Repeated measurement comparison of different protocols for anesthesia and analgesia consisting of Alfaxalone, Meloxicam, and Butorphanol or Tramadol IM in Leopard Geckos (*Eublepharis macularius*)

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Summary
Ten adult Leopard Geckos (*Eublepharis macularius)* about one year old, seven female and three male, consecutively underwent three different anesthesia protocols, one control protocol and two test protocols, to see if these protocols would give an anesthetic and analgesic effect that is sufficient for painful stimulation from firmly pinching the skin.
First, all animals underwent the control protocol of anesthesia with Alfaxalone (15 mg/kg IM). Then the animals were divided into two groups, and one group was treated with the Butorphanol protocol (Alfaxalone, Meloxicam (1 mg/kg IM) and Butorphanol (2 mg/kg IM)) and the other group was treated with the Tramadol protocol (Alfaxalone, Meloxicam (1 mg/kg IM) and Tramadol (10 mg/kg IM)). At last, both groups were treated with the other, remaining protocol. Between each experiment was a wash-out period of two weeks.
To measure the effect of the different protocols on anesthesia (loss of consciousness and muscle relaxation), the time to loss of righting reflex and palpebral reflex from the moment of injection of Alfaxalone and the duration of the anesthesia from the loss of righting reflex to the moment the animal woke up was compared. For the effect of the protocols on analgesia (loss of pain perception), reaction to painful stimuli (firmly pinching with forceps to simulate cutaneous tissue damage caused by cutting or puncturing the skin for small surgeries) was measured. One animal died after undergoing the control protocol and the Tramadol protocol, thus no information from this animal is available for the Butorphanol protocol. For extra reference, information about the body temperature was collected.
The loss of reflexes was not significantly different between protocols, but use of the Butorphanol protocol, and plausibly also the use of the Tramadol protocol, did significantly increase the anesthetic duration, from approximately 21 minutes for the control protocol to 35 and 31 minutes for respectively the Butorphanol and Tramadol protocol. The number of animals that lost pain perception during the experiment did not differ significantly between protocols (2/10 with the control protocol, 3/9 with the Butorphanol protocol, 4/10 with the Tramadol protocol), the duration of loss of pain ranged from 2,3 to 19,5 minutes and there was no significant difference between protocols. Thus, none of the used protocols is a reliable protocol for analgesia in Leopard Geckos. The fact that the animals could still display reaction to painful stimuli shows that all three protocols, although usable for sedation/immobilization for minor acts like handling or imaging, are not suitable to be used for surgery without additional means to ensure a deeper anesthetic plane in addition to adequate pain relief.
The mean body temperature of the animals was with approximately 32°C on the high side of the preferred range which might cause a shorter and generally lighter anesthesia. It might be interesting to control or monitor this in further research or clinical cases.
More research on analgesia in reptiles is necessary. For further research it is advised to not anesthetize the animals if the goal is to measure pain perception.

Introduction

The demand for save and easy anesthetics and analgesics for reptiles increases. In many reptiles, IV injections are not possible or hard to achieve because of the size of the animals, the possibilities to reach the veins, and in some animals the risk of autotomy. Although used a lot in reptiles[1], the effect of intramuscular injection of ketamine, with or without other agents, varies within and amongst species. Propofol has less side-effects, but has to be administered intravenous and thus its usage is very limited in most reptiles.

Alfaxalone on the other hand can be injected intramuscular.[2] In dogs and cats, it facilitates a smooth induction and recovery and no significant change in heart rate. In cats, there likely is no significant difference in respiratory rate and heart rate between induction with Alfaxalone or Propofol. Often used for induction, its rapid elimination likely prevents accumulation.[3] Thus, Alfaxalone might be a simple, effective and safe anesthetic for reptiles.

Pain is hard to measure and monitor in reptiles, because they often don't show signs that we can easily recognize as being a pain reaction, like signals we recognize in cats and dogs for instance. However, most mechanisms involved in pain perception and transmission seem to be similar to mammals'.[4,5] For this reason, we can and should assume that reptiles do feel pain, and that analgesics during surgery are necessary.

Butorphanol is frequently used as an opioid analgesic in reptiles. However, according to recent studies it appears to have no more effect than saline in reptiles; there were no significant thermal withdrawal latencies in red-eared sliders (*Trachemys scripta elegans*) or bearded dragons (*Pogona vitticeps*)[6,7] or changes in thermal thresholds in green iguanas (*Iguana Ignuana*)[8]. Thus, research into other analgetic possibilities is required.

Tramadol seems to be more promising for use in reptiles, although not much is known about its effects in reptiles. The application route used now is mostly per os.[9,10] Because of its lower affinity, it has the potential for producing fewer mu-opioid-induced adverse effects than morphine, like respiratory depression.[10] Administration per os seems more effective than subcutaneous administration, as it gives higher withdrawal latencies, quicker onset and a longer duration. With per os administration, the drug gets directly delivered to the liver by

Alfaxalone

Alfaxalone is a neuroactive steroid. In the 1980's, Alfaxalone was found to have an enhancing effect on activity of the inhibitory neurotransmitter GABA, reducing the neuronal excitability throughout the nervous system. It provides synaptic inhibition by binding to the fast acting $GABA_A$ receptors in the brain. In 1986, Baker et al. suggested that Alfaxalone works by enhancing the time that GABA-activated chloride (CL^-) channels are opened. Even low doses of Alfaxalone amplify the Cl^- conductance responses elicited by GABA and prolong the GABA-mediated postsynaptic potentials.[3,16-19] Alfaxalone is soluble in water and has an equilibrium between a free fraction and molecules that are bound to plasma proteins and cell membranes. There is a rapid metabolic clearance. Although the exact way of elimination is unknown, the liver and kidneys likely play an important role.[19]

Butorphanol

Butorphanol is a kappa-opioid agonist and partial mu antagonist.[10,12,20] It has low oral availability in mammals. The effect is dose-dependent, but Butorphanol has an effect plateau where increasing the dosage does not increase analgetic efficacy. Increasing the dose does not increase the effect duration, which in mammals is approximately an hour. Butorphanol can be used for mild to moderate pain but is not advised for severe pain or when analgesia is desired for more than one hour.[20] Although reptiles do have opioid receptors and endogenous opioids, their exact role, receptor subtypes and their locations are not completely clear.[5] This makes extrapolating information from mammals to reptiles difficult.

