Assessment of behaviour in groups of zebrafish (*Danio rerio*) using an intelligent software monitoring tool, the Chromatic Fish Analyser

Jack S. Thomson[a], Ali A. Al-Temeemy[b,c], Helen Isted[d], Joseph W. Spencer[c] and Lynne U. Sneddon[d]*

[a]School of Environmental Sciences, University of Liverpool, Liverpool, L69 3GP, UK

[b]Department of Laser and Optoelectronics Engineering, College of Engineering, Al-Nahrain University, Baghdad, Iraq

[c]Department of Electrical Engineering and Electronics, University of Liverpool, Liverpool, L69 3GJ, UK

[d]Institute of Integrative Biology, University of Liverpool, Liverpool, L69 7ZB, UK

**Corresponding author at Institute of Integrative Biology, The BioScience Building, Crown Street, Liverpool, United Kingdom, L69 7ZB*

E-mail address: lsneddon@liverpool.ac.uk

ABSTRACT

Background: Zebrafish (*Danio rerio*) are an increasingly popular model species within a variety of biomedical and neurobiological contexts. Researchers are required to prevent any negative states, such as pain, when using experimental animals to optimise their welfare but analysis tools for zebrafish are lacking.

New method: The chromatic fish analyser (CFA) is a computer-based monitoring system that has the potential to identify changes in fish behaviour via spatial chromatic analysis of video images. The CFA was used to monitor the behaviour of groups of six fish, where none, one, three or six fish were given a fin clip. Additionally a drug with pain-relieving properties, lidocaine, was administered to determine if this ameliorated any alterations in behaviour. The CFA measured hue horizontally and vertically reflecting the position of the fish in their tank and saturation (indicates clustering distribution) and lightness as a measure of overall zebrafish activity.

Results: Changes in vertical hue demonstrated that all fin clipped animals were closer to the bottom of the tank relative to pre-treatment; this was not observed in control groups, and was alleviated in those treated with lidocaine. Saturation (clustering) and lightness alterations indicated fin clipped groups reduced activity after receiving the fin clip. Lidocaine was effective in preventing the behavioural changes when 1 or 3 fish were clipped.

Conclusions: The CFA proved powerful enough to identify significant changes in behaviour taken directly from video images. With further development this monitoring tool represents a step forward in detecting behavioural changes in groups of zebrafish and could allow carers to intervene to improve welfare.

Keywords: Zebrafish, nociception, pain, fishes, behaviour, animal welfare.

1. Introduction

Fish are increasing in their popularity for use in scientific procedures globally. For example, approximately half a million fish are used annually in the UK alone, and they are now the second most popular experimental animal model, behind mice with the model species, the zebrafish (*Danio rerio*), estimated to account for 50% of these numbers (UK Home Office 2018). The desirability of zebrafish in experimentation has recently increased due to their rapid development, reproductive success, their high genetic homology to humans (80-85%) and the lack of ethical restriction to the use of zebrafish embryos (Valentim *et al.*, 2016). Zebrafish are an accepted model for research in developmental genetic studies, human diseases and drug discovery (Clark & Ekker 2015). During many of these procedures the fish are subject to invasive techniques which may result in stress and pain through tissue damage (e.g. Schweitzer et al. 2003; Chablais & Jaźwińska 2012; Lemmens et al. 2016). Recent empirical evidence has shown that zebrafish subject to a potentially painful event alter their normal behaviour for a prolonged period: rather than swimming constantly and using the entire tank space painfully treated zebrafish reduce activity and spend most of their time on the bottom of the tank which can be prevented by use of effective pain-relieving or analgesic drugs (Reilly et al 2008; Ashley et al. 2009; Sneddon 2015; Lopez-Luna et al. 2017a,b,c,d; Schroeder & Sneddon 2017; Taylor et al. 2017; White et al. 2017; Costa et al. 2019; Deakin et al. 2019a). Much of this data is collected by human observers directly or by video or by commercially available tracking software with only a few pain behaviour specific monitoring tools developed so far. For example, fractal dimension analysis of complex swimming trajectories can differentiate between painful and non-painful treatment in zebrafish (Deakin et al. 2019a) and the Fish Behaviour Index combines space use with distance travelled to gauge responses to painful treatment (Deakin et al 2019b). However, these have only been developed for individual fish. Thus, the automated analysis of the behaviour of groups of zebrafish remains a challenge.

