RESEARCH ARTICLE

Postoperative pain behaviours in rabbits following orthopaedic surgery and effect of observer presence

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Abstract

Rabbits are widely used in studies focusing on pain. However, pain is undertreated in this species and one possible factor to explain this is the lack of evaluation methods. The objective of this study was to identify behaviours related to orthopaedic pain in rabbits and to evaluate the influence of the presence of an observer on these behaviours. Twenty-eight rabbits undergoing orthopaedic surgery and filmed 24 hours before surgery, and 1 hour (before rescue analgesia), 4 hours (3 hours after rescue analgesia), and 24 hours post-recovery were observed in the presence and absence of an observer. The frequency and/or duration of behaviours were compared over time and between the presence and absence of an observer using the Friedman and Wilcoxon tests respectively. Data are expressed as median and interquartile range and a significant difference was considered when p < 0.05. At 1 hour post-recovery, the rabbits showed reduced activity, hopping, change posture, position in the cage, explore, and open eyes in both the presence and absence of the observer. In the absence of the observer, quadrupedal posture, interact with pinecone, and eat carrot also decreased, while wince behaviour increased. In the presence of the observer, before surgery, the rabbits were less active (Presence-280; 162–300, Absence-300; 300–300) and presented a lower duration of explore (Presence-3; 0–32, Absence-40; 4–63). Post-recovery the rabbits flinched less (Presence-0; 0–0, Absence-0; 0–1) and suspended the affected limb less (Presence-0; 0–0, Absence-0; 0–65). After rescue analgesia the rabbits put weight on and raised the affected limb less (Presence-0; 0–0, Absence-0; 0–2) and licked the affected area less (Presence-0; 0–0, Absence-0; 0–2). These findings demonstrate that the presence of the observer inhibited pain-free behaviours in the rabbits, leading to a false impression of pain, and after the surgery the rabbits masked some pain signs related to the affected area.
**Introduction**

Pain presents sensory, emotional, cognitive, and social components [1]. Rabbits (*Oryctolagus cuniculus*) are widely used worldwide as experimental models. In the UK alone, more than 11,000 experimental procedures involving rabbits were performed in the year 2018 [2].

Although rabbits and other laboratory animals are fundamental for translational medicine, paradoxically, pain in these species is still neglected [3]. Postoperative administration of analgesics to rodents and lagomorphs following experimental procedures reaches only 10.4% of the studies [4], and one possible reason for this is the difficulty in identifying and quantifying pain [5, 6], due to the lack of a simple and accurate method to evaluate acute pain in laboratory animals [7].

Methods routinely used to evaluate pain in rabbits include those based on evoked responses to a mechanical, thermal, or electrical nociceptive stimulus [8], however, these are not normally applicable to clinically evaluate postoperative pain, since they do not reflect spontaneous pain and are hard to implement. Although other methods, such as a reduction in body weight, and feed and water intake can also reflect pain states [7], these are retrospective indicators demonstrating that the animal has felt pain and, as they do not represent the animal’s current state, and so do not serve as a guide to the necessity to administer analgesics [9].

Thus, the most applicable methods for real-time clinical pain measurement are based on evaluation of behaviour. The Rabbit Grimace Scale (RbtGS) [10] is a facial expression based scale developed and evaluated in rabbits submitted to ear tattooing. A recent scale was developed to assess pain in pet rabbits by merging this facial scale with physiological and behavioural parameters [11].

An ethogram offers an appropriate starting point to identify pain behaviours [9, 10, 12–14], as it is a record of terms and descriptions of behaviour of an individual during a given period and situation [14]. In order to develop an ethogram to identify pain behaviours, one must compare behaviours present in situations that incur pain with behaviours present in a pain-free state, usually before surgery [9, 13]. Ethograms have only been developed in rabbits suffering soft tissue surgery [9, 12, 13, 15], and to our knowledge, only activity levels, not behaviour, have been evaluated in rabbits undergoing orthopaedic surgery [8]. Orthopaedic pain is expected to induce more intense pain [16, 17] and to be associated with specific behaviours (e.g. lameness) [18] in addition to those already observed [9, 12, 13, 15]. The impact of orthopaedic surgery is of particular concern and requires investigation as rabbits are frequently subjected to this procedure both clinically as pets [17] and in research [16].

Changes in general behaviour that have been associated with pain in rabbits and can include: reductions in activity [8, 9], moving around [9, 13, 15], hopping [9, 13], exploration [9, 13], and interaction with objects and other animals [9] as well as increased lying posture [9, 12]. Changes in behaviours considered to be more specific to abdominal pain have also been observed, such as, skin twitches, writhing and pressing the abdomen against the floor [9].

Although behaviour is a well-established method for evaluating acute pain in animals and widely used in many species [6, 19–25], the interaction or presence of a human observer may interfere with evaluations [26], and rabbits, like ruminants and rodents, tend to mask their pain behaviours [3, 5, 6, 19, 27]. To date, pain behaviour has often been described in rabbits [9, 12, 13] and guinea pigs [19] in the absence of a human observer, however, no studies have compared the effect of observer presence and absence on the exhibition pain behaviour in rabbits. In rats, scores based on facial expressions decreased in the presence of a male observer [28], while the latency to exhibit behavioural expression of pain increased in the presence of a cat [29]. Some authors have inferred that rabbits avoid demonstrating pain behaviour in the presence of an observer [3, 9], however this supposition has not been scientifically evaluated.
Consequently, it is crucial to identify the degree to which the presence of an observer influences the exhibition of rabbit behaviours associated with pain and whether it is possible to assess pain in-person or if a remote assessment is necessary to effectively identify pain behaviours in this species.

The current study aimed to determine the frequency and duration of acute pain-related behaviours in rabbits undergoing orthopaedic surgery and to compare them when a human observer was present or absent, based on the hypothesis that pain modifies the behavioural state of normality of rabbits and the presence of a human observer reduces the expression of pain-free and painful behaviours.