Tramadol

Tramadol has a high oral availability. Tramadol and its metabolite cause analgesia in mammals by non-selective activation of mu-, kappa- and delta-receptors and inhibiting serotonin and norepinephrine reuptake in the central nervous system. It has 6000 times less affinity than morphine, which lowers the risk for mu-induced adverse effects (e.g. respiratory depression). In the liver, tramadol is converted to its way more potent metabolite, O-desmethyl-tramadol. It is excreted by the kidneys.[10-12,20] Just like with Butorphanol, extrapolation of these results should be done with great care, and more research is needed.

the portal vein, leading to quick conversion to its more potent metabolite. With subcutaneous injection, a part of the dose may be excreted by the kidneys before reaching the liver. A maximum safe dosage is not known. A dose of 10 mg/kg PO in red-eared sliders is proven to be effective for up to 48 hours but 25 mg/kg is not clinically safe.[10-12]
However, administration per os is not always achievable or practical, and chances for regurgitation or spilling during administration make it somewhat unreliable. This is why the option of intramuscular administration is interesting and will be used in this research.

To reduce the necessary dose of Alfaxalone and thus reduce possible side-effects of Alfaxalone and Butorphanol like tachypnoea or bradypnoe[2,7,13], it is advisable to keep the body temperature on the lower side of the temperature range for the specific reptile. However, reptiles with a lower body temperature have a longer recovery time, thus increasing the temperature towards the end of a surgery accelerates recovery. The dosage of Alfaxalone that is used also affects the recovery time. A higher dose gives a deeper anesthesia and a longer recovery.[2,13,14] In this study, the temperature was not regulated (although animals were places above a heating pad), but the body temperature of the animals was measured several times during anesthesia (while testing the analgesic protocols) using a thermal camera. This context might be important to understand differences between these and other cases.

At the University of Brno, Czech Republic, the standard protocol for anesthesia and analgesia for small procedures in reptiles is 15 mg/kg Alfaxalone IM, 1 mg/kg Meloxicam IM and 2 mg/kg Butorphanol IM in the front legs. Because of recent research about the efficacy of Butorphanol, this protocol will be compared to a protocol containing Tramadol as replacement for Butorphanol.[6-8]

AIM OF THE STUDY:

"Does the administered dosage of Alfaxalone during surgery on reptiles, in combination with the supplemented Meloxicam and either Butorphanol or Tramadol, give an anesthetic and analgesic effect that is sufficient for painful stimulation from firmly pinching the skin?"

Materials and methods

Materials

Skinmed® chlorhexidin spray (Cymedia (chlorhexidin diacetat 10,84 mg/g))
BBraun Omnican® 100 needles, U-100 Insulin/100 I.U., 1 ml (0,3mmx12mm, 30Gx1/2")
Alfaxan® injection for dogs and cats, Vétoquinol (Alfaxalone 10,0 mg/ml)
Torbugesic® Small Animals and Horse, Pfizer (butorphanoli hydrogenotartas 10mg/ml)
Tramal® injekcí roztok [injection fluid] 50mg/1ml, Stada (tramadoli hydrochloridum 50mg/ml, diffused 1:1 with saline)

Methods

Ten Leopard Geckos (*Eublepharis macularius*) (7 female, 3 male) from about one year old were used. The animals were acquired from a breeder at a few months of age and were taken care of by employees of the University of Brno. In this period they did not display any signals of sickness. As far as known to the author, no additional faecal exam was done. The housing consisted of a cage of $1m^2$ with unlimited water supply and cardboard boxes and rolls for housing. The animals were fed with crickets, mealworms and greens. The crickets and mealworms were gut-loaded with bread, greens, fruit and calcium powder.

First, all animals underwent the control protocol. After a wash-out period of two weeks, the animals were divided into two groups. One group received the Butorphanol protocol, the other group received the Tramadol protocol. After another two week wash-out period, the groups switched protocols, so the group that was earlier treated with the Butorphanol protocol received the Tramadol protocol and vice versa. Thus, we collected data from all animals for the "Control" protocol, the "Butorphanol" protocol and the "Tramadol" protocol.
With the control protocol, the animals received only Alfaxalone (15 mg/kg IM). In the Butorphanol protocol, first the animals received analgesia consisting of Meloxicam (1 mg/kg IM) and Butorphanol (2 mg/kg IM) and after 45 minutes anesthesia with Alfaxalone (15 mg/kg IM). The Tramadol protocol received analgesia consisting of Meloxicam (1 mg/kg IM) and Tramadol (10 mg/kg IM) and after 45 minutes also anesthesia with Alfaxalone (15 mg/kg IM).

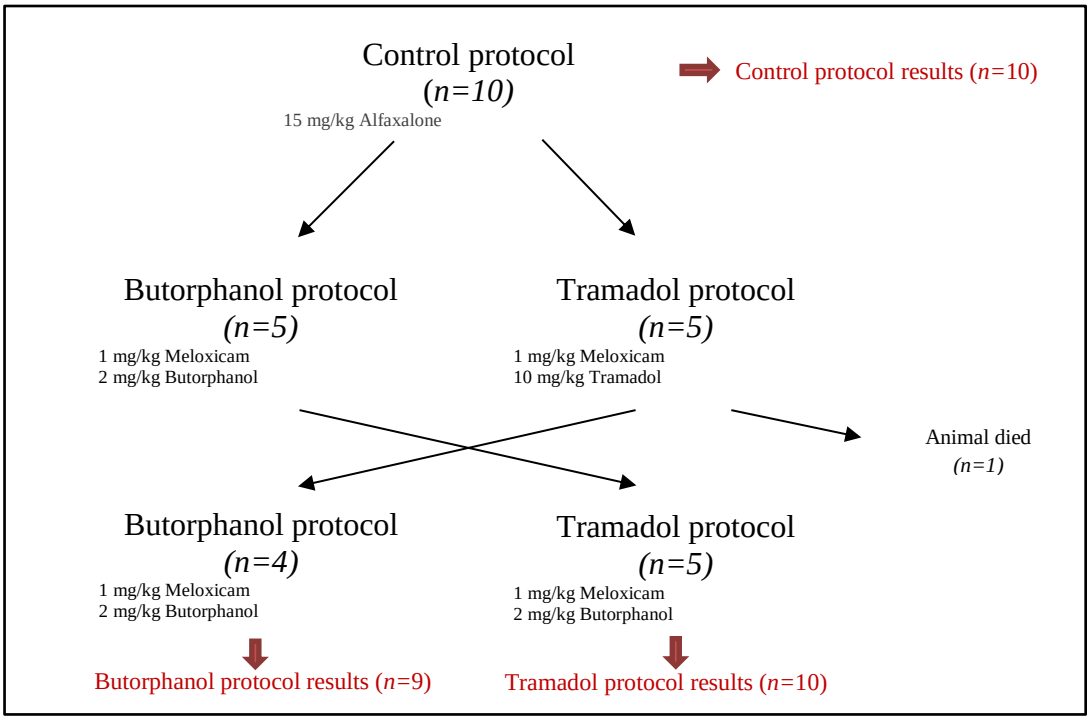

Figure 1: Study design. Ten Leopard Gecko's underwent treatment with the control protocol. After a wash-out period of two weeks, the group was split and received treatment with either the Butorphanol or the Tramadol protocol. After another two weeks, each group received treatment with the last protocol. Between the second and the last period, one animal died, resulting in results from ten animals for the control protocol, from ten animals for the Tramadol protocol and from nine animals for the Butorphanol protocol.