Zebrafish are legally protected in some countries. For example the European Directive on the protection of animals used for scientific purposes (Directive 2010/63/EU) has a legal requirement to avoid or minimise pain in adult fishes. Ethically we need to prevent or alleviate any negative states such as pain when subjecting fish to potentially damaging events. This requires us to be able to easily detect abnormal behaviour so that interventions can minimise any pain where the study of pain is not the objective of the study. This is difficult because the ability to identify abnormal behaviour in one or a few fish within a larger group is challenging and thus only information on individual zebrafish exists (Deakin et al. 2019a,b). However, zebrafish are generally housed in groups, but identifying a single individual in discomfort may be difficult to pinpoint with the human eye. Here, we apply spatial chromatic analysis to images obtained directly from video recordings of groups of zebrafish. This has been successfully deployed in a human care home where it had superior discrimination capabilities, was robust to events that normally disturb monitoring systems, and required less computational time and memory storage space (Al-Temeemy 2018; 2019). Thus human patients displaying behaviour outside of the measured norms can be identified and could receive care without verbal communication between them and carers. To validate our approach we developed the chromatic fish analyser (CFA) which processes 2D video images to analyse the overall average behaviour of a group of fish. The CFA uses the chromatic methodology to assign four parameters to the average group behaviour for zebrafish; these are related to the group position within the tank (horizontal and vertical hue), activity level (lightness) and the distribution of the group (saturation indicative of clustering). Measurements using the CFA allowed comparison of the behaviour of undisturbed groups of zebrafish (control) to those who were experiencing a potentially painful event, to determine how groups were affected by fin clipping where part of the tail (caudal) fin is removed. This routine laboratory procedure is performed to allow genomic screening. Groups comprised six individuals where one, three or all

individuals were fin clipped. A drug with pain-relieving properties (lidocaine) was administered to a further set of groups to determine if this prevented any behavioural responses to the fin clip. A sham treatment group was also included, where individuals were anaesthetised but not fin clipped as a control for the impact of anaesthesia and handling. The aim of this research was to show that this novel CFA is a valid approach to detect differences in behaviour between groups of zebrafish experiencing pain to those that are not.

2. Methods

2.1 Zebrafish

Experiments were conducted humanely and were ethically approved by the University of Liverpool and the Home Office UK (project license no. PPL 70/9005). All regulated procedures were conducted by licensed personnel.

AB **z**ebrafish (n = 288; 8 months; mean (se) weight = 0.30g±0.01g) were obtained from the University of Liverpool breeding programme and were selected at random from one stock. The fish were kept in groups of six in a sex ratio of 3 males to 3 females in a glass tank (30 x 20 x 15cm) at 28±0.1°C. Behavioural responses to painful treatment do not differ between the sexes in zebrafish (Taylor et al. 2017; Costa et al. 2019). Tanks were kept on a semi-closed water recirculation system, with constant aeration and biological and mechanical filtration on a 14:10h light:dark regime. Fresh water was added regularly (~33% water change per week) and water quality was kept optimal for this species (pH 7.2; ammonia <0.1mg/L; nitrite <0.1mg/L; nitrate <20mg/L). Prior to the experiment, fish were allowed to acclimatise to their tanks for at least two weeks and were fed twice a day *ad libitum* using commercial tropical fish flakes (Aquarian, Tetra, Melle, Germany).

2.2 Treatments

Each of the 48 tanks of zebrafish were assigned to eight different treatments at random (n = 6 per treatment; Table 1). The fish were not fed on the day of experiment and the flow into the tank was turned off 30 minutes before each experiment, to prevent water movement and to avoid any chemical communication or transfer of lidocaine between tanks. The previous night, the cameras were positioned in front of the tank and opaque dividers were put between adjacent tanks to prevent visual disturbance. All experiments commenced at the same time (~10am) to prevent any diurnal variation in behaviour.

For each group of zebrafish, a 25 minute pre-treatment video was firstly recorded to obtain data on normal behaviour. This video was of a full lateral view of the tank using an IDS USB 3.0 colour video camera (IDS; Obersulm, Germany) fitted with a 25mm monofocal lens and connected to a computer (HP compact elite 8300; Palo alto, Ca), placed opposite the fish tank (distance 1m). Fish were then netted at random to select either one or three fish to be used for the treatment. All fish were netted in the case of the 6 fin clip. Fish were placed into a glass beaker (500ml) covered with opaque material which contained system water prior to anaesthesia and transported to the anaesthetic tank.

In treatments including anaesthesia i.e. any treatments involving a fin clip or the SHAM handled treatment (Table 1), the individuals were chosen at random and moved to a separate tank (20 x 10 x 10cm) with aeration provided via airline and an air stone linked to a compressed air supply. The zebrafish were anaesthetised using benzocaine ($33mgL^{-1}$) to deep plane anaesthesia and then weighed to 0.01g. For fish that were treated using a fin clip, 40% of their caudal fin was measured using callipers and removed using a sterile scalpel as outlined in the Zebrafish Handbook (http://zfin.org/zf_info/zfbook/chapt7/7.8.html). The fish were then returned to their home tanks to recover from the anaesthesia. For the SHAM handled group, one fish was anaesthetised, handled and returned to their home tank, without any fin clip treatment. Preliminary analysis demonstrated that there was no significant difference in the

CFA values for these fish thus it was deemed not necessary to increase the sample size to include a further 6 groups of both 3 and 6 fish sham handled.