**Materials and methods**

The study was approved by the Ethical Committee for the Use of Animals in Research, of the School of Veterinary Medicine and Animal Science, of São Paulo State University (Unesp) and School of Agricultural and Veterinary Sciences, São Paulo State University (Unesp), under protocol numbers 0156/2018 and 019155/17 respectively. The study follows the Brazilian Federal legislation of CONCEA (National Council for the Control of Animal Experimentation) and covered two of the principles of the 3 R’s (reduction and refinement), referring to good practice in animal experimentation [30]. This is an opportunistic study (reduction) conducted in conjunction with another unrelated study, in which the animals underwent partial radius ostectomy, to identify pain behaviours in rabbits (refinement).

**Animals**

Twenty-eight New Zealand rabbits (*Oryctolagus cuniculus*) from the Central Vivarium of UNESP (São Paulo State University, Botucatu) were used, including 11 females and 17 males, 159±5 days old, and mean weight of 3.7±0.38kg.

As inclusion criteria, healthy animals were selected after laboratory (haemogram) and physical exams, including inspection, cardiac and respiratory auscultation, and rectal temperature measurement. Animals would be excluded from the study if they presented complications during surgery, such as bleeding, hypotension (systolic arterial blood pressure < 80 mmHg) or bradycardia (heart rate < 130 bpm) not resolved by reducing the concentration of the volatile anaesthetic or by fluid administration, arrhythmias, or, after the surgical procedure, complications such as intestinal motility disorders.

The animals were allocated to an experimental rabbit shelter adapted to house experimental animals, with natural ventilation controlled by curtains. Each rabbit was kept in an individual stainless steel cage, 60cm³ in size, with a grid floor litter tray which was cleaned twice a day. The study was conducted between August and September, with a local natural photoperiod of approximately 12 hours of light per day and a mean temperature of 21°C. The adaptation of the housing occurred approximately 40 days before surgery. Four days before the video recording, the animals were housed in specific cages within the same shelter to acclimate.

Water and dry feed (Fri-Coelho, FRI-RIBE, Trouw Nutrition, São Paulo, SP, Brazil) were supplied *ad libitum* in open dish drinkers and feeders, as well as carrot pieces daily and Tifton hay (*Cynodon spp.*) was provided twice a week. To supply the rabbits’ needs for interaction and chewing, eucalyptus pinecones were also provided.

**Anaesthesia and surgery**

Two days before surgery, the animals were anaesthetized through isoflurane inhalation (Isoforine 100%, Cristália Pharmaceutical Products LTDA, São Paulo, SP, Brazil) at a concentration of 4–5 Vol% diluted in oxygen 4L/min administered in an induction chamber, followed by a
face mask. The superficial anaesthetic plane was maintained, confirmed by the presence of foot reflexes and ear pinching for a period of three to five minutes, to allow shaving the surgical region (right thoracic limb), the ears (for venous access), and the distal region of the left thoracic limb (for monitoring arterial blood pressure with a Doppler ultrasound).

On the day of surgery, the non-fasting rabbits were treated with 5 mg/kg pethidine (Dolosal 50mg/mL, Cristália Pharmaceuticals LTDA, Sao Paulo, SP, Brazil) via the lumbar epaxial intramuscular (IM) route. Next, a 24G catheter was inserted into the marginal ear vein and fluid therapy was instigated with lactated Ringer’s solution (RL) at a rate of 3mL/kg/h administered by a syringe infusion pump. Oxygen therapy was instituted at 2L/min via face mask for three minutes and anaesthetic induction with 3 to 5 Vol% isoflurane diluted in 2L/min oxygen. Following the absence of interdigital and ear pinching reflexes, 0.1 mL of lidocaine (Xylestesin 2% without vasoconstrictor, Cristália Pharmaceuticals LTDA, São Paulo, SP, Brazil) was instilled into the arytenoids region, maintaining inhalation of isoflurane for a further minute. Capnography-guided orotracheal intubation (Capnostat 5, Digicare Animal Health, Rio de Janeiro, RJ, Brazil) was performed with an endotracheal tube of internal diameter 2.5–3.5mm, compatible with the size of the animal. If, after three attempts, intubation was not possible, anaesthesia was maintained via the face mask. The inspired isoflurane concentration was adjusted to maintain the surgical anaesthetic plane and 2 μg/kg of fentanyl (Fentanest 0.0785 mg/mL, Cristália Pharmaceuticals LTDA, Sao Paulo, SP, Brazil) was administered intravenously (IV) immediately prior to the start of surgery.

Anaesthetic monitoring was recorded on an appropriate chart every five minutes, consisting of heart rate, peripheral haemoglobin oxygen saturation, respiratory rate, expired CO$_2$ fraction (EtCO$_2$) (LifeWindow LW9x, Digicare Animal Health, Rio de Janeiro, RJ, Brazil), rectal temperature using a digital thermometer, and non-invasive systolic arterial blood pressure with a Doppler ultrasound (Vascular Doppler model 811-B. Parks Medical Inc, OR, USA), in addition to identifying the beginning and end of surgery and possible anaesthetic complications.

Prior to surgery, limb antisepsis was performed with 2% chlorhexidine followed by 0.5% alcohol chlorhexidine. An incision of approximately 2.5 cm was made in the skin of the craniolateral region of the radius, followed by blunt dissection of subcutaneous tissue and musculature, which allowed exposure of the distal radius diaphysis. Partial ostectomy was performed of 1 cm extension in the radial diaphysis, 1.5 cm distant from the radiocarpal joint, using an oscillatory saw and a spectrometer for measurements. The segment of the radius was removed together with the periosteum and the wound was sutured. The surgeries were performed between 8am and 4pm and each surgical procedure took between 10 and 30 minutes. Anaesthetic recovery was defined as when the rabbits, positioned in sternal recumbency, were able to support the head.