The animals were injected with BBraun Omnican® U-100 Insulin needles (0,3mmx12mm, 30Gx1/2") in the front limbs. Beforehand, the place of injection was disinfected with Skinmed® chlorhexidin spray (Cymedia, chlorhexidin diacetat 10,84 mg/g). Analgesia was delivered in both front limbs, one for Meloxicam and one for the other analgesic. Alfaxalone was injected in the left front limb, except when that seemed unfit (e.g. in case of subcutaneous bleeding). In the time between handling, the animals were placed in their plastic transport boxes underneath a cloth, to keep the stress as minimal as possible.
When the animal was anesthetized (measured by checking the righting reflex), they were placed on top of two foam blocks that were placed on top of a heat pad, with the idea that the warm air around the animal might slow cooling of the animal.

To measure the effect of these protocols on anesthesia (in this case loss of consciousness and muscle relaxation, not including analgesia), initially three reflexes (body posture, righting reflex and palpebral reflex) and the duration of the anesthesia were tested. The time after administering Alfaxalone at which body posture, righting reflex and palpebral reflex were lost, was written down. Losing body posture was marked when the animal couldn't hold the head high and rested the chest on the table. However, from experience during the experiment, it appeared that body posture loss was very hard to measure, often went unnoticed or was manipulated by the handling of the animal to test the righting reflex. Animals often had a muscle tone that held the head high, but as soon as they were manipulated, this muscle tone was gone. Also, for a lot of animals the moment of loss could not be determined, because they lost their righting reflex and thus would lay on their back, at which point the body posture was not measurable anymore. The loss of body posture was considered unreliable and is not used in the statistical analysis. The righting reflex was lost when the animal, when turned on its back by a researcher, did not make an effort to turn around to get back on the feet. This was done approximately every 1-2 minutes. Palpebral reflex was measured by softly touching the eyelid from above with a small metal rod, while being careful that the animal did not see the rod. This was done as often as possible to be as close to the actual time of loss as possible. The duration of the anesthesia was calculated by measuring the time between loss of the last reflex (loss of righting

Control protocol: Alfaxalone (15 mg/kg IM)
Butorphanol protocol: Alfaxalone (15 mg/kg IM), after 45 minutes followed by Meloxicam (1 mg/kg IM) and Butorphanol (2 mg/kg IM)
Tramadol protocol: Alfaxalone (15 mg/kg IM), after 45 minutes followed by Meloxicam (1 mg/kg IM) and Tramadol (10 mg/kg IM)

reflex or palpebral reflex) and the moment they responded again, for example if the reflexes returned or the animal began to move.
To measure the effects of the analgesia, the animals were pinched with small forceps in between the toes, in the skin on the belly and just behind the hind limbs in the lateral part of the tail. Careful observation was needed to check if the stimulus was high enough to not only trigger the skin-pinch reflex and the toe-pinch reflex, but also elicit another reaction that could be classified as a reaction to pain (e.g. sharply retracting the leg). With every animal, the same person was present and supervising this action to try and standardize the stimulus.
When the animal recovered the righting reflex or otherwise gave the impression to be awake ("response", for example showing convincing movements like walking movements, or blinking), the experiment was ended and the animal would be placed back in the plastic box and later in the own terrarium.
During the anesthesia, the body temperature of the animals was measured with a thermal camera, as a non-invasive way to monitor the animals. This was done in the context of another study by Simona Rusu. Some of her results are obtained to give extra information about the circumstances for this experiment. The temperature just before anesthesia, after ten to fifteen minutes and just before or after the animal woke up is given as a mean with the upper and lower limit. If more than one measurement was made during the ten-to-fifteen period, the average would be used. If no data were obtained during this period, the data was listed as 'missing' and will not be taken into account in the calculation.

In between the two analgesia protocols, one animal died and because of that, did not undergo the Butorphanol protocol. The animal became anorectic and quickly lost weight, and attempts to manually feed it did not have the desired effect. No underlying disease could be established and the cause of death could not be determined.

Statistics
To test for the effects of the anesthesia and the interaction with the different analgesia, the time at which the righting reflex and the palpebral reflex were lost, was compared between protocols. The duration of anesthesia was also compared, with the time starting at the moment of loss of the righting reflex and ending at the time of response (as described above). To analyse the data, repeated measurements ANOVA, paired T-tests and the non-parametric Friedman test were done. Repeated measurements ANOVA and paired T-test both assume normality of the data but because of the small group sizes, normality is hard to determine. To compensate for this, the non-parametric Friedman test was also conducted. If the results were significant, follow-up tests were conducted. For the Friedman test the Wilcoxon test was used. Because of the small group size, post hoc tests for repeated measurement ANOVA's are to be interpreted with care, and thus in this case are not used. The different tests were compared to each other to get a good indication about the effects of the different analgesics on the anesthesia.

To test the differences between the effects of analgesia for the three protocols, first, a McNemar test was carried out on the data. The data were made binary: when the animal did not react to painful stimuli during some time of the experiment they were considered to have lost pain perception, the animals that did not stop reacting did not lose pain perception. Second, the onset and duration of loss of pain perception was tested. For interpreting the differences in onset time and duration between protocols, only the results from animals that lost pain perception were used, and because of that, all results are used as if from individual animals. Results were compared with a one-way ANOVA.
Because the temperature of the animals was only measured and not managed, only descriptive details are given about the temperature during the anesthesia and no further statistics have been conducted to differentiate

between protocols. A repeated measurement ANOVA was used to check for differences over time.
P-values < .05 were considered significant, except for the T-test and the Wilcoxon test where a Bonferroni correction was used and consequently p-values of < .018 were considered significant. Results are presented as MEAN +/- SD.
If necessary and possible, cases are excluded case-wise because of the limited amount of data.

Results

Loss of righting reflex

The Kolmogorov-Smirnov test indicated an approximately normal distribution after a natural log transformation and the sphericity has not been violated according to Mauchly's test. The time to the loss of righting reflex was not significantly affected by the different treatments and no significant correlation existed between protocols (see Table 1, 2). The mean time to loss of righting reflex was 134,30 +/-23,542 seconds (n=10) for the control protocol and 140,67 +/- 22,542 (n=9) and 221,10 +/- 71,711 seconds (n=10) for the Butorphanol and the Tramadol protocols respectively (Figure 2).

