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Comparison of Thermal and Mechanical Noxious Stimuli for Testing Analgesics in White's Tree Frogs (*Litoria caerulea*) and Northern Leopard Frogs (*Lithobates pipiens*)

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Determining the clinical efficacy of analgesic drugs in amphibians can be particularly challenging. The current study investigated whether a thermal nociceptive stimulus is useful for the evaluation of analgesic drugs in 2 amphibian species. The objectives of this study were 2-fold: 1) compare 2 models of nociception (thermal and mechanical) using 2 frog species; White's Tree Frogs (*Litoria caerulea*; WTF) and Northern Leopard Frogs (*Lithobates pipiens*; NLF) after administration of saline or morphine sulfate; and 2) evaluate antinociceptive efficacy of morphine sulfate at 2 doses in a common amphibian research species, the NLF, using a mechanical stimulus. Neither WTF nor NLF displayed consistent drug-dependent changes in withdrawal responses to a noxious thermal stimulus applied using the Hargreaves apparatus, but NLF exposed to the noxious mechanical stimulus demonstrated a significant dose-dependent antinociceptive response to morphine sulfate. These results indicate that morphine is not antinociceptive in WTF, supporting previously reported results, and demonstrate the importance of using an appropriate experimental antinociceptive test in amphibians. Our data suggest that nociception in amphibian species may be best evaluated by using mechanical nociceptive models, although species differences must also be considered.

Abbreviations: AAT, acetic acid test; NLF, Northern leopard frogs; PVC, polyvinyl chloride; vFF, von Frey filaments; WTF, White's tree frogs; SC, subcutaneous

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Little is known about clinical analgesia for amphibians, indicating the need for validated approaches for the evaluation of pain and analgesic efficacy in these species. While systematically-derived data are sparse, 2 tests have been used in amphibian research: the acetic acid test (AAT) and, less commonly, the noxious thermal limb withdrawal test using the Hargreaves apparatus.[2,12] Amphibian antinociception is most commonly studied in *Rana pipiens* (now *Lithobates pipiens*) and *Xenopus laevis* due to their frequent use in biomedical research.[4,5,7,12] Most published amphibian nociceptive data are derived from a single laboratory that used the AAT in Northern Leopard Frogs (NLF); a wide variety of opioids, nonsteroidal anti-inflammatory drugs, and other agents were administered to the frogs to determine drug efficacy.[12-14] However, the AAT causes tissue damage and inflammation and is associated with infection, sepsis, and death.[2] Thus, safer methods of evaluating pain in amphibians are needed. The Hargreaves apparatus and von Frey filament tests are possible alternatives to the AAT.[6]

Mechanical nociceptive tests, such as the use of von Frey filaments (vFF), cause much less tissue damage than does the acetic acid wipe test. These filaments are used to test nociception in many mammalian species; application of these filaments exerts a specific amount of pressure, based on Euler's buckling law of physics.[3] When applying vFF to a limb, a positive nociceptive response occurs if the animal exhibits a withdrawal reaction. To date, only 2 amphibian pain studies have performed using vFF, including one study evaluating the efficacy of bath sedation with butorphanol-morphine-alfaxalone combinations in fire-bellied toads (*Bombina orientalis*)[1] and another evaluating morphine sulfate and dexmedetomidine analgesic effects in NLF (*Lithobates pipiens)*.[13] Other than these publications, the use of vFF is largely absent within amphibian research. Similarly, only 2 studies used the Hargreaves apparatus in amphibians, with mostly disappointing results.[2,7]

The objective of this study was to compare the Hargreaves and modified vFF methods in a common research amphibian species, the NLF (*Lithobates pipiens*) and a common pet amphibian species, the White's Tree Frog, WTF (*Litoria caerulea*). This objective was developed based on a companion study in which we were unable to document analgesic efficacy of morphine or tramadol in WTF using the Hargreaves apparatus.[7] In the current study, our hypothesis was that morphine would provide measurable, dose-dependent antinociception in both NLF and WTF when assessed using either of the 2 nociceptive tests.