One hour after anaesthetic recovery, all animals received rescue analgesia with 2 mg/kg morphine (Dimorf 1%, Cristália Pharmaceuticals LTDA, Sao Paulo, SP, Brazil) and 1 mg/kg meloxicam (Maxicam 2%, Ourofino, Sao Paulo, SP, Brazil) IM. Eight hours after anaesthesia recovery the animals were evaluated according to the researcher’s clinical assessment and if necessary, an extra dose of 1 mg/kg of morphine was administered, IM. From 24 hours after anaesthetic recovery, the animals received 5 mg/kg tramadol hydrochloride (Tramadol hydrochloride, Teuto, Goiás, Brazil) IM every 8 hours for three days.

**Video recordings**

The twenty-eight animals were filmed at I) 24 hours before surgery (Baseline), II) 1 hour after recovery from anaesthesia (Pain), III) 3 hours after rescue analgesia, i.e. 4 hours after recovery.
from anaesthesia (Analgesia), and IV) 24 hours after recovery from anaesthesia (24h post) (Fig 1).

The same female researcher (RHP), who was familiar to the rabbits, made all of the recordings. After positioning the camera, a piece of carrot, approximately 2 x 2.5 cm, was offered, the pinecone already present was removed and replaced with a new one, and then the observer left the room. The observer waited in silence in another room about three meters away from the room housing the rabbits. After five minutes (to allow the rabbit to acclimate to the researcher’s absence), the camera was triggered using a mobile app (GoPro App. GoPro Inc., California, USA) and filming continued for five minutes (absence of observer). At the end of this five minutes of filming, the researcher returned to the filming room, sat in a chair in front of the cage behind the camera and filming continued for another five minutes (presence of the observer).

Ethogram

The ethogram was developed and adapted according to the pain behaviours described after ovariohysterectomy (OVH) [9] as a reference. The video sequences recorded were first analyzed to identify whether the same pain behaviours as observed after OVH were present in this study. This initial analysis also allowed the identification of new pain behaviours that were not previously reported after OVH [9], these included: ‘scissor’ ears, press limbs, scratch ears, punch, eat a carrot, interact with the pinecone, ingest cecotropes, tremble, suspend limb, put weight on and raise the affected limb, and try to get up. Semantical adaptations were also made to the original ethogram [9]. Some descriptions were altered according to the state and are described in Table 1.

The following behavioural categories were considered: activity, position in cage, posture, position of the ears in relation to the body, eye opening, typical behaviours, interaction with motivational items, physiological behaviours (eat, drink, and ingest cecotropes), self-cleaning, and pain-related behaviours (see Table 2).

Table 1. Modifications from the original ethogram developed for rabbits submitted to OVH [9].

| Behaviour | Original description | Current description |
|-----------|----------------------|---------------------|
| Normal posture | Sitting relaxed with hind limbs tucked under the rump and fore limbs underneath | Flexes pelvic limbs under the hip, thoracic limbs under the body; abdomen and chest not supported on cage floor |
| React | Originally named “alert”: Immediate reaction of individual to being disturbed; animal momentarily looks around | Moves head and/or body sharply in response to environmental stimulus (e.g., sound stimulus) |
| Wince | Rocking motion accompanied by eye closing and swallowing action | Shrinks back and closes eyes |
| Twitch | Rapid movement of fur on back | Presents spasms in the skin of the back |
| Behaviour                                      | Description                                                                 | F | D |
|------------------------------------------------|-----------------------------------------------------------------------------|---|---|
| **1. Activity**                                |                                                                             |   |   |
| Active                                         | Moves and/or moves around                                                  | ✓ |   |
| Inactive                                       | Remains stationary                                                        |   | ✓ |
| **2. Position in the cage**                    |                                                                             |   |   |
| Front                                          | Front of the cage                                                          |   | ✓ |
| Back                                           | Back of the cage                                                           |   | ✓ |
| Change position                                | Moves around the cage: front to back or back to front                      |   | ✓ |
| **3. Posture**                                 |                                                                             |   |   |
| Normal                                         | Flexes pelvic limbs under the hip, thoracic limbs under the body; abdomen   | ✓ |   |
|                                               | and chest not supported on cage floor                                       |   |   |
| Seated                                         | Sits with vertically extended thoracic limbs                               |   | ✓ |
| Lying down                                     | Lies with the abdomen and chest in contact with the cage floor and         | ✓ |   |
|                                               | horizontally extended thoracic limbs                                       |   |   |
| Lying on one side                              | Lies on one side of the body with horizontally extended thoracic limbs     |   | ✓ |
| Quadrupedal                                    | In the quadrupedal position with the four limbs extended vertically;       | ✓ |   |
|                                               | abdomen not in contact with the cage floor                                |   |   |
| Bipedal                                        | Supported on both pelvic limbs; thoracic limbs not touching the floor       | ✓ | ✓ |
| Change posture                                 | Alternates between different postures                                      | ✓ |   |
| **4. Position of the ears in relation to the body** |                                                                             |   |   |
| Completely lowered                            | Parallel to the vertebral column                                           |   | ✓ |
| Semi-lowered                                   | Semi-lowered (positioned between fully lowered and erect)                  | ✓ |   |
| Erect                                          | Erect, perpendicular to the spine                                          |   | ✓ |
| ‘Scissor’ ears                                 | Each ear moves to different positions                                      |   | ✓ |
| **5. Eye opening**                             |                                                                             |   |   |
| Open                                           | Fully open                                                                 | ✓ |   |
| Semi-closed                                    | Semi-closed                                                                |   | ✓ |
| Closed                                         | Closed                                                                     |   | ✓ |
| Not visible                                    | Not possible to visualize the eyes                                         |   | ✓ |
| **6. Typical rabbit behaviours**               |                                                                             |   |   |
| Hop                                            | Hops to move around with both pelvic limbs at the same time                 | ✓ |   |
| Rotating jump                                  | Jumps performing a 180° or 360° rotation with both pelvic limbs at the     | ✓ |   |
|                                               | same time                                                                  |   |   |
| React                                          | Moves head and/or body sharply in response to environmental stimulus        | ✓ |   |
| (e.g., sound stimulus)                         |                                                                           |   |   |
| Shake body                                     | Shakes the whole body                                                      |   | ✓ |
| Shake the head                                 | Shakes only the head                                                       | ✓ |   |
| Dig                                            | Digs the floor                                                             | ✓ | ✓ |
| Explore                                        | Sniffs the cage floor and/or walls and/or bars curiously                   | ✓ | ✓ |
| Gnaw                                           | Gnaws the floor or bars of the cage                                        | ✓ | ✓ |
| Press limbs                                    | Presses limbs strongly against the cage floor                              | ✓ |   |
| Stretch                                        | Stretches the body                                                         | ✓ |   |
| Scratch ear                                    | Scratches the ears with the limbs                                          | ✓ | ✓ |
| Punch                                          | Extends the thoracic limbs horizontally, quickly alternating between right  | ✓ |   |
|                                               | and left                                                                   |   |   |
| **7. Interaction with motivational items**      |                                                                             |   |   |
| Interact with pinecone                         | Interacts with the pinecone (chews, gnaws, pushes with the thoracic limbs) | ✓ | ✓ |
| Eat carrot                                      | Eats the carrot                                                            | ✓ | ✓ |
| **8. Physiological behaviours**                |                                                                             |   |   |
| Drink                                          | Drinks water from the water trough                                        | ✓ | ✓ |