Loss of palpebral reflex

A normal distribution can be assumed and the sphericity has not been violated. The time to loss of palpebral reflex was not significantly affected by the different treatments and no significant correlation existed between protocols (Table 1, 2). The mean time to loss of palpebral reflex was 377,60 +/- 61,203 (n=10), 470,33 +/- 81,609 (n=9) and 432,80 +/- 54,803 seconds (n=10) for the control, Butorphanol and Tramadol protocol respectively (Figure 3).

Duration of anesthesia

A normal distribution can be assumed and the sphericity has not been violated. The duration of the anesthesia was significantly affected by the different protocols, calculated with the repeated measurement ANOVA, the Friedman test and the T-test (Table 1, 2). With the T-tests and Wilcoxon tests (Table 3), the difference between the control protocol and the Butorphanol protocol is significant and the difference between the Butorphanol protocol and the Tramadol protocol is not (with p< .018 after Bonferroni correction). However, the correlation between the control protocol and the Tramadol protocol is technically not significant when using the T-test (p= .018, Table 2) but significant when using the Wilcoxon signed-rank test (p= .017, Table 3). Taking into account that the Bonferroni correction is very conservative, and thus increases the chances of a Type II error (further explained in the discussion), it is plausible that this difference is also significant.
Mean anesthesia time was 1234,10 +/- 96,507, 2115,89 +/- 208,004 and 1886,80 +/- 226,717 seconds (approximately 21, 35 and 31 minutes) for the control, Butorphanol and Tramadol protocols respectively (Figure 4).

	Repeated measurement ANOVA		Friedman test	
Time to loss of righting reflex	F(2,16)= 0.835	p= 0.452	$chi^2(2)$ = 0.889	p= 0.641
Time to loss of palpebral reflex	F(2,16)= 0.381	p= 0.689	$chi^2(2)$ = 0.743	p= 0.690
Duration of anesthesia	F(2, 16)= 7.748	p= **0.004**	$chi^2(2)$ = 11,556	p= **0.003**

Table 1: Effect of treatment on the parameters measured for anesthesia, results from the repeated measurements ANOVA (normality assumed) and Friedman test (normality not assumed). With both tests, the time to loss of righting reflex and palpebral reflex did not differ significantly. The duration of anesthesia is significantly different between the three protocols (p<0.05), although these tests do not specify between which protocols the significant difference exists (See Table 2).

	Control to Butorphanol		Control to Tramadol		Butorphanol to Tramadol	
Time to loss of righting reflex	$t(8)=$ 0.022	$p=$ 0.983	$t(9)=$ -1.199	$p=$ 0.261	$t(8)=$ -1.361	$p=$ 0.211
Time to loss of palpebral reflex	$t(8)=$ -0.952	$p=$ 0.369	$t(9)=$ -0.780	$p=$ 0.456	$t(8)=$ 0.162	$p=$ 0.875
Duration of anesthesia	$t(8)=$ -5.010	$p=$ **0.001**	$t(9)=$ -2.899	$p=$ **0.018**	$t(8)=$ 1.257	$p=$ 0.244

Table 2: Effect of treatment on the parameters measured for anesthesia, results from the T-tests. A T-test compares two protocols and checks for a significant difference. Similar to the results from the ANOVA and the Friedman test (see Table 1), no significant difference was found between all protocols concerning Time to loss of righting reflex and palpebral reflex. There is a significant difference between the control and Butorphanol protocols for duration of anesthesia and no significant difference between the Butorphanol and Tramadol protocols. The difference between the control and Tramadol protocols is technically not significant when a Bonferroni correction is used (significance when $p<0.018$). However, the Bonferroni correction is very conservative and one can argue that the difference between the control and Tramadol protocol is also significant.

Control to Butorphanol	Control to Tramadol	Butorphanol to Tramadol
Z= -2.666 **0.008**	Z= -2.395 **0.017**	Z= -1.125 0.260

Table 3: Result of Wilcoxon Signed Ranks Test for duration of anesthesia. With the Wilcoxon Signed Ranks Test, there is no significant difference between the Butorphanol and the Tramadol protocols. The differences between the control and the Butorphanol protocols and between the control and Tramadol protocols are significant ($p<0.018$ after Bonferroni correction).

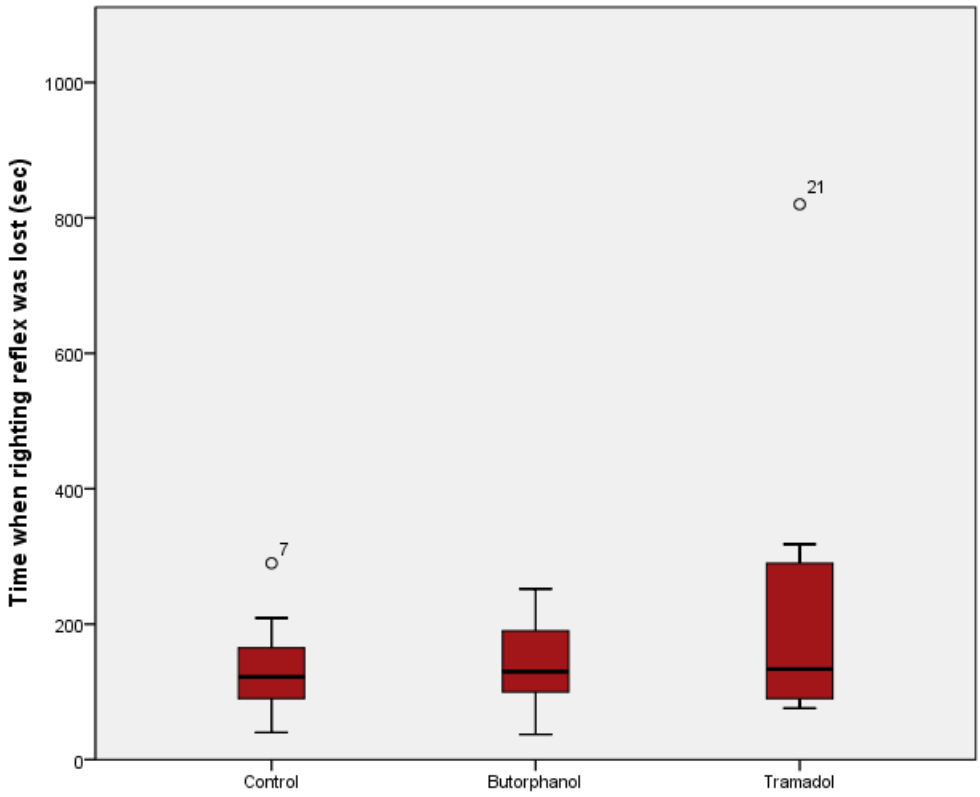

Figure 2: Boxplot showing the time to loss of righting reflex after injection with Alfaxalone. There is no significant difference between protocols.