For the treatments involving the administration of an analgesic, 1mL of 5mgL^{-1} lidocaine was added to the home tank water before the fin clipped fish were returned to the tank after the fin clip procedure (Table 1). Lidocaine at this dose not affect normal behaviour in intact zebrafish (Schroeder & Sneddon 2017; Deakin et al. 2019a,b). For the control, the fish were left undisturbed within their home tank for the entire experiment.

2.3 Post treatment video recording and CFA analysis

For each experiment, four more 25 minute videos were recorded using the same UDS camera at 1, 2, 3 and 6 h after the fish had been returned to their tanks following their treatment. For the control group, these were recorded at these time intervals at the same time points as treatment groups. The videos were then processed after each experiment using the CFA software which measured average horizontal and vertical hue, average lightness and average saturation (Table 2). Averages for each parameter within each replicate of treatment were calculated for each video and downloaded into an Excel file by the CFA. At the end of the experiments all fish were humanely killed by overdose in anaesthetic followed by brain destruction to ensure death.

2.4 Development of the CFA

Chromatic monitoring or analysis of complex conditions can be applied to behaviour; chromatic processing is analogous to human vision yet also extends into a wide range of non-optical domains as applied in the present study (see Jones et al. 2008; Al-Temeemy & Spencer

Commented [A1]: I added two appendices A & B;

Appendix A, presents all the required equations used for extracting the chromatic parameters from the activity image.

Appendix B; presents the chromatic transformation algorithms.

2014; 2015a,b; Al-Temeemy 2018). Applying the chromatic methodology for monitoring the fish activities is implemented with three stages; these are activity image calculation stage, chromatic processing and transformation stages. Figure 1 presents the block diagram of the algorithmic flow for these stages; the first stage is responsible for calculating the activity image from the input video frames, while the last two stages are responsible for applying the chromatic processors on these activity images and extracting the chromatic parameters that reflect the average group behaviour for zebrafish.

In the chromatic processing stage, two sets of spatial chromatic processors (vertical and horizontal processors shown in Fig.1) are applied on the zebrafish activity images. The chromatic processors arrangement makes their triangular responses overlap with each other, allowing continuous sensitivity across the entire video frame (see Appendix A for more details). This makes these processors not only describe the activity and the distribution for the zebrafish cluster but also its location within the tank. The processors' outputs are then transformed by the chromatic transformation stages to chromatic parameters that reflect the average group behaviour for zebrafish (see Appendix B for the chromatic transformation algorithms).

The performance for these monitoring stages is evaluated by using LASER imaging technology. Figure 2 shows the experimental setup used in this task, which consists of DepthSence^{TM} Time-of-Flight (TOF) LASER Imaging System (LIS), testing bench with moving object, and personal computer with the required evaluation programs.

The evaluation procedure includes recording the moving object by the LIS, processing the recorded videos using the chromatic monitoring stages after applying different noise levels, and finally comparing the resultant object's locations (hue-horizontal and verticals) with the

calculated locations by the 3D LIS. The root mean square error (RMSE) between these locations ranged from 0.86% when there is no noise applied to 8.09% with applying noise. This revealed a good monitoring performance for these stages.

The chromatic fish analyser CFA is designed to process the recorded videos for zebrafish behaviour inside their tank. Figure 3A shows the CFA's graphical user interface (GUI) which contains two screens, one is used to display the video file and the other screen is used with the threshold slider to help the user to adjust the threshold level required for calculating the activity image. This image (colour coded image displayed in the activity screen), is calculated by finding the absolute difference between the successive frames for the recorded video, and then by enhancing the resultant difference using hard-thresholding technique (based on the selected threshold value).

After processing the activity images, the software calculates the following chromatic parameters for each frame:

- Hue_horizontal: horizontal location for the cluster.
- Hue_vertical: height for the cluster.
- Saturation: indicates the cluster distribution, which is equal to the multiplication between the calculated saturation values for both processors' sets divided by two.

In additional to these values, the software also calculates the average values for the above parameters and also the average values for the Lightness (Table 2). Figure 3B shows the parameters' 3D representations for each frame (red point cloud) and also their average values (cyan circles).

2.4 Statistical tests

Chromatic values for horizontal position, vertical position, activity and clustering were analysed using repeated measures ANOVA, with time (h), treatment, and their interaction as explanatory variables (see Appendix C for raw data). Individual fish IDs were included as an error term. Treatment was coded as a factor with eight levels: control, sham, and six levels corresponding to groups containing 1, 3 or 6 treated fish, either with or without analgesia. The response variables were normalised values generated by subtracting the pre-treatment value from the post-treatment value. Negative values therefore reflect greater time spent on the left hand side (horizontal position), at depth (vertical position), or less time being active or clustering. Data were analysed by comparing reduced models to full models using likelihood ratio tests. All analyses were conducted in R using the package lme4 (Bates et al, 2015).

