality of the femur. Magnusdottir et al. reported mechanical hyperalgesia and cold sensitivity in male mice after sham surgery (no external fixator and osteotomy) and femur fracture over 6 weeks while the sham group significantly improved after 3 weeks (Magnusdottir et al., 2021). This indicates that the wound healing itself already plays an important role in potential pain sensitization after osteotomy. A reduction in the stride length over 14 weeks was also observed by Histing et al. (Histing et al., 2010). In addition, limited mobility and changed gait are also common in human patients with e.g., proximal femur fractures or the femoral diaphysis and are not directly related to pain (Bizzoca et al., 2020; Wong et al., 2004).

Of note, the sensitive and in-depth analyses of gait pattern and velocity used in the present study (as opposed to simple limp scores) is important since most gait parameters correlate to velocity (Batka et al., 2014; Jacobs et al., 2014). Failing to address possible impacts of changes in velocity can affect the outcome of gait related data (Batka et al., 2014; Cendelín et al., 2010).

Unexpectedly, at 24h and 48h, the delta composite pain scores of male mice receiving Tramadol after osteotomy with a more flexible fixator showed statistically significant higher scores than all other groups. Together with more pronouncedly altered changes in relative velocity, our data suggest a clinically relevant degree of discomfort/pain which has not been described previously. One explanation could be that the effective Tramadol dose was not sufficiently achieved due to the higher body weight of male mice. Moreover, Evangelista et al. showed that male mice had lower serum concentrations of Tramadol than female mice when applying Tramadol (0.2 mg/ml) in the drinking water for up to 30h, although the analgesic effective M1 metabolite was similar between male and female mice (Evangelista Vaz et al., 2018) which is in line with a previous study performed in rats (Matthiesen et al., 1998). In contrast, other studies report lower sensitivity of female mice or rats to Tramadol (Dai et al., 2008; Liu et al., 2003). With respect to the influence of the body weight on the local degree of interfragmentary movement, Röntgen et al. characterized two configurations of the external fixator, the rigid one (18.1 N/mm) and a more flexible one (0.82 N/mm) calculating an interfragmentary strain for a 25 g mouse with 2.8% and 61%, respectively (Röntgen et al., 2010). However, when applied to female C57BL/6 mice, they did not find differences in body weight, ground reaction force and motion over 18 days when treated 3d post-surgical with 0.025 mg/ml Tramadol in the drinking water (Röntgen et al., 2010). This additionally highlights potential sex-specific differences in pain perception but also the response to analgesic medication (Fillingim and Gear, 2004). Sex-specific adaption of pain management regimes might be advisable in future studies.

In terms of fracture healing outcome analyzed by *ex-vivo* µCT and histomorphometry, we found no differences between the analgesic regimens, indicating safe use of the newly developed BUP-Depot. A more flexible fixation allows higher interfragmentary movements and, therefore, promotes cartilage rather than bone formation, as seen in the histomorphometric analysis, as well as formation of a larger periosteal callus (Röntgen et al., 2010). Staining for endomucin, representing vessels, revealed no

difference between fixations or analgesic in male mice However, female mice with more flexible stabilized osteotomies showed a reduced Emcn-positive area as well as lower cellularity, regardless of their analgesic regime. Besides mechanical hinderance of revascularization due to higher strains, the higher proportion of cartilage observed in female mice with flexible stabilized osteotomies might prevent revascularization due to the intrinsic anti-angiogenic nature of cartilage e.g., due to chondromodulin-1 (Patra and Sandell, 2012; Shukunami and Hiraki, 2001). This reduced vascularization is in accordance with earlier observations in sheep (Lienau et al., 2005). Revascularization occurs in a two-waved fashion during fracture healing (Bahney et al., 2015; Schmidt-Bleek et al., 2015). In the first wave, hypoxia, and invading immune cells secreting pro-angiogenic factors such as vascular endothelial growth factor and platelet-derived growth factor, induce angiogenesis, predominantly in the marrow space. The second wave of revascularization goes along with the conversion of the avascular hypertrophic cartilage to bone. The different patterns of Emcn staining in males and females might also hint on a more advanced callus remodeling and therefore a more rapid healing progression in males than females (Haffner-Luntzer et al., 2021).