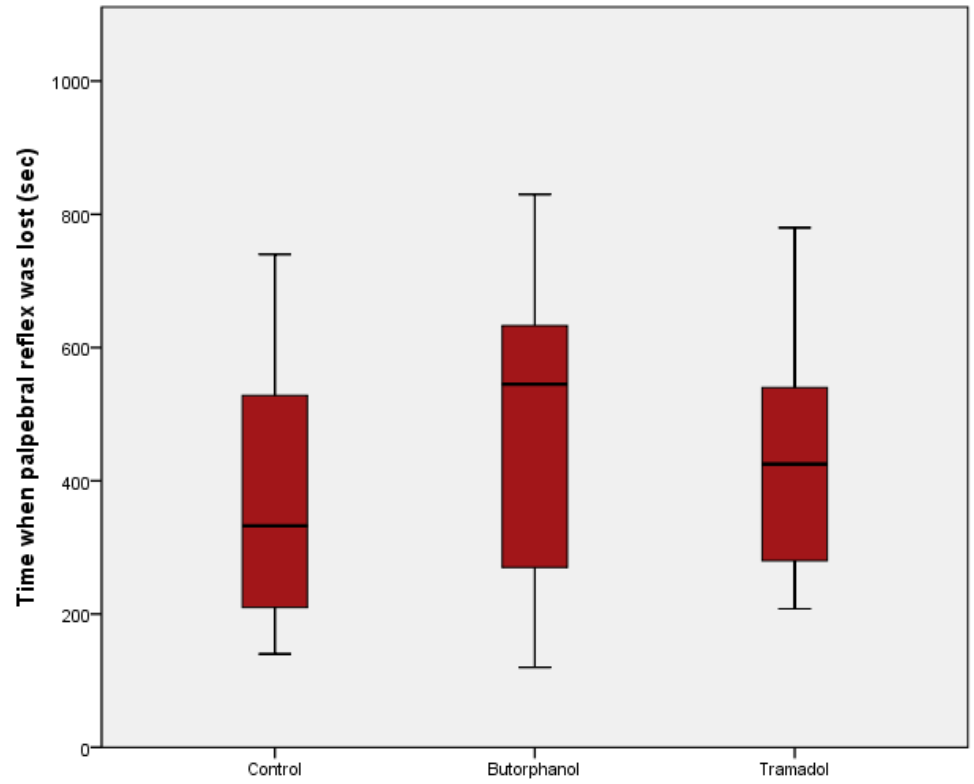

Figure 3: Boxplot showing the time to loss of palpebral reflex after injection with Alfaxalone. There is no significant difference between protocols.

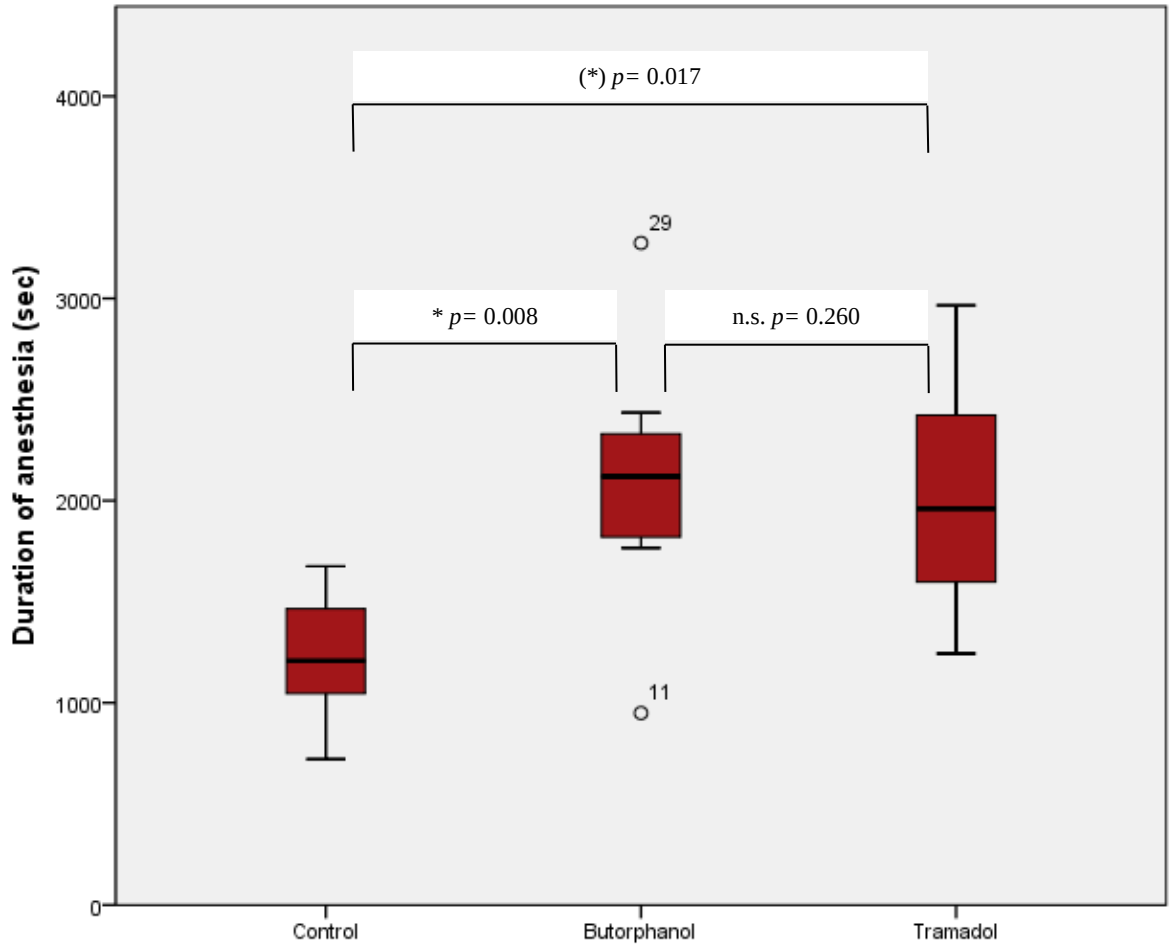

Figure 4: Boxplot showing the duration of anesthesia in seconds. P-values in this image are derived from the Wilcoxon test. With the Bonferroni correction, $p<0.018$ is considered significant. The difference between the Butorphanol and the control protocol is significant, the difference between the Butorphanol and the Tramadol protocol is not. According to the Wilcoxon test, the difference between the Tramadol and the control protocol is significant. However, results from the T-test suggest this difference is not significant (but just barely, $p= 0.018$). Taking into account that the Bonferroni correction is very conservative, the difference is probably significant.