3. Results

3.1 Position in tank

There was no significant effect of treatment (χ_7^2=13.68, p=0.06), time (χ_1^2=0.533, p=0.47) or their interaction (χ_7^2=2.70, p=0.91) on horizontal position (Fig. 4; Appendix D: Fig. 1). Whilst the interaction of time and treatment did not influence vertical position (χ_7^2=8.99, p=0.25), main effects of time (χ_1^2=6.91, p=0.009) and treatment (χ_7^2=39.56, p<0.0005) did. Fish receiving fin clip alone were observed closer to the bottom of the tank relative to pre-treatment; this behaviour was not observed in control or sham-handled fish, and was alleviated in those that had received lidocaine (Fig. 5; Appendix D: Fig. 1). When all 6 fish were fin clipped they tended to spend more time deeper in the tank regardless of the use of analgesia. Across all fish average vertical position was lowest immediately after the treatment but increased with time (Fig. 5).

3.2 Activity

Activity levels also changed depending on main effects of treatment (χ_7^2=28.76, p<0.0005) or time (χ_1^2=8.12, p=0.004). In a similar fashion to vertical position, reduced activity relative to pre-treatment was observed in groups which received a fin clip alone, but this effect

Commented [TJ[2]:
Reviewer 1 suggests consistently using “fin clip” rather than mixing “fin clip” and “treatment”. My response is:

“Treatment” is a factor which contains control, sham-handled and fin-clipped animals, and both those which received and did not receive lidocaine. It’s important to therefore retain this term as distinct to “Fin clip”. However, the reviewer is correct that there are points where the terminology are confused. We have gone through and made changes where this is the case, but also attempted to improve clarity throughout the Results section.

was alleviated in those that had received lidocaine and not apparent in control and sham-handled fish (Fig. 6). Again, activity was reduced in groups of six fin-clipped fish even with analgesia applied. Overall, activity levels declined with time post-exposure. However, there was no significant interaction (χ^2_7=5.42, p=0.61).

When plotting change from pre-treatment values for each group, clearly vertical distribution and activity values differentiate fin clip groups from the control groups and demonstrates that the dose of lidocaine is effective for groups with one or three fish fin clipped but less so when all six fish receive a fin clip (Fig. 7).

3.3 Clustering

The clustering of zebrafish appeared to be most strongly explained by treatment (χ^2_7=50.81, p<0.0005; Fig. 8). Control and sham fish, and those where only one fish was fin-clipped, did not adjust their behaviour, whereas clustering increased in groups where three or six fish had been fin-clipped. This effect was ameliorated by analgesia when only half the group had been treated, but not when all six had been treated. There was however little evidence of an effect of time (χ^2_1=3.10, p=0.078) or of an interaction between the two (χ^2_7=5.17, p=0.64).

4. Discussion

The Chromatic Fish Analyser (CFA) software was successfully deployed to discern differences between zebrafish treatment groups in their behaviour. The values obtained by the CFA did not differ over time in undisturbed control groups nor in the sham handled group subject to anaesthesia only. However, the CFA values obtained from groups that had been fin clipped were different to behaviour before the treatment. Specifically fin clipped zebrafish groups spent more time at the bottom of the tank, activity was reduced and groups became more clustered. Lidocaine appeared effective at preventing these changes in fin clipped groups when one or three individuals were clipped but was less effective when the entire group received a fin clip.

4.1 Tank Position

After fin clipping, all treatment groups moved closer to the bottom of the tank and their position remained lower for the rest of the experiment. This lowering of group position, was not seen in Sham or Control groups, thus it can be concluded that this was a response to tissue damage that may give rise to pain. Previous studies have found similar behavioural changes in zebrafish following a fear induced response such as simulated predator attach (Speedie and Gerlai, 2008; Levin *et al.*, 2007; Waldman, 1982) and in individual zebrafish subject to painful treatment (Taylor et al. 2017; Costa et al. 2019) including fin clipping (Schroeder & Sneddon 2017; White et al. 2017; Deakin et al 2019a,b). Fin clipping involves the severing of nerves and blood vessels and also damage to the fish skin that releases an alarm substance that elicits an innate anti-predator response (Huang *et al*., 2003). This may be the case for the fin clipped fish, and one could speculate the amount of alarm substance is greater in the groups with 6 fish fin clipped where lidocaine has less of an impact in reducing this behavioural change. It may be interesting to test this further in future studies by having a constant flow through so the alarm substance would be removed from the tank or adding alarm substance when only one fish has been fin clipped within a group to determine if the behaviour is comparable with whole group fin clipping. Zebrafish do increase their use of the bottom area of a tank when exposed to alarm substance in other studies and this is inferred as an innate anti-predator response (e.g. Mezzomo et al. 2019). However, exposure to alarm substance in larval zebrafish (Lopez-Luna et al. 2017d) and in piaçu fish (Alves et al 2013) actually acts as an anti-nociceptive via stress induced analgesia (Wolkers et al. 2013) thereby reducing behavioural responses to painful treatment. An alternative explanation is that when only one or three fish are clipped, there may not be a large enough change in group behaviour for the CFA to detect. However, this can be dismissed since the data clearly show fin clip groups without lidocaine exhibit a very similar decrease in vertical position in the tank.

4.2 Activity and clustering

The control and sham groups did not alter activity over the experiment but there was a significant decrease in the fin clip groups. This reduction in activity follows confirms what has been found frequently within research studies involving individual zebrafish subject to painful treatment (Reilly *et al.,* 2008; Correia *et al.*, 2011; Schroeder & Sneddon 2017; Taylor et al. 2017; White et al. 2017; Costa et al. 2019 Deakin et al 2019a,b). Reduced activity could indicate that after fin clipping, the activity of swimming is more painful, causing the fish to become more reluctant to swim (Schroeder and Sneddon, 2017). It may also represent a state of depressive activity or guarding behaviour following trauma, to prevent further damage and pain and to promote healing (Schroeder and Sneddon, 2017). The reduction in activity was greater when all fish had been fin clipped in a group compared with groups with one or three fish clipped. This seems intuitive that if all fish are affected similarly then overall activity will decline in relation to the number of fish affected. In that case it would be expected that there would be little change if only one fish is clipped within a larger group. However, this may be a consequence of the fin clipped fish displaying unusual behaviour related to the fin clip, causing it to be ostracised by the group, such that the other fish clustered together to avoid that one fish and thus moved around the tank less. Therefore, clustering of the other group members resulted in lower activity. Future studies should perhaps test this theory by employing larger tanks where zebrafish could move more freely, however, our stocking density is lower to that recommended at 5 fish per litre (Review in Graham et al 2018) whereas we held our fish at 2 fish per 3 litres to allow unrestrained expression of behaviour and zebrafish could choose to remain at a distance from injured individuals. The increased clustering does give weight to the idea that the fin clipped individuals are emitting alarm substance since this does elicit increased shoal cohesion in zebrafish (Spence et al. 2008) and as such groups with even one clipped fish would perceive any alarm substance as a potentially dangerous situation. However, our results

do not support this since lidocaine did prevent the reduced activity and increased clustering but only in the one and three fin clipped groups and further the fin clipped group with only one individual did not show significantly increased clustering.

In terms of number of number of fish receiving a fin clip, only the group with one individual clipped displayed a discernible difference but that was only seen in the amount of clustering. Time spent at the bottom and activity were the same as the 3 and 6 fin clipped groups. Recent evidence suggests that individual zebrafish recover more quickly in a group (White et al. 2017) and this has been related to the phenomenon termed social buffering where social support or being in a familiar group assists in reducing responses to threatening stimuli or events and has been observed in studies investigating fish through to mammals (Faustino et al. 2017; Oliveira & Faustino 2017). In a study by Deakin et al. (2019a) individual zebrafish displayed reduced complexity of swimming after a range of painful treatments including fin clipping and this was characterised by increased use of the bottom of the tank and a reduction in activity (Deakin et al. 2019b). The authors suggested that these behavioural responses may be reduced by social buffering and when one fish in the present study was fin clipped it would seem that clustering was reduced but no other behaviours were modified. Thus being in a social group may assist one individual recover but may not have an impact if more individuals are affected.

4.3 Impact of lidocaine

Lidocaine is a local anaesthetic with pain relieving properties and a number of studies have demonstrated that it is effective in reducing pain-related responses in fish (e.g. Mettam et al 2011) including zebrafish (Lopez-Luna et al 2017a,b,c,d; Schroeder & Sneddon 2017; Deakin et al. 2019a,b). We used a dose of 5mg/L dissolved in the tank water which is reported to be effective for individual zebrafish (Schroeder & Sneddon 2017; Deakin et al. 2019a,b). In

the case of vertical distribution, activity and clustering, lidocaine was effective in groups with one or three fish fin clipped but much less so for the groups with six fish. Lidocaine is a local anaesthetic that provides local or regional anaesthesia and is used in the management of acute and chronic pain (Shephard & Anandampillai 2019). The mechanism of action lidocaine exerts is by temporarily inhibiting voltage gated sodium channels in neuronal plasma membranes thus nociceptors do not convey information about painful stimuli (Sneddon 2012; Sloman et al. 2019). Very little is known about the pharmacokinetics or uptake of lidocaine in fishes and we assume that it exerts its action on the severed nociception neurons in the tail fin. Nociceptors have been described in another species of fish, the common carp (*Cyprinus carpio*) (Roques et al. 2010), and fin clipped zebrafish have a detectable whole body concentration of lidocaine 24 hours after administration using the same approach as the present study (Schroeder & Sneddon 2017). However, it may be that due to uptake rates of this small amount, 5mg/L, which were effective when one to three fish are clipped but not for 6 fish and thus higher doses may be required for more than 3 fish. Increased doses should be investigated when applying analgesia to groups greater than 3. There are a number of other analgesic drugs validated for use in fish and it might be more prudent to inject these directly into the fish to ensure effective analgesia is provided for each individual (review in Sloman et al. 2019). However, zebrafish are a relatively small species making injection problematic and logistically it would be easier to administer via immersion in the tank water. Any future research should take this into consideration and may wish to investigate other types of analgesics such as opioids and non-steroidal anti-inflammatory drugs which have shown to be effective in other studies (Taylor et al. 2017; Costa et al. 2019; Deakin et al. 2019b).

4.4 Future directions and limitations

The CFA demonstrated that chromatic analysis of video images was powerful enough to detect behavioural changes of groups of zebrafish when subject to fin clipping. However,

the system as it is may require further modifications before it can be used in the context of laboratory aquaria with existing tank set-ups and husbandry practices. However, this may be a useful tool in generating data on group behaviour in neurobiological research exploring the impact of a variety of pharmaceutical treatments relevant to neuroscience and possibly other fields. Light intensity would need to remain the same since the CFA measures this directly and any changes would affect the CFA values. Future studies should investigate larger stock sizes since the stocking density we chose is lower than the recommended density for zebrafish. However, since this approach does not measure individual behaviour and instead measures a group average increasing group size should be feasible. Studying other common laboratory fish species would allow extrapolation to other popular models (e.g. salmon, trout, stickleback, minnow and guppy). Further development is required to produce a real time monitoring system that employs alerts to notify researchers and animal technicians that there is a welfare issue within a group of fish, so that they can intervene to improve welfare.

4.5 Conclusions

Chromatic analysis does allow the identification of those groups of zebrafish subject to a potentially painful procedure and can be used as a tool to determine analgesic efficacy in future studies. To our knowledge this is the first application of this type of analysis to groups of fish and thus the CFA could be developed and used to improve the welfare assessment of groups of zebrafish with the aim of refining our existing protocols. Currently, fin clipping is seen as a mild procedure under the Home Office licensing legislation, however, mild procedures should only result in acute pain over a few hours (EC Severity Assessment, 2018). These results demonstrated that by 6 h zebrafish have not recovered and thus perhaps fin clipping is actually of moderate severity unless analgesia is provided.

Acknowledgements

L.U.S and J.W.S are grateful for research grant NC/K000888/1 from the National Centre for the Replacement, Refinement and Reduction of Animals in Research, UK (NC3Rs). Thank you to Samantha Moss, Rhys Sweeney and Barry Cotterill for technical assistance.

Conflict of Interest

The authors declare no conflict of interest.

Appendix A

The responses for the horizontal and the vertical sets of the spatial chromatic processors (shown in Fig. 1) for a (discrete) image $I(x, y)$, are obtained using the following equations (Al-Temeemy 2018; 2019):

$$P_{\Re 1}(l_{xy}) = \begin{cases} \frac{-l_{xy}+C_1}{C_2-C_1} & l_{xy} \in [2C_1 - C_2, C_1) \\ \frac{l_{xy}-C_2}{C_2-C_1} & l_{xy} \in (C_2, 2C_2 - C_1] \\ 0 & \text{Otherwise} \end{cases} \quad (1)$$

$$P_{\Re 2}(l_{xy}) = \begin{cases} \frac{l_{xy}-2C_1+C_2}{C_2-C_1} & l_{xy} \in (2C_1 - C_2, C_1) \\ \frac{-l_{xy}+C_2}{C_2-C_1} & l_{xy} \in [C_1, C_2) \\ 0 & \text{Otherwise} \end{cases} \quad (2)$$

$$P_{\Re 3}(l_{xy}) = \begin{cases} \frac{l_{xy}-C_1}{C_2-C_1} & l_{xy} \in (C_1, C_2) \\ \frac{-l_{xy}+2C_2-C_1}{C_2-C_1} & l_{xy} \in [C_2, 2C_2 - C_1) \\ 0 & \text{Otherwise} \end{cases} \quad (3)$$

Where $C_n = 1 + \left\lceil \frac{n(\ell_{xy}-1)}{3} \right\rceil, n = 1,2$

These responses $(P_{\Re 2}, P_{\Re 2}, P_{\Re 2})$ are functions of location parameter l_{xy} and can be used to represent both types of chromatic processors sets (horizontal and vertical). With the horizontal set representation, the responses length ℓ_{xy} is equal to the image $I(x, y)$ width, while its value is equal to the image height if these responses are used for presenting the vertical set.

The processors' outputs for both the horizontal $(HPo_{1,2,3})$ and the vertical $(VPo_{1,2,3})$ sets of the chromatic processors can be calculated using the following equations (Al-Temeemy 2018; 2019):

$$HPo_n = \sum_x P_{\Re n}(x) \sum_y I(x, y), n = 1,2,3 \quad (4)$$

$$VPo_n = \sum_y P_{\Re n}(y) \sum_x I(x,y)\,, n = 1,2,3 \quad (5)$$

Appendix B

The transformation of the processors outputs (for horizontal ($HPo_{1,2,3}$) and vertical ($HPo_{1,2,3}$) sets) to the chromatic coordinates (hue H, lightness L, and saturation S) is performed using the following relationships (Al-Temeemy 2018; 2019):

$$H = \begin{cases} 0.667 - 0.333\left(\frac{po_2}{po_2+po_3}\right), & po_1 = 0 \\ 1.000 - 0.333\left(\frac{po_3}{po_3+po_1}\right), & po_2 = 0 \\ 0.333 - 0.333\left(\frac{po_1}{po_1+po_2}\right), & po_3 = 0 \end{cases} \quad (6)$$

Where $po_n = Po_n - min\,, \quad n = 1,2,3$

$$L = \sum_{n=1}^{3} Po_n \;\; /3 \quad (7)$$

$$S = (max - min)/(max + min) \quad (8)$$

Where *max* and *min*, represents the processors' outputs ($Po_{1,2,3}$) having the highest and lowest values, respectively (Al-Temeemy 2018; 2019). In case of transforming the outputs for the vertical set of processors, the vertical hue value is equal to $1 - H$.

Appendix C: Raw data

Appendix D: Figure of Hue Vertical data

References

Al-Temeemy, A.A. 2019 Multispectral imaging: Monitoring vulnerable people. Optik, Elsiver, 180 (2019) 469 – 483 (2019). https://doi.org/10.1016/j.ijleo.2018.11.042.

Al-Temeemy, A.A. 2018 Human region segmentation and description methods for domiciliary healthcare monitoring using chromatic methodology. Journal of Electronic Imaging, SPIE, 27(2), 023005 (2018). https://doi.org/10.1117/1.JEI.27.2.023005.

Al-Temeemy, A.A. & Spencer, J.W. 2014 Laser radar invariant spatial chromatic image descriptor. SPIE- Optical Engineering, 53(12), 123109 (2014). doi:10.1117/1.OE.53.12.123109.

Al-Temeemy, A.A. & Spencer, J.W. 2015a Invariant chromatic descriptor for LADAR data processing.Machine Vision and Applications, Springer (2015), doi:10.1007/s00138-015-0675-0.

Al-Temeemy, A.A. & Spencer, J.W 2015b Chromatic methodology for laser detection and ranging (LADAR) image description. Optik - International Journal for Light and Electron Optics, Elsevier, Volume 126, Issue 23, December 2015, Pages 3894-3900, ISSN 0030-4026.

Alves, F L., Barbosa, A. and Hoffmann, A. 2013. Antinociception in piauçu fish induced by exposure to the conspecific alarm substance. *Physiology & Behavior*, **110–111,** 58–62.

Ashley, P.J., Ringrose, S., Edwards, K.L., Wallington, E., McCrohan, C.R. & Sneddon, L.U. Effect of noxious stimulation upon antipredator responses and dominance status in rainbow trout. Animal Behaviour 77, 403–410 (2009).

Bates, D, Machler, M, Bolker, B & Walker, S (2015) Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software 67: 1-48.

Chablais, F. and Jaźwińska, A. Induction of myocardial infarction in adult zebrafish using cryoinjury. Journal of Visualised Experiments 62, e3666, doi:10.3791/3666 (2012).

Clark, K.J.& Ekker, S.C How Zebrafish Genetics Informs Human Biology. Nature Education 8(4):3 (2015).

Correia, A. D., Cunha, S. R., Scholze, M. and Stevens, E. D. 2011. A Novel Behavioral Fish Model of Nocic eption for Testing Analgesics. *pharmaceuticals*. **4**, 665-680.

Costa. F. V., Rosa, L. V., Quadros, V. A., Santos A. R. S., Kalueff, A. V. & Rosemberg, D.B. Understanding nociception-related phenotypes in adult zebrafish: Behavioral and pharmacological characterization using a new acetic acid model. Brain and Behavioural Research 359, 570-578 (2019).

Deakin, A.G., Cossins, A.R., Spencer, J.W., Young, I.S. & Sneddon, L.U. 2019a Welfare challenges influence the complexity of movement: Fractal analysis of behaviour in zebrafish. Fishes, 4, 8.

Deakin, A.G., Buckley J., AlZu'bi, H.S., Cossins, A.R., Spencer, J.W., Al-Nuaimy, W., Young, I.S. Thomson, J.S. & Sneddon, L.U. 2019b Automated monitoring of behaviour in zebrafish after invasive procedures. Scientific Reports, MS under review.

EC Severity Assessment. 2018. Available online: http://ec.europa.eu/environment/chemicals/lab_animals/pdf/report_ewg.pdf (accessed on 18 December 2018).

Faustino, A.I.; Tacão-Monteiro, A.; Oliveira, R.F. Mechanisms of social buffering of fear in zebrafish. Sci. Rep. 2017, 7, 44329.

Graham, C, von Keyserlingk, MAG & Franks, B 2018. Zebrafish welfare: Natural history, social motivation and behaviour. Appl. Anim. Behav. Sci. 200, 13-22.

Huang, C. C., Lawson, N. D., Weinstein, B. M. and Johnson, S. L. 2003. reg6 is required for branching morphogenesis during blood vessel regeneration in zebrafish caudal fins. *Developmental Biology*, **264**(1), 263-274.

Jones, G.R., Deakin, A.G. & Spencer, J.W. Chromatic monitoring of complex conditions. Boca Raton ; London : CRC Press, 2008. 281pp.

Lemmens, K., Bollaerts, I., Bhumika, S., de Groef, L., Van Houcke, J., Darras, V. M., Van Hove, I. & Moons L. Matrix metalloproteinases as promising regulators of axonal regrowth in the injured adult zebrafish reti notectal system. Journal of Comparative Neurology 524, 1472–1493 (2016).

Levin, E. D., Bencan. Z. and Cerutti, D. T. 2007 Anxiolytic effects of nicotine in zebrafish. *Physiology and Behaviour*, **90**(1), 54–8.

Lopez-Luna, J., Al-Jubouri, Q., Al-Nuaimy, W. and Sneddon, L.U. Activity reduced by noxious chemical stimulation is ameliorated by immersion in analgesic drugs in zebrafish. Journal of Experimental Biology 220, 1451-1458 (2017a).

Lopez-Luna, J., Al-Jubouri, Q., Al-Nuaimy, W. & Sneddon, L.U. (2017b). Impact of analgesic drugs on the behavioural responses of larval zebrafish to potentially noxious temperatures. Applied Animal Behaviour Science 188, 97-105.

Lopez-Luna J. Canty MN, Al-Jubouri Q, Al-Nuaimy W, Sneddon LU. (2017c) Behavioural responses of fish larvae modulated by analgesic drugs after a stress exposure. Appl Anim Behav Sci, 195, 115–120.

Lopez-Luna, J., Al-Jubouri, Q., Al-Nuaimy, W. & Sneddon, L.U. (2017d). Impact of stress, fear and anxiety on the nociceptive responses of larval zebrafish. PLoS One 12, e0181010.

Mettam, J. J., Oulton, L. J., McCrohan, C. R. & Sneddon, L. U. (2011). The efficacy of three types of analgesic drugs in reducing pain in the rainbow trout, Oncorhynchus mykiss. Applied Animal Behaviour Science 133, 265-274.

Mezzomo, NJ, Fontana, BD, Muller, TE, Duarte, T, Quadros, VA, Canzian, J, Pompermaier, A, Soares, SM, Koakoski, G, Loro, VL, et al. 2019. Taurine modulates the stress response in zebrafish. Hormones and Behavior, 109, 44-52.

Oliveira, R.F.; Faustino, A.I. Social information use in threat perception: Social buffering, contagion and facilitation of alarm responses. Commun. Integr. Biol. 2017, 10, e1325049.

Reilly, S.C., Quinn, J. P., Cossins, A. R. and Sneddon. L. U. 2008. Behavioural analysis of a nociceptive event in fish: Comparisons between three species demonstrate specific responses. *Applied Animal Behaviour Science.* **114**(1-2), 248-259.

Roques, J. A. C., Abbink, W., Geurds, F., van de Vis, H. and Flik, G. 2010. Tailfin clipping, a painful procedure: Studies on Nile tilapia and common carp. *Physiology & Behavior,* **101**(4)**,** 533-540.

Schroeder, P. G. and Sneddon, L. U. 2017. Exploring the efficacy of immersion analgesics in zebrafish using an integrative approach. *Applied Animal Behaviour Science,* **187**, 93-102

Schweitzer, J., Becker, T., Becker, C. G. & Schachner, M. Expression of protein zero is increased in lesioned axon pathways in the central nervous system of adult zebrafish. Glia 41, 301–317 (2003).

Sheppard, HN & Anandampillai, R 2019. Systemic toxic effects of local anaesthetics. