Stiff as a Board: Handling Frequency in Zebrafish and its Effect on Rigor Mortis

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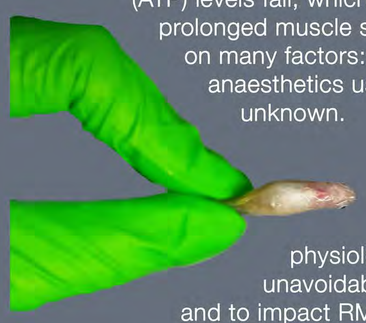
Aim: To determine the impact of handling frequency on rate of rigor mortis

Introduction

Zebrafish Schedule 1 Killing (S1K) includes the humane method of death (anaesthetic overdose), and a confirmation of death, such as confirmation of rigor mortis (RM). Currently, the Code of Practice advises minimising handling during S1K for all species, but does not quantify it; this can and does lead to variations and interpretations of handling frequency based on experience and logistics. A previous trial suggests RM may be used as an index to comparatively indicate high levels of pre-mortem stress. With this background, we conducted another trial to identify if pre-mortem stress due to handling frequency can also impact this onset.

Rigor Mortis

Rigor mortis (RM) is a stage of death characterised by muscle stiffness. Following death, lactic acid accumulates, decreasing cellular pH (1). This disrupts glycolysis and in turn adenosine triphosphate (ATP) levels fall, which breaks actin-myosin cross-bridges. Its depletion results in prolonged muscle stiffness (fig. 1). The rate of onset and duration of RM is dependent on many factors: size; temperature; and pre-mortem exhaustion. Studies on anaesthetics used in zebrafish are limited, and the effects on RM are mostly unknown.



Handling is a known stressor for animals, and whilst some species may become accustomed to it, zebrafish typically do not. Handling and emersion can cause stress that is physiologically measurable for approximately an hour (3). As handling is an unavoidable part of the S1K process, it is likely to be a pre-mortem stressor and to impact RM.

Fig. 1: a zebrafish that is in rigor mortis

Methods

Two strains were used for the trial: the hybrid AB;TupLF (n=57; 10 months old) and the wildtype Wik (n=54; 11 months old). The fish from each strain were from the same respective stock. All fish were maintained in recirculating 10 L tanks, 28.2 – 28.4°C, pH (7.1-7.3), in a 14:10 hour light/dark cycle and fed on a combination dry food diet. All fish used were of similar size for each respective sex, albeit AB;TupLF fish are typically larger than Wik. The fish were scheduled for S1K, as found in Animals (Scientific Procedures) Act 1986 (ASPA). The 2-PE dose followed UCL protocol, and the benzocaine dosage based on published recommendations (table 1).

Anaesthetic	Dosage
Benzocaine	0.7 g/L
2-PE	6 mL/L

Table 1, left: Anaesthetic doses. The the time of no observed movement is the time of assumed death, which occurs after respiration ceases.

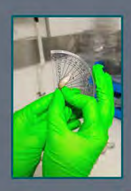
All fish were handled either x1, x3, or x5, with the x3 being the current protocol.The steps involved in x5 frequncy reflect all movements that a fish would experience during a 24 hour period prior to S1K. Each handling occurred as the following:

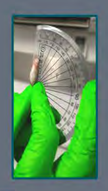
x1	x3	x5
Net from home tank into S1K anaesthetic	Net from home tank into transport bag	Net from home tank into breeding box
	Transport in bag into cull area	Net from breeding box into ‘cull’ tank; wait 5 minutes
	Net from transport bag into S1K anaesthetic	Net from ‘cull’ tank into breeding box
		Net from breeding box into transport bag
		Net from transport bag into S1K anaesthetic
		

Fig.1. The frequency of handling the fish was either x1, x3, or x5.

For each treatment, three fish were added to the pre-dosed water and left for ten minutes to ensure death. A stimulus, or ‘knock test’ was performed to ensure death at which point individual fish were transferred to labelled petri dishes. At 30 minute intervals, each fish was held by the caudal perduncle and measured against a protractor to measure the angle.

Fig.3. Every 30 minutes, the angle of each fish was measured using a protractor to determine onset/stage of RM

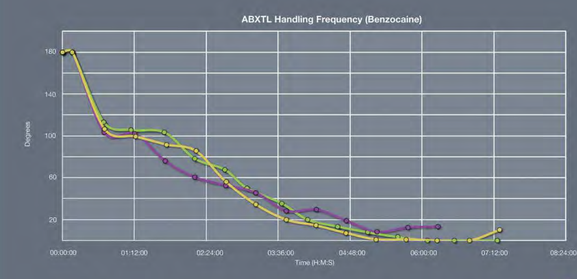




Results

The time points for the measured angles for each treatment were averaged and plotted to compare the rate of RM in each anaesthetic and strain. Data points from one fish (WIK Benzocaine, x3) was not collected after 2:30:00 (H:M:S) due to human error.