Analgesia

An exact McNemar's test determined that there was no statistically significant difference in the proportion of animals that lost pain perception during the anesthesia between the control and the Butorphanol protocols, the control and the Tramadol protocols and the Butorphanol and the Tramadol protocols ($p= .625$, $n=9$, $p= .625$, $n=10$ and $p= 1.000$, $n=9$ respectively, Figure 5).

For the duration of the loss of pain perception, only data of animals that lost pain perception are used and because just two animals lost pain perception in two instances (Table 4), data are used as if from a non-repeated design.

Animal	Control	Butorphanol	Tramadol
I	No	Yes	No
II	No	No	Yes
III	No	Yes	Yes
IV	No	No	Yes
V	No	No	No
VI	Yes	No	No
VII	No	No	No
VIII	No	No	No
IX	Yes	-	Yes
X	No	Yes	No

Table 4: Information about loss of pain from individual animals. "Yes" means the animal lost pain sensation during any amount of time during the test, "No" means they did not. If an animal lost pain perception seems completely random. Only two animals lost pain perception in more than one instance. Animal IX died between undergoing the Tramadol and the Butorphanol protocol, so no information is available for the Butorphanol protocol for this animal.

The time during which the animals lost pain perception, if they did, ranged from 140 seconds (2,3 minutes) to 1170 seconds (19,5 minutes) with a mean of 492,50 seconds for the control protocol (8,2 min, *n*=2), 678,33 seconds for the Butorphanol protocol (11,3 min, *n*=3) and 673,75 seconds for the Tramadol protocol (11,2 min, *n*=4), and no significant difference was found between protocols (F(2,6)= .264, *p*= .777) (Figure 6). The onset of the loss of pain perception was between one to twelve minutes after administration of Alfaxalone with no significant difference between protocols (F(2,6)= 2,059, *p*= .209).

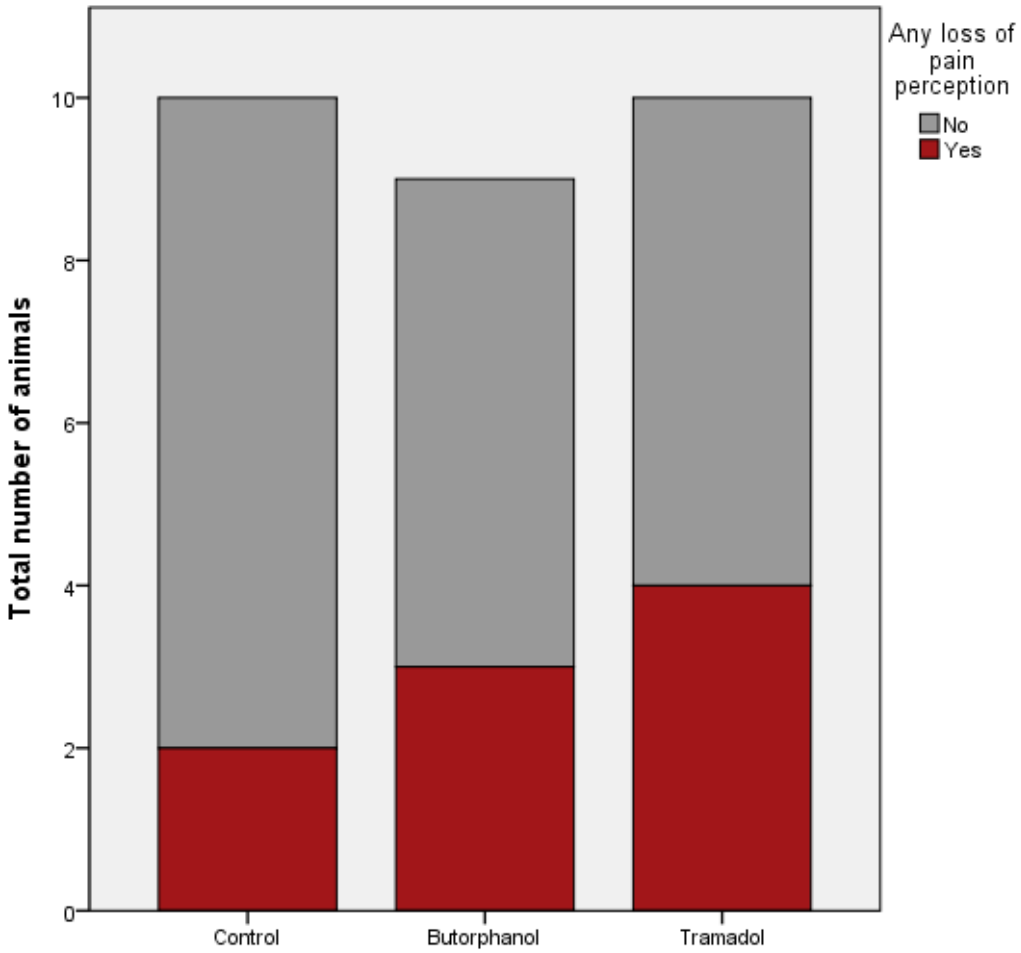

Figure 5: Binary illustration of loss of pain perception during anesthesia. For each protocol is shown how much animals have lost pain perception for any amount of time during the anesthesia (red) and how many animals did not lose pain perception at all (grey). There is no significant difference between protocols, and it is obvious that none of the protocols has reliable analgesic properties in the way that it is used now.

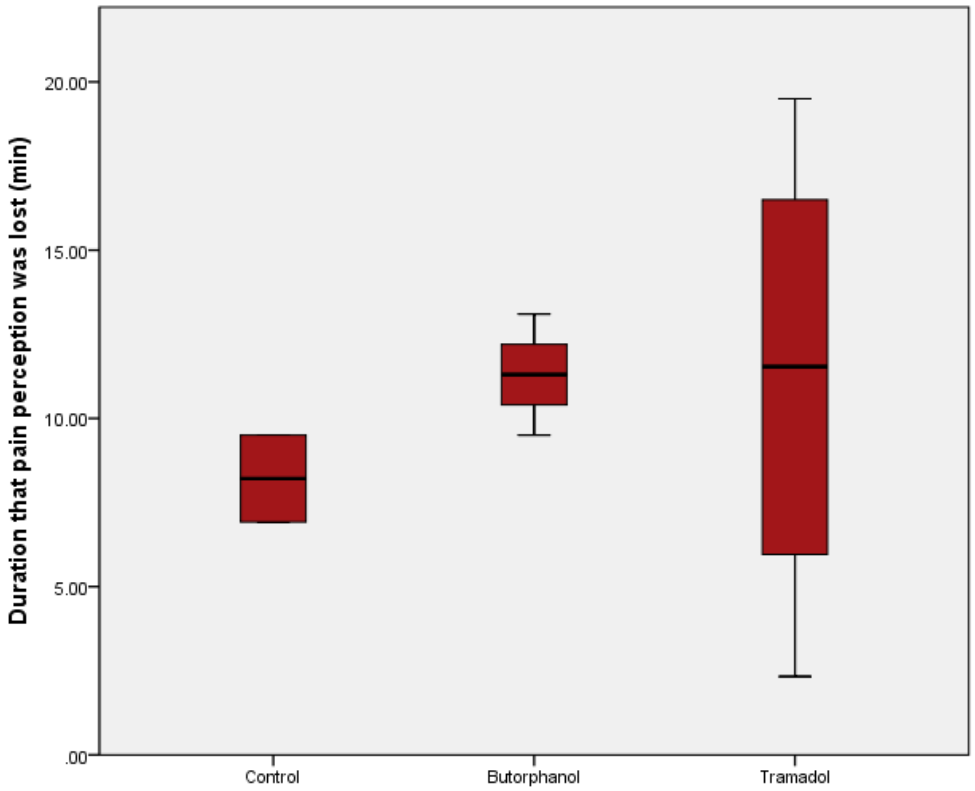

Figure 6: Duration that pain perception was lost in minutes. The time ranged from 2,3 to 19,5 minutes and there was no significant difference between the protocols.

Temperature

The body temperature, measured with a thermal camera, was only measured, not managed, except for the heat pad under the block of foam on which the animals were lying. The body temperature was not measured in the control protocol (equipment was not available at the time) and no distinction between the other two protocols could be made because of the way this information was provided. Room temperature was not measured, but is estimated to have been around or just above 25°C. The first measurement was made just before injection with Alfaxalone. The last measurement was made just before or after the animals woke up, which was on average 36 minutes after injection of Alfaxalone (ranging from 19 to 56 minutes). Body temperature of the animals at the start of the anesthesia ranged from 26,6 to 36,4°C, with a mean of 31,9°C. Between 10-15 minutes, it ranged from 29,0 to 35,2°C with a mean of 31,8°C, and at the last measurement it ranged from 30,3 to 33,1°C with a mean of 31,6°C. (Table 5, Figure 7)

No significant difference between these points in time was discovered using a repeated measurement ANOVA ($F(1,321)= .069$, $p= .933$).

	N	Minimum	Maximum	Mean	Std. Deviation
Start	19	26,60	36,40	31,8526	2,65861
10-15 minutes	17	29,00	35,20	31,8441	1,24661
End	19	30,30	33,10	31,6263	,79989

Table 5: Descriptive statistics about the body temperature, measured by infra-red camera. The data from all animals during the two analgesia protocols is used (9 animals for the Butorphanol protocol, 10 animals for the Tramadol protocol). For two animals, no information was available for the 10-15 minute mark. The first measurement was made just before injection with Alfaxalone. The last measurement was made just before or after the animals woke up, which was on average 36 minutes after injection of Alfaxalone (ranging from 19 to 56 minutes). No significant difference was found between these moments.

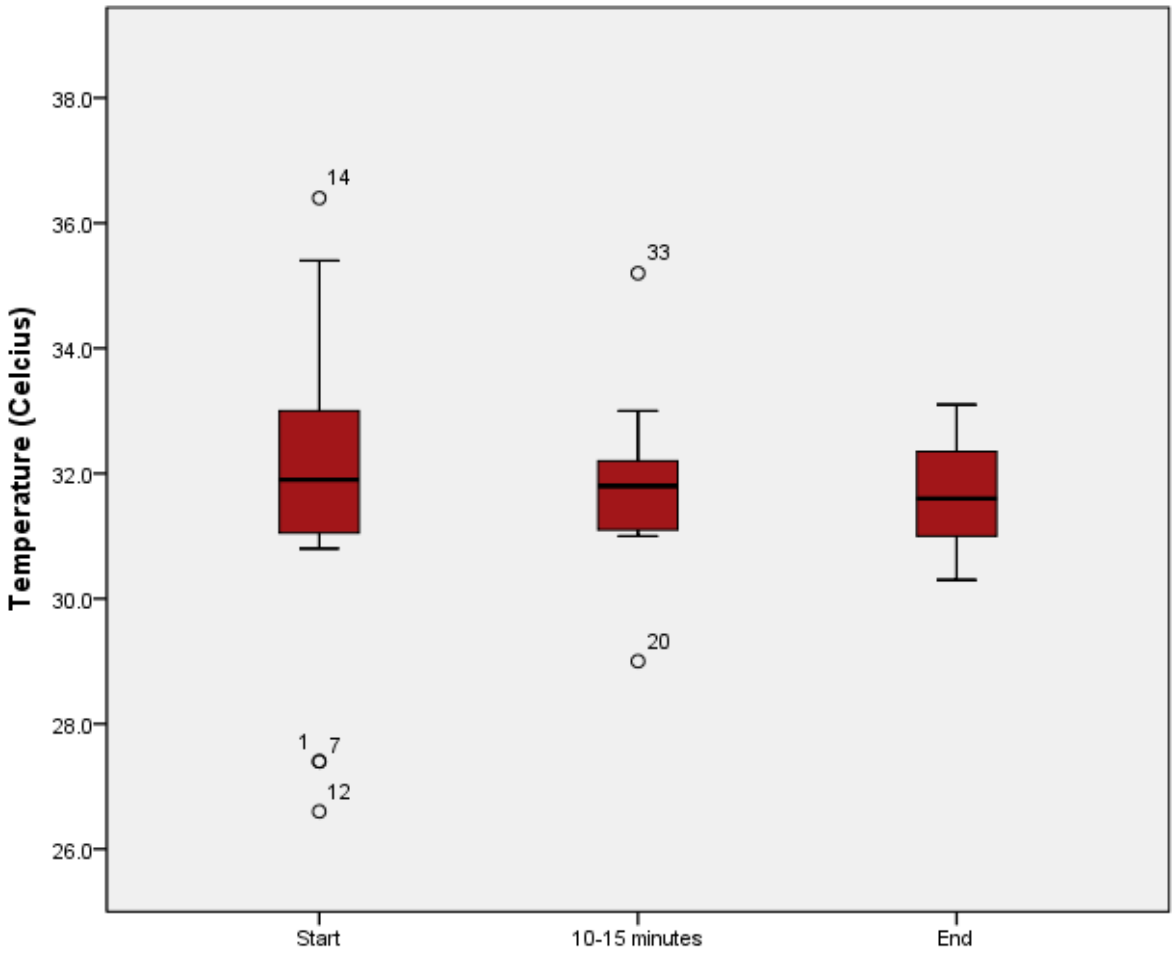

Figure 7: Body temperature during the test, measured with a thermal camera. This was measured while using the Butorphanol and Tramadol protocols (not the control protocol, equipment was not available during that time). No distinction between the protocols was made. Room temperature was estimated around 25 degrees Celcius. Body temperature of the animals at the start of the anesthesia ranged from 26,6 to 36,4°C, with a mean of 31,9°C. Between 10-15 minutes, it ranged from 29,0 to 35,2°C with a mean of 31,8°C, and at the last measurement it ranged from 30,3 to 33,1°C with a mean of 31,6°C. No significant difference was found between these points in time.