(Continued)
Once the behaviours had been identified in the first analysis of the videos, the footage was evaluated again to calculate the frequency of ‘event behaviours’ (number of occurrences started) and the duration (in seconds) of all behaviours (i.e. ‘state’ behaviours). The duration was recorded in seconds for behavioural states and only frequency was reported for event behaviours. Duration and frequency were assessed for some behaviours, such as self-cleaning and interaction with pinecones and carrots (Table 2).

### Statistical analysis

Taking into account a test power of 90% with an alpha of 5% (http://biomath.info/power), 21 rabbits were necessary to observe differences in duration of activity at baseline, when the observer was present compared to when the observer was absent, and 11 rabbits were necessary to compare duration of activity between the baseline and 1h post-recovery time points in the presence of the observer.

Analysis were performed using Graph Pad Prism 5.0 software considering an $\alpha$ of 5%. According to the Shapiro Wilk test and histogram distribution analysis, data were defined as non-normal. To compare the time-points (Baseline, Pain, Analgesia, and 24h post) Friedman’s test was used followed by Dunn’s multiple comparison post-hoc test. To compare the behaviours between the presence and absence of the observer, the Wilcoxon test for paired samples was used. Differences were considered statistically significant when $p<0.05$.

### Results

None of the rabbits were excluded from the study. It was not possible to perform endotracheal intubation in three animals and, therefore, they were kept under inhalation anaesthesia via a
face mask. EtCO$_2$ was greater than 45mmHg in all rabbits at some time-point during anaesthesia, which was resolved by manual assisted ventilation or by reducing isoflurane concentration. Nine rabbits had transient hypotension (systolic arterial blood pressure $< 80$ mmHg) which was successfully treated by reducing the concentration of isoflurane in eight rabbits or by administering fluids (10 ml/kg of Lactated Ringer in 10 minutes) in one rabbit.

Rabbits recovered 3±2.8 (1–13) minutes after the end of anaesthesia. There were no cases of postoperative hypothermia or other complications. Three rabbits received an additional administration of 1mg/kg of morphine eight hours after recovery from anaesthesia.

In total, 50 behaviours were identified. Thirty nine behaviours had been previously described in rabbits undergoing OVH [9] and an additional 11 new behaviours were identified in the current study (‘scissor’ ears, press limbs, scratch ears, punch, eat a carrot, interact with the pinecone, ingest cecotropes, tremble, suspend limb, put weight on and raise the affected limb, and try to get up) (Table 2).

The frequency and/or duration of 19 and 13 of these 50 behaviours respectively changed over time and from the absence to presence of the observer. The medians and interquartile range of the duration and frequency of occurrence of each behaviour that altered over time or was affected by the presence of the observer are described in Table 3.

**Pain behaviours related to surgery**

Both in the presence (Pr) and in the absence (Ab) of the observer, one hour post-recovery (Pain), the rabbits became less active (Pr and Ab—$p < 0.0001$), and therefore more inactive (Pr and Ab—$p < 0.0001$), changed their cage position (Pr and Ab—$p < 0.0001$), and posture less (Pr and Ab—$p < 0.0001$), hopped (Pr and Ab—$p < 0.0001$), and explored (Pr and Ab—$p < 0.0001$) less, kept their eyes open for a shorter time (Pr—$p < 0.001$ and Ab—$p < 0.0001$), and semi-closed their eyes (Pr—$p < 0.001$ and Ab—$p < 0.0001$) for longer compared to baseline. Four hours post-recovery (Analgesia) the rabbits remained lying down for longer (Pr and Ab—$p < 0.0001$), compared to the baseline and 24h post. Putting weight on and raising limbs was more frequent at 24h post (Pr—$p < 0.0001$ and Ab—$p < 0.01$) compared to baseline (Table 3).

The rabbits remained less in the quadrupedal position ($p < 0.001$), ate less carrots ($p < 0.0001$), interacted less with the pinecone ($p < 0.0001$), and winced more ($p < 0.001$) only in the absence of the observer at one hour post-recovery (Pain) (Table 3).

In the presence of the observer, 4h post-recovery (Analgesia), compared to Baseline and 24h post, the rabbits remained more frequently at the back of the cage ($p = 0.0058$) and less frequently at the front ($p = 0.0058$) and remained seated for a shorter time ($p < 0.0001$). The duration of body self-cleaning was shorter at the 1h post-recovery (Pain) than at 24h post ($p < 0.001$). When the observer was absent the rabbits remained less frequently in the normal posture after 4h post-recovery (Analgesia) compared to Baseline ($p < 0.05$) (Table 3).