Materials and Methods

Animals. Young adult, captive-bred NLF of unidentified sex were obtained from a commercial supplier (Niles Biologic, Sacramento, CA); young adult, captive-bred WTF of unidentified sex were obtained from a reptile retailer (Reptile Rapture, Monona, WI). For convenience, the NLF were housed in pairs

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in 10-gallon glass aquariums, while the WTF were housed individually in 2-gallon clear plastic enclosures. The WTF enclosures were transparent amber-tinted plastic with a floor space of approximately 143 cm^2 per frog (measuring 10 3/4″W × 19 1/4″D × 10 3/4″H). Enclosures for the NLF were 10-gallon glass aquariums (12.25″L × 10.5″W × 12.57″H) with a floor space of 685 cm^2 per frog.

Both species had ad libitum access to dechlorinated water; housing included artificial foliage and a hide structure for the WTF and large, natural-looking water basins for the NLF. Frogs were exposed to a UVB spectrum light (Reptisun UVB310 tube light Zoo Med Laboratories, San Luis Obispo, CA) on a 12:12 h light:dark cycle. Temperatures in the room ranged from 26 to 30 °C, and temperatures in the laboratory where the 2-wk acclimation period, experiment 1, and experiment 2 were performed ranged from 25 to 27 °C. Both species received either crickets, mealworms, or soldier fly larvae, or a combination of these for daily feeding. Insects were maintained on invertebrate diets (Fluker Farms Hi-Cal cricket diet and Orange cube cricket diet, Port Allen, LA) before being fed to the frogs. Physical examinations were performed on entry into the study, and all frogs were treated with a single dose of oral fenbendazole (100 mg/kg; Fenbendazole 10% suspension, Intervet Millsboro, DE 19966) as an anti-parasitic preventative measure. Frogs were weighed each week during the experiment. All protocols and procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison, School of Veterinary Medicine (IACUC V005683).

Experiment 1. WTF ($n = 6$) and NLF ($n = 6$) were randomized to receive either subcutaneous injections of either morphine sulfate (50 mg/kg) or saline (Morphine sulfate Inj., USP 50 mg/mL, Hospira, Lake Forest, IL 60045) (0.9% Sodium Chloride Injection, USP, Hospira, Lake Forest, IL 60045) via a random number generator in a complete cross-over experimental design, with the observer blind to treatment. The Hargreaves apparatus and vFF were used based on availability in the laboratory, with both used for 3 consecutive weeks in a row to allow for 1 wk washout periods. A standard Hargreaves apparatus[6] was used; the thermal component was placed just below a row of 3 acrylic compartments (8.75 in W × 6.75 in L × 5.5 in D). Subjects were acclimated to the observer and the apparatus for a minimum of 2 wk prior to any experiment. Typically, 3 frogs were placed in the Hargreaves apparatus, each in separate containers, and allowed to explore their surroundings while the observer was present and mimicked the movements used during the actual experimental process. Frogs could not see one another but could see the observer. During the experiment, all data collection started between 0700 and 0900. Frogs were given 5 min for acclimation to the device, in a manner identical to the 2-wk acclimation period, before recording the baseline data. After the 5-min acclimation period, a series of 3 baseline measurements were taken per frog, with approximately 5 min between measurements. For these measurements, the thermal component was placed directly beneath the plantar surface of either right or left distal hind limb, specifically the metatarsus or phalanges. The maximum threshold temperature of the noxious thermal stimulus was approximately 45.2 °C. Consecutive measurements were alternated between right and left hindlimb whenever possible. The apparatus automatically measured the time latency between contact with the thermal stimulus and frog limb withdrawal. The mean of the 3 baseline data points determined a final cumulative mean withdrawal latency. After baseline data were collected, a blind observer injected either morphine (50 mg/kg) or an equivalent volume of physiologic saline (0.9%) subcutaneously on the dorsum in the cranial half of the body under manual restraint, and the same procedure for collecting hind limb withdrawal latencies was performed at 1, 3, 6, and 24 h after the injection. A one-week washout period was permitted between trials. An identical data collection process was used for the vFF apparatus portion of Experiment 1. Details of the vFF method are described below in Experiment 2 and directly apply to Experiment 1.

Experiment 2. NLF ($n = 12$) were used to evaluate the analgesic efficacy of morphine sulfate at 50 mg/kg and 100 mg/kg as compared with saline, using the same products and injection method as used in Experiment 1. A vFF assessment was conducted in a complete crossover, observer-blind design. An apparatus for vFF application was designed with a series of small acrylic compartments (2.25 in W × 3.75 in L × 3.25 in D) raised up 18 in on a supporting stand made of PVC. The bottom of the compartments consisted of a padded, wire mesh surface to reduce skin abrasions and permit vFF access. Frogs could see conspecifics in the adjacent compartments and the observer. Frogs were acclimated to the apparatus and observer for a minimum of 2 wk prior to the start of the experiment. During the experiment, the frogs were given a 5-min acclimation period after placement in the compartment, followed by collection of 3 baseline measurements, with approximately 5 min between measurements. For these measurements, a 4.74 vFF (6.0 g) (20-piece Touch Test Kit, North Coast Medical, 780 Jarvis Drive, Suite 100, Morgan Hill, CA 95037) was applied to the caudal ventrum or plantar surface of the hind limb using a mirror for better visualization. If flinching or movement away from the stimuli occurred before the vFF bent, the response was classified as a "0". If movement occurred after the applied filament was bent or if no flinching or movement occurred at all, the response was classified as a "1". The 3 baseline measurements were averaged for a cumulative evaluation of vFF sensitivity. After obtaining the baseline data, a blind observer administered either saline or morphine (50 mg/kg or 100 mg/kg) subcutaneously on the dorsum in the cranial half of the body under manual restraint. The same procedure was used to collect vFF sensitivities at hours 1, 3, and 6 after the injection. A 1-wk washout period was permitted between trials.

Statistical analysis. The compiled data were analyzed using commercially available software SigmaPlot 11.0 (Systat Software San Jose, CA 95131). All data passed normality and equal variance assumptions, according to Shapiro–Wilk normality evaluation. Hind limb withdrawal latencies and vFF sensitivity scores were evaluated using a 2-way repeated-measures ANOVA. Pairwise multiple comparison procedures were performed using a Student–Newman–Keuls method. Descriptive data are expressed as mean ± SEM. Statistical significance was classified at $P < 0.05$.

Results

Experiment 1. *Hargreaves test.* No statistically significant differences in mean hind limb thermal withdrawal latencies were found between morphine and saline treated groups for WTF or NLF. However, evaluation of only the non-24-h means of the WTF revealed a near-significant hyperalgesic reaction to morphine ($P = 0.07$) (Figure 1). Statistically significant differences were present between mean baseline and morphine at 1 h ($P \leq 0.001$), 3 h ($P = 0.034$), and 6 h ($P = 0.035$) for WTF's receiving morphine at 50 mg/kg (Figure 1). For NLF, hind limb thermal withdrawal latencies were similar for morphine and saline at all timepoints (Figure 2). Baseline hind limb latencies ranged between 5.5 to 12.9 s for untreated WTF and between 5.6 to 10.7 s for NLF. Hind limb latencies for WTF receiving morphine

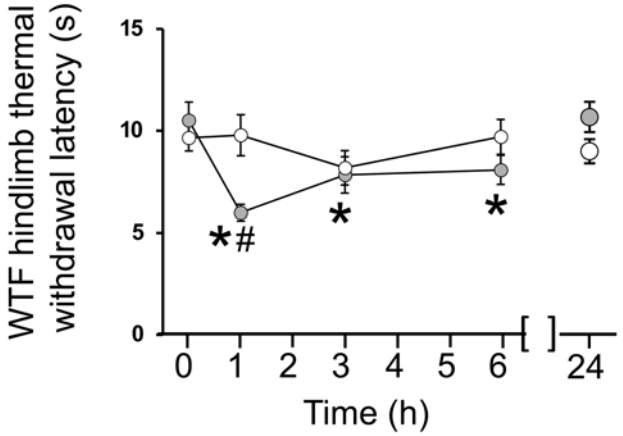

Figure 1. Mean (± SE) hindlimb withdrawal latencies to a noxious thermal stimulus (Hargreaves model) for WTF (n = 6). Gray circles represent morphine (50 mg/kg, SC) and white circles represent saline controls (0.9%, isovolumetric SC). * indicates $P < 0.05$ for data point compared with baseline (0 h); # indicates $P < 0.05$ compared with saline at that time point.

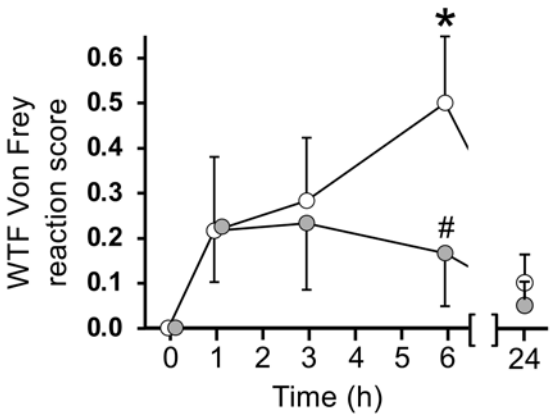

Figure 3. Mean (± SE) hindlimb withdrawal latencies to a noxious mechanical stimulus (von Frey model) for WTF (n = 6). Gray circles represent morphine (50 mg/kg, SC) and white circles represent saline controls (0.9%, isovolumetric SC). * indicates $P < 0.05$ for data point compared with baseline (0 h); # indicates $P < 0.05$ compared with saline at that time point.

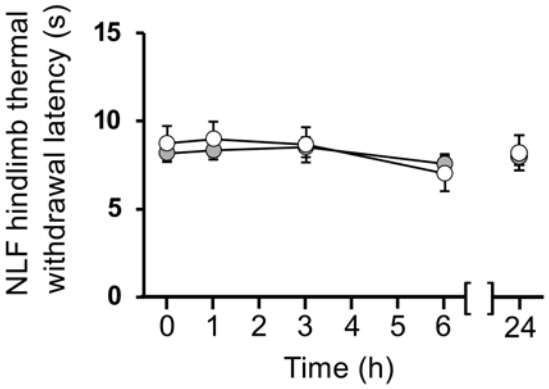

Figure 2. Mean (± SE) hind limb withdrawal latencies to a noxious thermal stimulus (Hargreaves model) for NLF (n = 6) Gray circles represent morphine (50 mg/kg, SC) and white circles represent saline controls (0.9%, isovolumetric SC). There were no significant differences in saline- compared with morphine-injected frogs.

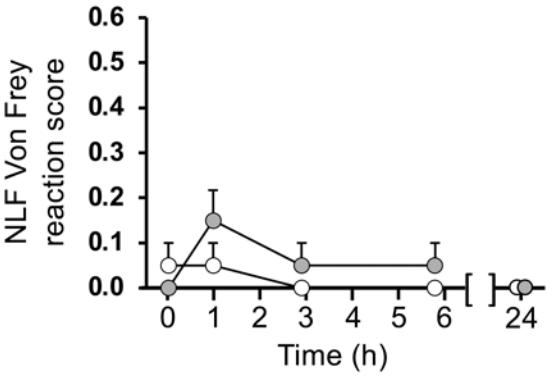

Figure 4. Mean (± SE) hindlimb withdrawal latencies to a noxious mechanical stimulus (von Frey model) for NLF (n = 6). Gray circles represent morphine (50 mg/kg, SC) and white circles represent saline controls (0.9%, isovolumetric SC). * indicates $P < 0.05$ for data point compared with baseline (0 h); # indicates $P < 0.05$ compared with saline at that time point.

(50 mg/kg) ranged between 5.0 and 13.2 s and for NLF receiving morphine (50 mg/kg) between 5.8 to 10.0 s.

von Frey test. No statistically significant differences were detected in overall treatment versus saline in either WTF or NLF. Statistical significance was achieved between baseline means and means at the 6-hour time point for WTF's receiving morphine (50 mg/kg) (Figure 3). No significance effects were detected for time points or treatment for NLF (Figure 4).

Experiment 2. NLF (n = 12) that received morphine (100 mg/kg) did not show a significant drug effect (P = 0.089). Mean response times to vFF application were significantly longer for frogs administered morphine compared to baseline values at 1 h (P = 0.006) and 3 h (P = 0.001). No treatment effect was detected for NLF (n = 6) receiving morphine (50 mg/kg).

In the observer-blind, complete crossover portion of Experiment 2, NLF (n = 12) receiving morphine (50 mg/kg) showed a significant drug effect as compared with baseline at 1 h only (P = 0.011; Figure 5). Those receiving morphine (100 mg/kg) showed a significant drug effect as compared with baseline at 1 h (P = less than 0.001) and 3 h (P = less than 0.001) but not at

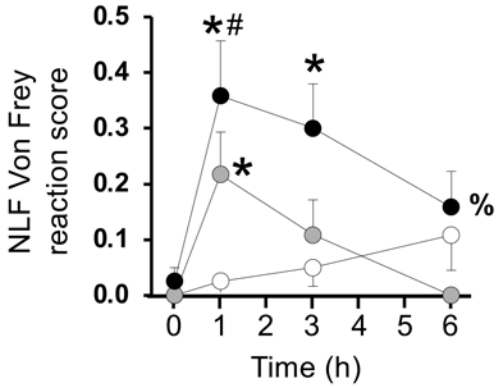

Figure 5. Mean (± SE) von Frey filament reaction scores for NLF (n = 12). Black circles represent morphine (100 mg/kg, SC), gray circles represent morphine (50 mg/kg, SC), and white circles represent saline controls (0.9%, isovolumetric SC). * indicates $P < 0.05$ for data point compared with baseline (0 h); # indicates $P < 0.05$ compared with saline at that time point. %indicates a significant drug effect ($P < 0.05$).

6 h ($P = 0.071$). A comparison of morphine (50 mg/kg) to saline revealed a significant increase in response times to vFF only at 1 h ($P = 0.013$). A comparison of morphine (100 mg/kg) to saline revealed significant increases in response times to vFF at 1 h ($P \leq 0.001$) and 3 h ($P = 0.004$). Evaluation of the data overall after administration of morphine revealed that the vFF method provided a statistically significant, dose-dependent increase in response times ($P = 0.002$).

Discussion

We originally hypothesized that administration of morphine would provide measurable, dose-dependent antinociception in both NLF and WTF regardless of nociceptive model used. However, in our experiments using the thermal nociceptive model, WTF demonstrated hyperalgesia to morphine, while the NLF responded similarly to either saline or morphine administration. This was somewhat similar to our previous data in NLF, but unexpected in WTF, and counter to our hypothesis. However, using vFF mechanical nociception, NLF demonstrated an antinociceptive response after receiving morphine. These data suggest that a thermal nociceptive test may not be ecologically appropriate for use in some amphibian species and, instead mechanical or surgical models should be used to evaluate analgesic efficacy in amphibian species. Species differences in response to noxious stimuli should also be considered.

In our study, morphine was antinociceptive in the vFF mechanical nociception test, but not the Hargreaves thermal nociception test. This result highlights the importance of the application of species-relevant nociceptive models in amphibian pain and analgesia research. Mechanical and thermal noxious stimuli are thought to be modulated by slightly different neuropathways. It is hypothesized that thermal (and chemical) stimuli are processed by pathways that use opioid and adrenergic receptors as inhibitory mechanisms, while mechanical stimuli are processed by pathways that use only opioid receptors as inhibitory mechanisms.[10] This suggests that frog limb withdrawal responses after application of both the Hargreaves and the vFF tests should be similar after morphine administration, as morphine would inhibit both thermal and mechanical stimulus signaling. However, the results of application of the vFF indicate that mechanical nociception may be more ecologically relevant in certain frog species than thermal nociception. In addition, the results suggest that different species may have specific nociceptors (for example, mechanical, thermal, multimodal, chemical) concentrated in different anatomic regions.

The unexpected result of hyperalgesia in WTF may be explained by considering morphine-induced hyperalgesia in other species, including cats, horses, swine, and cattle.[10] In mammals, this effect was hypothesized to be due to morphine or its metabolites antagonizing the action of glycine at the spinal level, thereby removing its inhibitory effect at the dorsal horn cells and leading to neuroexcitation.[9] The doses of morphine used in our study were high as compared with published doses used in reptiles.[11] The high dose may have contributed to a neuroexcitatory effect in the WTF. The absence of the same effect in NLF may suggest differences in species sensitivity to morphine, highlighting the problems with extrapolating drug effects or doses between species due to potential pharmacokinetic and pharmacodynamic differences.

We used a binary scoring system for frog responses to the vFF nociception, scoring a "0" if the frog withdrew in response to application of the filament and a "1" if the frog did not withdraw. Our use of a binary scoring system limited statistical comparisons and our ability to consistently detect antinociception. An alternative method would adopt the use of a vFF attached to an electronic system that directly measures the level of pressure at which a reaction to the filament occurs, thus using a continuous, rather than binary, measure. While the hand-held vFF may be useful as a clinical tool and has been used in mammalian species for decades, it may not provide optimal results in this specific research setting.

Whether morphine is an effective analgesic in amphibians is unknown. Some contradictory evidence with respect to morphine antinociceptive efficacy exists for reptiles, such that withdrawal latencies increase after morphine administration in a dose-dependent manner in bearded dragons and red-eared slider turtles, but not in corn snakes.[11] Similarly, morphine has no effect on withdrawal latencies in ball pythons.[8] These conflicting results may highlight species differences or differences in the ecological relevance of a given modality for the species, as we saw in the frogs in this and our companion study.[7] In addition, different analgesics may have different antinociceptive effects. In our companion manuscript,[7] we found no significant change in limb withdrawal latencies to a noxious thermal stimulus in WTF that received subcutaneous morphine at 5 mg/kg or 10 mg/kg. In contrast, 2 previous studies found that NLF exhibited a dose-dependent response to morphine sulfate administered in the lymph sac (10, 30, 100 and 300 nmol/g) and via intrathecal route (30 nmol/g) using the AAT model, the Hargreaves model, and the vFF model.[13,14] The results of our study agreed with the positive morphine vFF results, but were not consistent with the positive morphine Hargreaves results. Perhaps the differences in drug doses, concentrations or routes of administration contributed to the different results obtained in these studies.

In summary, our data showed that morphine at both 50 and 100 mg/kg SC provided antinociception in NLF using a modified vFF test, but results were less clear with the Hargreaves test, indicating that amphibians may respond more consistently to mechanical noxious stimuli as compared with thermal noxious stimuli. In addition, effective opioid doses appear to be significantly higher in amphibian species than in other animal orders that have been studied. This is true of other sedative drugs as well. In poison dart frogs, sedation was achieved only after using high doses of dexmedetomidine combinations.[15] These combined drug doses are dramatically higher than sedating doses of the same drug combinations in typical mammals or reptiles. Obviously, more studies are needed to assess pain management in amphibians. Future pain and analgesia studies should focus on the appropriate application of antinociceptive tests, justified sample sizes, choice of opioid drugs and doses, and consideration of species differences.

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