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Hargreaves does not evaluate nociception following a surgical laparotomy in *Xenopus leavis* frogs

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ABSTRACT

The present study was performed to determine the effectiveness of the Hargreaves test for the evaluation of nociception in frogs, more precisely to determine if cutaneous thresholds to a radiant heat stimulus would increase with analgesics following an abdominal laparotomy performed under general anaesthesia. Non breeding female *Xenopus leavis* frogs (3 groups (non-anaesthetized, anaesthetized with tricaine methanesulfonate (MS222), with or without an abdominal laparotomy) were used to evaluate the effectiveness of the Hargreaves test. Cutaneous thresholds were evaluated at baseline and following anaesthetic recovery (over 8 h) at six different body locations. Increased reaction times were observed in the gular area only at 1 h post-recovery following a MS222 bath immersion in frogs with ($p < 0.02$) and without the abdominal laparotomy ($p < 0.002$). In conclusion, the Hargreaves test does not provide an adequate test to evaluate nociception induced by an abdominal laparotomy and consequently cannot be used to evaluate analgesics in *X. leavis* frogs.

Analgesia is difficult to evaluate in frogs and the most common test used to evaluate nociceptive thresholds is the acetic acid test (Stevens, 1992) which consists of placing single drops of gradually increasing concentrations of acetic acid, usually on the hind legs, until a wiping response from the opposite leg is observed (Hargreaves et al., 1988). A recent study used the Hargreaves test to evaluate analgesia in *Xenopus leavis* frogs since it would be less traumatic to the skin than the acetic acid test (Coble et al., 2011). In the Hargreaves test, a focused light-radiant heat source, increasing progressively in intensity, is directed to the skin of the animal and a reaction time is measured when a withdrawal or an escape movement occurs during its application. Findings from Coble et al. (2011) showed that the Hargreaves test could be used to determine cutaneous thresholds in *X. leavis* frogs up to 9 h following surgical recovery, using different analgesics. This study also suggested that peri-operative thresholds were not modified by analgesics, even following abdominal surgery. However, only one distant body location from the surgical incision was evaluated. The main goal of this experiment was to evaluate the effectiveness of the Hargreaves test by evaluating nociceptive thresholds at different body locations including the surgical incision site.

The first objective of the present study was to determine if a residual analgesic effect of tricaine methanesulfonate (MS222) was present following the administration of an anaesthetic dose by evaluating nociception in non-anaesthetized frogs as well as in frogs following recovery from anaesthesia. The second objective was to evaluate cutaneous nociceptive thresholds following an abdominal laparotomy performed under general anaesthesia, before different analgesics could be evaluated. MS222 was used since it is the most commonly used agent to induce anaesthesia for major surgical interventions in amphibians (Downes, 1995; Wright, 1996, 2001). Laparotomy is used to collect frog eggs in different areas of research including developmental studies and genetics (Beck and Slack, 2001).

Eighteen *X. laevis* frogs (nonbreeding females, Xenopus I, USA) with an average (±SD) body weight of 113 g (±11) were used for these studies. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine prior to animal use and all procedures were in accordance with the guidelines of the Canadian Council on Animal Care. Animal care, room temperature and water quality parameters were as previously published (Lalonde-Robert et al., 2012).

Three groups of six frogs each were used for this study. All frogs were acclimated to the Hargreaves apparatus (glass floor) for 15 min prior to the heat threshold evaluations. Experiments were performed on frogs that remained calm following the 15 min of acclimation to the enclosure and between heat stimulations. Frogs were contained in a square (25 cm × 25 cm floor surface; 25 cm high) Plexiglas enclosure. The floor surface temperature was regularly measured with an infrared temperature gauge (Duratrax, USA; accuracy 2%) before and after the evaluation of each frog, and remained constant (21 ± 0.2 °C) throughout the experiment.

The first group of frogs was not anaesthetized. These frogs were evaluated with the Hargreaves apparatus (IITC Life Science, USA;

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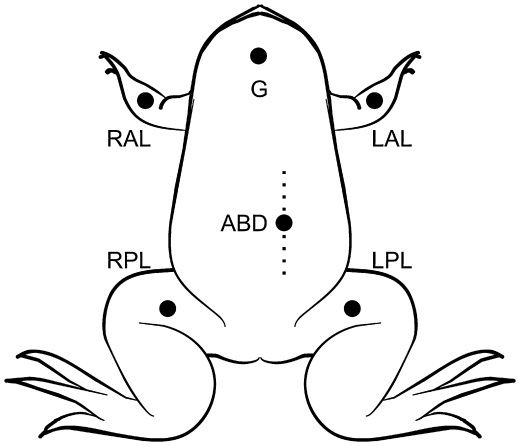

Fig. 1. Schematic ventral aspect of a *X. leavis* frog showing the different skin stimulation areas used for the evaluation of reaction times with the Hargeaves test. Locations (black dots) were stimulated in the following sequence: RPL right posterior leg, LPL left posterior leg, ABD abdomen, RAL right anterior leg, LAL left anterior leg, and G gular area. The doted line corresponds to the laparotomy incision (length 2.5 cm).