**Differences in pain behaviours between the presence and absence of the observer**

In the presence of the familiar female observer, compared to the absence of the observer, there was a higher frequency and/or duration of the following behaviours: inactive at all time-points (Baseline—$p < 0.0001$, Pain—$p < 0.01$, Analgesia—$p = 0.0195$; 24h post—$p < 0.05$) and lie down posture ($p < 0.01$), react ($p < 0.05$) and wince ($p < 0.05$) at 24h post recovery. Otherwise, in the presence of the observer, at all time-points the rabbits remained active (Baseline—$p < 0.0001$, Pain—$p < 0.01$, Analgesia—$p < 0.05$; 24h post—$p < 0.05$), in normal posture (24h post) ($p < 0.05$) and seated ($p < 0.05$) and lying down (Analgesia) ($p < 0.001$) for a
| Behaviour                          | F/D | Observer | Baseline | Pain | Analgesia | 24h post |
|-----------------------------------|-----|----------|----------|------|-----------|----------|
| **Activity**                      |     |          |          |      |           |          |
| Active                            | D   | Pr       | 280 (162–300)† | 0 (0–78)† | 0 (0–16)† | 300 (92–300)† |
| Inactive                          | D   | Pr       | 20 (0–138)† | 300 (222–300)† | 300 (284–300)† | 0 (0–208)† |
| Position in cage                  |     |          |          |      |           |          |
| Front                             | D   | Pr       | 204 (91–289)† | 0 (0–300)† | 0 (0–139)† | 229 (62–291)† |
| Back                              | D   | Pr       | 96 (12–209)† | 300 (300–300)† | 300 (161–300)† | 71 (9–238)† |
| Change position                   | F   | Pr       | 2 (1–5)† | 0 (0–0)† | 0 (0–0)bc | 1 (0–2)ab |
| Posture                           |     |          |          |      |           |          |
| Normal                            | D   | Pr       | 91 (8–199)† | 63 (0–300)† | 15 (0–237)† | 50 (2–143)† |
| Seated                            | D   | Pr       | 128 (10–220)† | 0 (0–212)ab | 0 (0–0)† | 131 (44–236)† |
| Lying down                        | D   | Pr       | 57 (3–162)† | 0 (0–216)† | 0 (0–170)† ab | 111 (44–253)† |
| Quadrupedal                       | D   | Pr       | 0 (0–0)† | 0 (0–262)ab | 241 (300–300)† | 0 (0–64)‡ |
| Change posture                    | F   | Pr       | 2 (1–6)† | 0 (0–1)ab | 0 (0–1)ab | 3 (1–8)‡ |
| Eye opening                       |     |          |          |      |           |          |
| Open                              | D   | Pr       | 293 (237–300)† | 57 (7–194)c | 60 (12–282)bc | 255 (57–300)ab |
| Semi-closed                       | D   | Pr       | 0 (0–0)† | 94 (2–208)† | 69 (0–208)ab | 0 (0–87)ab |
| Typical rabbit behaviours         |     |          |          |      |           |          |
| Hop                               | F   | Pr       | 2 (1–6)† | 0 (0–0)† | 0 (0–0)† | 1 (0–4)† |
| React                             | F   | Pr       | 0 (0–1)† | 0 (0–0)† | 0 (0–0)† | 0 (0–1)† |
| Shake body                        | F   | Pr       | 0 (0–0)† | 0 (0–0)† | 0 (0–0)† | 0 (0–0)† |
| Explore                           | F   | Pr       | 1 (0–2)† | 0 (0–0)† | 0 (0–0)ab | 0 (0–1)ab |
| Interaction with motivational items|     |          |          |      |           |          |
| Interact with the pinecone*       | F   | Ab       | 2 (0–4)† | 0 (0–0)† | 0 (0–0)† | 1 (0–2)† |
| Eat the carrot*                   | F   | Ab       | 1 (0–1)ab | 0 (0–0)† | 0 (0–0)ab | 1 (0–1)‡ |
| Self-cleaning                      |     |          |          |      |           |          |
| Body                              | F   | Pr       | 0 (0–2)† | 0 (0–0)† | 0 (0–0)† | 1 (0–4) |
| (Continued)                        |     |          |          |      |           |          |
shorter period compared to the absence of the observer (Table 3). When the observer was present, there was a lower frequency of explore (Baseline) \( (p < 0.01) \), flinch (Pain) \( (p < 0.05) \), put weight on and raise the affected limb (Analgesia) \( (p < 0.05) \), and suspended limb at 24h \( (p < 0.05) \). When the observer was present there was a lower duration of explore (Baseline) \( (p < 0.01) \), suspended limb (Pain) \( (p < 0.05) \), lick the affected area (Analgesia) \( (p < 0.05) \), and normal posture at 24h post \( (p < 0.05) \) (Table 3).

Behaviours that did not change over time or in the presence of the observer

Lying on one side and bipedal postures and the behaviours rotating jump, shake the head, dig, drink, eat feed, gnaw, press limbs, stretch, scratch ear, ingest cecotropes, punch, head self-cleaning, twitch, stagger, fall, tremble, try to get up, writhe, eyes closed and eyes not visible, ears completely lowered, semi-lowered, erect, and ‘scissor’ ears did not alter over time or between the presence and absence of the observer (S2 Table).