An effective sustained-release Buprenorphine in Europe would not only be a conceivable alternative to the application of Tramadol via the drinking water as investigated in this study, but also a potential alternative to repeated injections of Buprenorphine, which are still more frequently used for pain management in femur fracture models compared to Tramadol in the drinking water (Wolter et al., 2021). Assessment of the analgesic effect of Buprenorphine is most often based measurement of plasma or blood serum levels. Studies specified therapeutic effective concentrations of Buprenorphine in plasma at a threshold of around 1 ng/ml or 1 ng/g in mice and rats (Yassen et al., 2005; Yun et al., 2010; Guarnieri et al., 2012). However, Buprenorphine works through the μ-, κ- and δ-opioid receptors in the brain (Villiger and Taylor, 1981; Lutfy and Cowan, 2004) and it can be hypothesized that reliable pain alleviation is more reliant on specific binding concentration values in the brain than specific plasma concentrations (Schreiner et al., 2020). Ohtani et al. also demonstrated a correlation between analgesic effects and specific binding concentrations of Buprenorphine in the brain of rats (Ohtani et al., 1995). Schreiner et al. contemplated that specific binding concentrations of 5 ng/g in the brain might be needed for reliable pain relieve in mice, as observed 24h after injection of the BUP-Depot. Binding concentrations of less than 3 ng/g at 48h injection (BUP-Depot) – which is comparable to levels observed 12h after Temgesic injection – still resulted in high, but not significantly increased withdrawal latencies compared to a single injection with Temgesic or NaCl. Schreiner et al. therefore suggests that alleviation of strong pain through the BUP-Depot might require the administration every 24h (Schreiner et al., 2020). However, based on our assessment of the clinical, behavioral, and model-specific parameters, we can postulate that the analgesic properties of the BUP-Depot are sufficient for 72h post-operative analgesia in our specific mouse-osteotomy model of moderate severity. Nonetheless, the potential use of the BUP-Depot in other, more painful models still needs to be critically assessed and evaluated.

Generally, the use of a sustained-release Buprenorphine results in less handling induced stress and prevents potential gaps in analgesic coverage due to infrequent application intervals when injected repeatedly. Drug administration via the drinking water always bears the risk of insufficient water intake

after anesthesia/surgery and due to circadian rhythm (Sauer et al., 2016). Therefore, adequate pain management requires a constant reevaluation of the chosen protocol as well as the consideration of strain, sex, procedure, interindividual differences in animals, and more, and the use of the most suitable analgesic protocol must be separately evaluated and regularly reevaluated for each animal experiment. As not only sex, but also genetic heritability influences experienced pain and response to analgesia in mice (Smith, 2019), further studies using different strains are needed in the evaluation of commonly used analgesic regimens in laboratory rodents. However, it needs to be considered that animals were kept in IVC cages, leading to a naïve immune system that could not maturate accordingly, potentially leading to different bone healing properties under different housing conditions that could not be assessed within this study (Bucher et al., 2019).

Taken together, our assessment of clinical, behavioral, and model-specific parameters suggest that the analgesic properties of the BUP-Depot were sufficient for 72h post-operative analgesia in male and female C57BL/6J mice after femoral osteotomy. Therefore, the BUP-depot provides an excellent alternative for extended pain relief in preclinical studies. There is no question that the availability of such a sustained-release formulation of Buprenorphine in Europe would be beneficial for pain management in preclinical studies.

Conflict of interest statement

The authors declare that they have no conflict of interests.

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Data availability statement

The authors declare that all data supporting the findings of this study are available within the paper and its Supplementary Information file. Further information is made available by the authors upon request.

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