Both strains euthanised with benzocaine showed very little difference between handling frequencies, although x5 for the AB;TupLF strain was marginally faster to reach full RM (0 degrees). The only notable difference was in the 2-Phenoxyethanol-x3 treatment: both strains reached full RM (0 degrees) faster than any other groups, at approximately the 3:30:00 hour mark. All other groups reached full RM at approximately the 5:00:00 mark. Additionally, both the AB;TupLF and WIK x3 treatment were further into RM at the 1:12:00 mark the other two treatments.



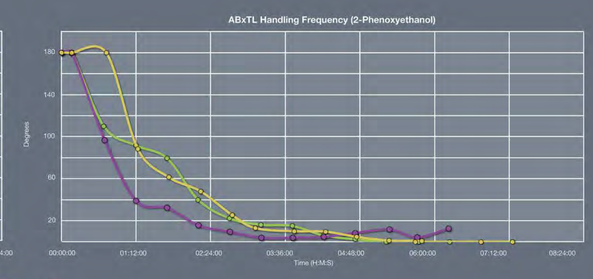
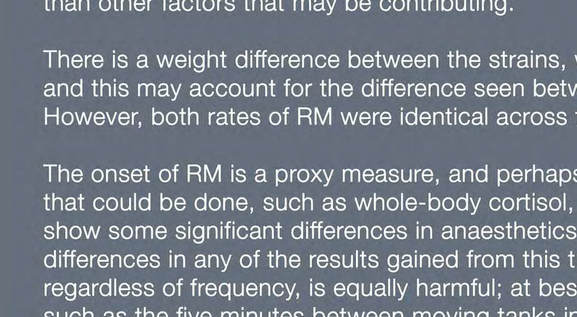
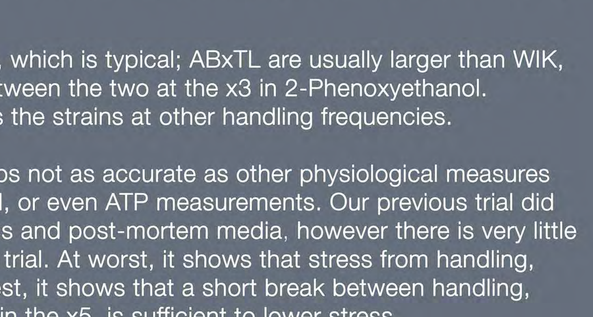


Fig. 4 above right and left: the different post-mortem media. The fish in 2-PE ‘Stay’ (right) reached the maximum angle at a faster rate than MS-222 or Benzocaine. Fig. 5 below: the three anaesthetics for ‘stay’ tested using a 2-way ANOVA test. Other variables were tested via the same method (data not

x1
x3
x5





Discussion

Handling is a proven stressor in fish, and pre-mortem stress can increase the onset of rigor mortis. It stands to reason then that excess handling pre-mortem would cause a higher onset of RM; however, this was not evident in the results.

Both strains showed no difference in the onset of RM with benzocaine; however, for those under 2-Phenoxyethanol, there is some difference between both the strains that were handled x3.

The data for all x3 handling came from a previous trial that looked at the impact of different anaesthetics (2-Phenoxyethanol, benzocaine, and MS-222) and different post-mortem media (staying or being removed from the anaesthetic post-mortem). Both trials were conducted within the same parameters (see ‘Methods’), using the same dosages, and the same strains of a similar age. However, the individual fish in this trial came from a different clutch than the initial trial, which may account for the difference. However, given that the results from benzocaine were nearly identical, this is less likely than other factors that may be contributing.

There is a weight difference between the strains, which is typical; ABxTL are usually larger than WIK, and this may account for the difference seen between the two at the x3 in 2-Phenoxyethanol. However, both rates of RM were identical across the strains at other handling frequencies.

The onset of RM is a proxy measure, and perhaps not as accurate as other physiological measures that could be done, such as whole-body cortisol, or even ATP measurements. Our previous trial did show some significant differences in anaesthetics and post-mortem media, however there is very little differences in any of the results gained from this trial. At worst, it shows that stress from handling, regardless of frequency, is equally harmful; at best, it shows that a short break between handling, such as the five minutes between moving tanks in the x5, is sufficient to lower stress.

Although the Code of Practice advises to minimise handling prior to S1K, the data here does not show a difference between most of the handling frequencies.

Conclusion

Whilst it is accepted that zebrafish experience stress during handling, this trial seems to suggest that it is not a main factor in the stress experienced during S1K. The previous trial suggested that anaesthetics were more likely to impact stress and welfare, and these should be the focus for investigating refinements in euthanasia for zebrafish.

References:

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2. Collymore, C., Tolwani, A., Lieggi, C., & Rasmussen, S. (2014). Efficacy and safety of 5 anesthetics in adult zebrafish (Danio rerio). *Journal of the American Association for Laboratory Animal Science* 53(2), 198-203.
3. Ramsay, J.M., G.W. Feist, Z.M. Varga, M. Westerfield, M.L. Kent, C.B. Schreck. (2009). Whole-body cortisol response of zebrafish to acute net handling stress. Aquaculture. 297(1–4) p157-162

Acknowledgements:

Many thanks to the UCL Fish Facility staff: H. Callaway, P. Barwood, E. Hitchcock, J. Upstone, J. Warmsley.