Table 3. (Continued)

| Behaviour | F/D | Observer | Baseline | Pain | Analgesia | 24h post |
|-----------|-----|----------|----------|------|-----------|----------|
| **Pain-related behaviours** | | | | | | |
| Lick affected area | F | Pr | 0 (0–1) | 0 (0–1) | 0 (0–0) | 1 (0–2) |
| | | Ab | 0 (0–1) | 0 (0–3) | 0 (0–1) | 0 (0–1) |
| | D | Pr | 0 (0–3) | 0 (0–2) | 0 (0–0) | 0 (1–12) |
| | | Ab | 0 (0–2) | 0 (0–111) | 0 (0–2)\(^{†}\) | 0 (0–7) |
| Flinch | F | Pr | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) |
| | | Ab | 0 (0–0) | 0 (0–1)\(^{†}\) | 0 (0–0) | 0 (0–0) |
| Wince | F | Pr | 0 (0–0) | 1 (0–1) | 0 (0–0) | 0 (0–1)\(^{†}\) |
| | | Ab | 0 (0–0)\(^{b}\) | 1 (0–1)\(^{a}\) | 0 (0–0)\(^{ab}\) | 0 (0–0)\(^{ab}\) |
| Suspend limb | F | Pr | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) |
| | | Ab | 0 (0–0) | 0 (0–1) | 0 (0–0) | 0 (0–1)\(^{†}\) |
| | D | Pr | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) |
| | | Ab | 0 (0–0) | 0 (0–65)\(^{†}\) | 0 (0–0) | 0 (0–1) |
| Put weight on and raise the affected limb | F | Pr | 0 (0–1)\(^{b}\) | 0 (0–0)\(^{b}\) | 0 (0–0)\(^{b}\) | 4 (1–11)\(^{a}\) |
| | | Ab | 0 (0–0)\(^{b}\) | 0 (0–1)\(^{ab}\) | 0 (0–2)\(^{ab}\) | 2 (0–7)\(^{a}\) |

Frequency (F) in number of occurrences and duration (D) in seconds in each of the 300 second (five minute) observations of behaviours in the twenty-eight rabbits in the moments before surgery (Baseline); 1 hour after recovery from anaesthesia (Pain); 4 hours after recovery from anaesthesia and 3 hours after rescue analgesia (Analgesia); and 24 hours after recovery from anaesthesia (24h post). Different letters indicate statistical difference between the time-points according to the Friedman test \( (p < 0.05) \), being \( a > b > c \). The outline highlighted in the table indicates difference between the observer’s presence (Pr) and absence (Ab); \(^{†}\) indicates significant difference in duration or frequency between Pr and Ab by the Wilcoxon test \( (p < 0.05) \).

“Data on the behaviours of “eat the carrot” and “interact with the pinecone” in the presence of the observer were not included, because these motivational items were not replaced when the observer entered and remained in the room; therefore the carrot could have already been eaten and the pinecone would not be a novelty factor.”

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Discussion

This study corroborated previously reported postoperative pain behaviours in rabbits after ovariohysterectomy (OVH) [9] and demonstrated the presence of different and apparently specific orthopaedic pain behaviours not identified in previous studies on soft tissue surgery [9, 12, 15]. Another finding that confirms the study hypothesis was that the presence of the observer influenced both the preoperative behaviour of the pain-free state and the postoperative pain behavioural expression.
The lack of support from the affected limb in the presence of musculoskeletal injuries is a common finding in rabbits and other species [31]. The movement of putting weight and raising up the limb was identified in the current study as a specific behaviour after orthopaedic surgery in rabbits. The lack of a number of pain behaviours that have been previously observed after soft tissue surgery, such as arching of the back and pressing the abdomen towards the floor [9] most likely indicated that these behaviours are probably specific to abdominal pain since they were not identified in this study.

Press limbs, suspend the limb, 'scissor' ears, scratch ears, punch, ingest cecotropes, tremble, and try to get up behaviours were identified in this study, but were not described in the previous OVH ethogram [9], however, there were no differences in their frequency of occurrence or duration over time in this study. Some of the behaviours observed, such as suspend the limb, try to get up, stagger, and close the eyes occurred exclusively following surgery (S2 Table), suggesting that their expression is related to postoperative pain, as reported for stagger and closed eyes [9].

In the immediate post-recovery period, both before and after the rescue analgesia, the rabbits were less active, and thus rarely hopped and changed position in the cage and changed posture. This is similar to that observed after soft tissue surgeries without the presence of an observer [9, 13]. Decreased activity after surgery minimizes pain and discomfort and potentially accelerates recovery [12], has a protective function [9], and is an important indicator of pain in this species [15, 27]. However, in rabbits evaluated in a previous study in the presence of an observer [8], there was no difference in activity scores between the immediate and late postoperative periods, contrary to the present study, where the activity was lower even with the observer present. This is likely to be because in the previous study the rabbits were under the effect of analgesics, as well as due to differences in acclimation period, housing, type of surgery, and anaesthetic protocol.

Following rescue analgesia, the rabbits remained in the back of the cage for longer when the observer was present, which may reflect a natural escape behaviour from a potential threat [32], since in both the current study and previous research [9, 12], when the observer was absent, there were no differences in cage positioning.

In previous studies, weight loss in the days following surgery has been related to pain [9, 27]. Although weight loss is a delayed evaluation [19], as it is directly related to food consumption, it can be evaluated in real time through appetite. However, if food is available ad libitum, the measurement of consumption over a short period of time may not be representative of the animals’ pain state. This limitation was observed in the current study, since the behaviour of eating the food did not change over time which demonstrates that evaluation of appetite may not differentiate pain-free vs painful states. Alternatively, the interest in a piece of carrot as a motivational item reduced during post-recovery in the absence of the observer. As carrot is more palatable, fresh, and less frequently offered than feed, it seems to stimulate the appetite and allows evaluation of appetite in real time. The same approach has been used in cats by offering more palatable and different food to what they were conventionally given [20].

In addition to eat the carrot, interact with the pinecone and explore appear to be important for defining pain-free rabbits, because they decreased postoperatively. Likewise, pain following soft tissue surgery in rabbits reduced exploratory behaviour [9, 13]. The presence of the observer reduced explore behaviour at baseline, showing the inhibitory influence of the observer even in pain-free animals.