intensity set at 90%) at 0, 2, 6 and 10 h to establish a baseline as well as reproducibility and variability of the test over time. This stimulation schedule respected the same stimulation schedule used in anaesthetized frogs, respecting the number of stimulations and the time intervals. The light beam was applied sequentially on the skin of both hind legs (always L then R), the abdomen (middle area), both fore legs (always L then R) and on the gular area (Fig. 1) with a two minute delay between each body stimulation site. Frogs were returned in their original water environment following each evaluation and no skin dehydration occurred since the duration of the evaluation was relatively short (approximately 10 min) (Guénette et al., 2007). The first voluntary limb (seen with anterior limbs following gular stimulation) or whole body (seen at other skin stimulation sites) movements following the onset of the heat source was noted as the reaction time. The second and third groups were evaluated at the same body locations at 1 h prior to and at 1, 4 and 8 h following the MS222 bath immersion. Importantly, the abdominal stimulation in Group 3 was directed on the surgical site. The second group received MS222 only and for the third group, an abdominal laparotomy of approximately 2.5 cm in length (1 cm parasagittal to the midline) was performed after (within 5 min) the MS222 bath immersion. The surgery was performed with sterile instruments under aseptic conditions. Single interrupted 3.0 monofilament nylon sutures (Ethilon, Ethicon) were used to close in two layers (abdomen and skin) since it causes the least tissue reaction in *X. leavis* frogs (Tuttle et al., 2006).

For the immersion bath, frogs were immersed for 15 min in 250 mL of a 1 g/L of MS222 solution (purified water buffered at a pH 7 ± 0.4 with sodium bicarbonate) (Downes, 1995; Lalonde-Robert et al., 2012). The container was covered to keep animals in full darkness. Following the immersion bath, frogs were thoroughly rinsed and placed in purified water leaving the nostrils in contact with air, until recovery.

Repeated measures ANOVA and Tukey's post-hoc analysis were performed with SAS (version 9.2, SAS Institute, Cary, NC, USA). Using data form a previous publication (Coble et al., 2011), animal numbers were selected to obtain a statistical power of 99%, setting the alpha error level at 5%. Statistical significance was set at $p < 0.05$.

All anaesthetized frogs recuperated well and were able to swim freely 30–40 min following MS222 anaesthesia. No significant differences in reaction time to the radiant heat stimulus were seen for any of the stimulated areas, in any group (non-anaesthetized, anaesthetized and anaesthetized with a laparotomy) except for the gular area (Fig. 2). Frogs receiving MS222 ($p < 0.002$), and MS222 followed by the laparotomy ($p < 0.02$), had a similar reaction time increase for the gular area only, and this only at the one hour time point following the recuperation from the MS222 bath.

Apart from the gular area, radiant heat stimulation on the different body locations of *X. leavis* frogs revealed that MS222 does not change skin sensitivity between 1 and 8 h following a MS222 bath immersion. More importantly no significant change of the reaction time occurred at the incision site, and therefore the Hargreaves test appears inadequate to evaluate nociception following an abdominal laparotomy. These results depend on environmental and skin conditions remaining relatively constant, which are suggested by constant reaction times at non-affected (without a skin incision) skin sites and the returned to normal reaction time at the gular site.

These results corroborate the findings of Coble et al. (2011) and further indicate that sensitized skin does not react to radiant heat stimulation. My original intent was to test different analgesics but these findings made the continuation of the study impossible. Our findings could be explained by the location of pain fibres in the skin of frogs. In this species, large and medium sensory fibres that discharge to mechanical stimuli are found in the superficial layers whereas small axons that respond to painful stimuli are found only in deeper dermal layers (Lindemann and Voûte, 1976). These small fibres, that terminate as free nerve endings, do not respond to light touch but to injurious stimuli. Pain fibres are sensitized following a local inflammation, but if they are found in deeper layers, this might explain the constant reaction times observed, even at the surgical site. This does not imply that frogs do not need an analgesic following surgery but that the nociceptive sensations originate from receptors in dermal layers not readily recruited by the stimulation of the superficial skin, as the case with the Hargreaves test.

The only stimulation site affected by MS222 was the gular area, and this was observed only at 1 h following exposure, suggesting that MS222 might desensitize frog skin. Frogs had a clear wiping response with one fore limb following the skin stimulation that strongly suggested a skin receptor response. Different explanations can be proposed for this finding. Firstly, only histological and physiological studies of the abdominal skin of frogs have been reported (Lindemann and Voûte, 1976) and there may be structural and functional differences that explain painful heat sensitivity in the skin of the head. If so, further work is required to confirm that the organization of nociceptive fibres might be different in different body areas of frogs. This might explain the delayed reaction time in the gular area following MS222 anaesthesia. Secondly, if gular skin sensory receptors were similarly organized in the abdominal skin, the delayed response observed might be related to other head structures, such as the tongue. Gular skin possesses nociceptors that are very sensitive to sudden warming (Spray, 1976) and a rapid reaction time of the gular area could be caused by the activation of these receptors, which in this case would seem to be affected by MS222 neural depression. No clear explanation can be given at the present time for the effect of MS222 on gular area reaction times. Although the skin of the frog's head appears more sensitive to the Hargreaves test, analgesics cannot be evaluated until histological and physiological studies are performed to better describe the sensory characteristics of the area.

In conclusion, the Hargreaves test did not evaluate pain induced by an abdominal laparotomy in *X. leavis* and consequently postoperative analgesics cannot be evaluated with this test. Further work on gular nociception may be used to investigate anaesthetic sensory perception in *X. leavis*. Findings could translate to other aquatic amphibians, and probably much less to terrestrial amphibians, however these considerations need to be evaluated in future studies.