Discussion

These animals were collected from a breeder, and their background was largely unknown. The animals were housed in group housing, which is not necessary in Leopard Geckos and makes observing individuals harder. The geckos were placed in their respective protocol groups by one person, based on which protocol was first and which animals were least shy/easiest to grab. This should not have an effect in this case because all animals underwent all protocols, but in general it is not a good way to make groups. The painful stimulus was applied by hand with a forceps, which makes the force and thus the stimulus that is used hard to estimate and standardize. More standardization and complete, blind, randomization would make experiments like these more reliable to draw conclusions from.

In this experiment, the time to the loss of the palpebral and the righting reflex is not significantly influenced by the additional use of an analgesic combination of either Meloxicam and Butorphanol or Meloxicam and Tramadol. In mammals, opioids tend to have a (slight) sedative effect, but this was not noticed in these Leopard Geckos. The duration of the anesthesia however is prolonged by the use of the Butorphanol protocol, and plausibly also by the Tramadol protocol. While using the Bonferroni correction (significance when $p < 0.018$ in this case), the results are barely significant with a Wilcoxon test ($p = 0.017$) and technically not significant with a T-test ($p = 0.018$). A Bonferroni correction is used to compensate for the increased chance of Type I errors (falsely rejecting the null-hypothesis and thus declaring a significant result when there is none) when testing multiple hypotheses. However, the use of a Bonferroni correction is very conservative, making it more likely to have Type II errors (missing a significant difference and not rejecting the null-hypothesis). Because of that, the Bonferroni correction is associated with a loss of statistical power. When taking this into account, one can argue that it is very plausible that the difference between the control protocol and the Tramadol protocol for the duration of the anesthesia is significant, and that the Tramadol protocol also has a prolonging effect on the anesthetic duration.

One very important note about the anesthetic effects of these protocols in general however, is that animals that are under sufficiently deep anesthesia for chirurgic manipulation, even without any analgesia, should not be able to react to external incentives or display reflexes like the skin pinch reflex. The fact that these animals still could display reaction to pinching of the skin, shows that all three protocols are not suitable to be used for surgery without additional means to ensure a deeper anesthetic plane. These protocols are usable for sedation/immobilization for minor acts like handling or imaging, but for more invasive acts, additional measures should be taken. For example, use of isoflurane after induction with Alfaxalone is be an option, or increasing the dosage of Alfaxalone could be investigated.

Most research done for pain perception in reptiles is done by thermal withdrawal. However, the pain that would be expected during surgery would be a very different type of pain than thermal pain. Because of that, scratching, cutting or pinching (as is done in this experiment), although more invasive, seems to be a more suitable way to measure this pain. However, during this experiment, it became clear that the reactions to skin and toe reflexes are hard to distinguish in anesthetized animals. At the very least, one constant observer with an experienced eye is important.

No statistically significant difference in proportion of animals that lost pain perception and the duration of that loss of pain perception was found when using the protocols with analgesia, compared to the control protocol. Besides, the animals that lost pain perception, did so in a very irregular and hard to predict way (during 2,3 to 19,5 minutes). Moreover, it could be that there was no analgesia altogether, but that the measured effect was due to a deepening in the aesthetic plane by Alfaxalone which made it so the animals could not react to the stimulus. Comparing these protocols, the protocols with additional analgesia are not adequate for analgesia for painful stimulation inflicted by pinching the skin with a forceps in Leopard Geckos. This does however not mean that Butorphanol and Tramadol do not work in Leopard Geckos. Most probable, the dose or route of administration should be changed. For instance, injection of Tramadol in the hind limb of yellow-bellied slider turtles (*Trachemys scripta scripta*) provides a 30% higher production of the active metabolite than

compared to injection in the front limb, possibly because the injected Tramadol gets to the liver quicker via the portal vein. This article also describes that the maximum effect was reached after 24 hours.[12] And although slider turtles might be significantly different from Leopard Geckos, it might be a promising possibility to check if Tramadol should be injected in the hind limb, maybe even the day before, or if it is more suited for use in the long term. A combination of Butorphanol and Tramadol or other analgesia might also be interesting for further research. Another possibility for the non-significant effects on pain perception measured in this article, is that this experiment was not adequate to measure these effects. The administration of the Alfaxalone could have interfered with the reaction to the stimuli, or the stimulus itself had a variable intensity that we did not monitor for. At the very least, more research is necessary to form a protocol with adequate and reliable analgesia.

The mean body temperature during the experiments is around 31,8°C. The spread in measurements is quite high at the start (lowest measured temperature being 26,6°C and highest being 36,4°C) but the range becomes smaller towards the end. The preferred temperature range of Leopard Geckos ranges from 25 to 35°C.[15] The mean temperature of the animals during this experiment is just below 32°C, which means that the agents could be eliminated quicker than when the animals would be at a lower temperature and this might influence the duration of the anesthesia to be shorter and generally lighter. The wide range in the beginning could be a factor in the differences between animals in other aspects. In this experiment, the duration of the anesthesia was not bound to the duration of a surgical procedure and thus the length of the anesthesia was not as important, but in cases where it is, further research or control of the temperature might be important to ensure an anesthesia that is as favorable as possible during surgery. It is also important to be aware of and/or control the body temperature in further research with reptiles, because this can influence the results.

Conclusions

The use of a protocol with an analgesic in addition to Alfaxalone has effect on the duration of the anesthesia. Animals who receive Meloxicam and Butorphanol sleep significantly longer than with the control protocol. It is plausible that this is also the case for the protocol with Meloxicam and Tramadol.
For both the protocol with Meloxicam and Butorphanol and the one with Meloxicam and Tramadol in these dosages, there is no significant effect in the number of animals who lost pain perception during the test in comparison to animals who received no analgesic. Animals who did loose pain perception, did so during 2,3 to 19,5 minutes, making none of the used protocols a reliable protocol for analgesia in Leopard Geckos.
The temperature of the animals did not change significantly over time, with a mean around 31,8°C, which might be relevant for interpreting results in comparison to other studies.

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