The introduction of a pinecone stimulated play behaviour and interaction with a new object, which resembles the interaction with toys [9, 13, 15]. The inclusion of new objects stimulates the innate activity of animals, even in environments with restricted space, such as cages [33]. However, it is important to replace objects frequently to maintain interaction and
positive effect, otherwise the interaction is reduced once the rabbit is familiar with the object [34]. In this study a new pinecone was offered at each recording period in the absence of the observer, to arouse the curiosity of the animals. The interactions with motivational items in the current study were good indicators of pain-free state and well-being, as playful behaviour represents potential positive emotions [35]. In contrast, the reduction in these interactions indicates painful states after surgery.

Although the interaction with motivational items occurred at low frequency and duration even at baseline, this behaviour is apparently sensitive and specific to pain, occurring in most rabbits at baseline, but in the minority of rabbits after surgery.

Suspend limb and lick the affected area were the most expected behaviours after limb surgery, however they were apparently masked by the presence of the observer. Wince behaviour is important to identify both orthopaedic, as in the current study, and soft tissue postoperative pain [9, 12].

Another indicator that forms part of the behavioural repertoire of painful rabbit seems to be semi-closed eyes, previously described in the Rabbit Grimace Scale [10], as well as other laboratory animal species [36, 37], cats [38, 39], horses [40], and sheep [41]. Similarly, as expected, the presence of open eyes decreased in the presence of pain in this study.

Ear position was important indicator of pain in rabbits when assessed only from images [10, 42] using a 3-point scale. However, like in previous studies [9, 12], the current study showed no pain-related changes in ear position when assessed by duration, even with the introduction of a catheter into the auricular right ear vein. Ear position is an apparently non-specific pain behaviour because it may be influenced by environmental stimuli and other motives, such as during self-cleaning behaviour.

As rabbits are sociable animals in nature and interact with each other, caged rabbits exhibit more frequent body self-cleaning due to lack of environmental stimulation [43]. In contrast to observations following other types of surgery [9, 12, 13], this behaviour did not reduce after orthopaedic surgery in this study, and the possible explanations may be because orthopaedic surgery performed in the limb does not prevent rabbits from grooming nor reduce the motivation to groom as seen with visceral pain [9, 13] and/or because grooming may be a displacement activity post-surgery that distracts rabbits from pain.

Licking of the affected area did not change over time, in contrast to previous studies in rabbits [10] and other species [20, 23] experiencing pain. Factors other than pain may influence this behaviour and the higher than expected occurrence at baseline could be due to the shaving performed the day before, as described in rats submitted to abdominal shaving [24] and rabbits with a jugular catheter [13].

React and shake the body behaviours were not altered in pain situations in the current and other studies [9, 12], but they were affected by the presence of the observer. Reactive behaviour is related to the animal’s reactivity to stimuli, which increased when the observer was present at 24 hours post-recovery. Otherwise the observer inhibited shaking, a behaviour related to comfort [43].

In addition to their presence, the observer’s sex may influence the behavioural expression of the animals. The expression of pain through facial expressions and the threshold of nociceptive tests were masked by the presence of a male observer, but not female observers in rodents [28]. This is apparently related to the presence of androstenone and androstadienone in axillary secretions found predominantly in males. The current study excluded this factor as the observer was female; however, different results could be found if the observer were male.

Another factor that may have attenuated the differences between the presence and absence of the observer was that the rabbits were familiar with the observer, who was responsible for the handling and feeding of the animals during the acclimation period. Rats demonstrate a
preference for familiar researchers [26, 44] and even superficial handling interaction between laboratory rats and researchers reduced rat anxiety scores [26].

Positive interactions between humans and other animals in experimental conditions reduce stress and facilitate identification of abnormal behaviours related to pain and disease [45]. In the current study, it is challenging to assess whether the interaction was positive or negative. Familiarity through feeding and animal care procedures prior to surgery can have a positive effect [26], however, in this study the interaction observed at 24 hours post-recovery, was aggressive, suggesting this interaction was apparently negative. After rabbits have been submitted to stressful situations such as surgery and administration of analgesics, the effect of the presence of the observer was more evident in a higher number of behaviours (active, inactive, react, shake body, wince and suspend limb behaviours and normal and lying down postures) at 24 hours postoperatively compared to the other moments.

As described in other species, appetite would be expected to increase after rescue analgesia, both due to reduced pain [21, 46, 47] and through the effect of opioids [48]. This was not the case in the current study, and was likely to be due to morphine and meloxicam were insufficient to relieve pain after surgery or because morphine caused sedation.

The time-point after rescue analgesia was defined as three hours after administration to avoid the effect of sedation observed post-morphine injection, since in a pilot study intense sedation of the animals was observed one hour after morphine. Therefore, 3h after morphine administration it would be expected that the analgesic effect would be present, but with little effect of sedation [49]. Sedation was not assessed because there are no validated tools to quantify sedation in rabbits as reported for dogs [50] and observation of this parameter was beyond the scope of this study. A possible effect of sedation influencing the expression of postoperative pain behaviour cannot be disregarded in rabbits; cats undergoing ketamine administration have been falsely diagnosed with pain [51]. Little is known about the effects of morphine on pain behaviours in leporine species. At doses of 3 to 5 mg/kg, morphine sedated rabbits [52]; the lower dose resulted in analgesia for 240 minutes and reduced activity for up to 300 minutes [49], when the postoperative rescue analgesia time-point was assessed in the current study.

Although buprenorphine is the most commonly used opioid in rabbits [16] and in behavioural studies [8, 13, 15], this drug is not commercially available in Brazil. Thus, the choice of analgesic protocol was based on the fact that the 1 mg/kg dose of meloxicam was the most effective dosage in rabbits submitted to OVH [9]. Since the orthopaedic surgery induces severe pain, morphine 2 mg/kg was included in the analgesic protocol, as this is an effective opioid analgesic [5, 46]. The analgesic protocol used in this study was similar to that described in other species to validate pain scales [20, 21].

Pre- and trans-operative analgesia was based on pethidine and fentanyl respectively. These opioids apparently guarantee sufficient trans-operative analgesia because there were no pain-related autonomic trans-operative changes. As pethidine and fentanyl are short acting opioids, their effects were abated shortly after surgery; this facilitated expression and identification of postoperative pain behaviours in this study. Morphine and meloxicam apparently prevented hyperalgesia up to 24h after anaesthetic recovery, because by this time behaviours were similar to baseline. Carprofen and indomethacin prevented experimentally induced hyperalgesia in cats and rabbits respectively [53, 54] and morphine reversed hyperalgesia in rabbits [54].

The maintenance of postoperative analgesia with meloxicam for a few days would be more appropriate than tramadol only. However, this was an opportunistic study and because one of the aims of the other study was to measure acute-phase protein, the anti-inflammatory effect produced by long-term treatment with meloxicam would interfere in this analysis. Dogs submitted to orthopaedic surgery exhibited the most intense degree of pain in the initial postoperative hours [18]. Considering that at 24h, the rabbits’ behaviours were similar to baseline, it
appears the rabbits were not suffering sufficient pain to require analgesia, other than tramadol, at 24h post-recovery.

In a previous study [9], rabbits were less active in the morning compared to the afternoon. In another study rabbit behaviours did not change over the course of the day [13]. In the current study, the animals underwent surgery from early morning until early afternoon, they acted as their own controls and time-point intervals were the same. In this study, this probably compensated for the effect of circadian rhythm on behavioural variables.

A 20-minute period has been suggested as representative for evaluating behaviour in rabbits [9], however this is too long and impractical for clinical evaluation. Brevity is an important quality of pain assessment tools [22]. Behaviour assessment of the pain-free state is essential to compare against the painful state and, therefore, identify pain-related behaviours. However, if there is no stimulus in the pain-free state, the animal may rest most of the time and this low activity may be falsely identified as pain. Alternatively, if environmental enrichment is provided to stimulate activity, the animal could become accustomed and lose interest, as reported in the previous study in rabbits submitted to OVH, where even when assessing the rabbit’s behaviour for 20 minutes, the interaction with the toy was sporadic [9]. Although the evaluation time was shorter in the current study, it seems that the inclusion of new motivational items in every behaviour assessment optimized the analysis of the playing behaviour and allowed expression of pain behaviours.

Limitations

One of the limitations of the current study was the different female-male ratio and the non-evaluation of the female estrous cycle. This was an opportunistic study, which followed the recommendations for developing pain evaluation methods [7], by using animals that had already undergone surgical stimulation, instead of promoting suffering in other animals for this exclusive purpose. Previous studies showed that due to their hormonal cycling, female rabbits are more likely to be antisocial compared to male rabbits [55]. Female mice showed a higher mechanical threshold than males [56], and bitches tend to protect the surgical wound area more than male dogs [46].

A control group of rabbits submitted only to anaesthesia could identify the exclusive anaesthetic effects on post-intervention behaviour [19]. Previous studies also did not include control groups, however, most of the behaviours identified in the current study were similar to those described previously in rabbits [9, 13, 15] and in other species suffering pain [18]. A future study should be performed to address possible anaesthesia-related behavioural changes.

Videos should ideally be assessed by a blind evaluator to avoid expectation bias [57], however this risk was minimized because among the methodologies used in behavioural studies, the recording of ethogram based on frequency and duration of behaviours is one of the least affected by expectation bias [58]. The order of filming in the absence and presence of the observer was not randomized. This decision was taken as it was impossible to know how much time was necessary for the rabbit to return to the original behaviour after the observer had left. Similarly, the five-minute period before filming began may not have been sufficient to adapt the rabbit to the absence of the observer.

Although in the absence of the observer there was no eye contact with the animal, as the observer was in another room, one cannot rule out the possibility that the rabbit could smell the observer. To minimize this limitation, the observer did not use any perfume during the filming period.
Conclusion
Orthopaedic surgery pain in rabbits reduces activity, changes in different postures and position around the cage, and behaviours of hopping and explore, as well as increasing the presence of semi-closed eyes in both the presence and absence of the observer. In the absence of the observer at the moment of likely greatest pain post-recovery (i.e. 1h), the rabbits remained for less time in the quadrupedal posture, ate less carrot, interacted less with the pinecone, and winced more frequently. The behaviours of suspend the affected limb and put weight on and raise the affected limb appear to be specific to orthopaedic pain. The presence of the female and familiar observer before surgery reduced activity and exploratory behaviour, leading to the false impression of pain in a pain-free state. After surgery, the frequency of wince increased and flinch, lick the affected area, suspend the limb, and put weight on and raise the affected limb decreased in the presence of the observer, which suggests that the rabbits were masking signs of pain, which is likely to affect sensitivity of these behaviours for the diagnosis of pain, possibly leading to false negative results.

Supporting information
S1 Table. Video examples of each perioperative behaviour in rabbits submitted to orthopaedic surgery.
(DOCX)

S2 Table. Median and interquartile range (Q1 –Q3) of behaviours of the 28 rabbits undergoing orthopaedic surgery that did not alter over time or between the presence and absence of observer. Frequency (F) in number of occurrences and duration (D) in seconds in each of the 300 second (five minute) observations of behaviours that were not different over time or between the absence and presence of the observer, observed in the 28 rabbits undergoing orthopaedic surgery in the moments before surgery (Baseline); 1 hour after recovery from anaesthesia (Pain); 4 hours after recovery and 3 hours after rescue analgesia (Analgesia); and 24 hours after recovery from anaesthesia (24h post).
(DOCX)

S1 Data. Data of the ethogram.
(XLSX)

S1 Fig.
(TIF)

S1 Video.
(MP4)

S2 Video.
(MP4)

S3 Video.
(MP4)

S4 Video.
(MP4)

S5 Video.
(MP4)

S6 Video.
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S7 Video.
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S8 Video.
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S9 Video.
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S10 Video.
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S11 Video.
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S49 Video. (MP4)
S50 Video. (MP4)

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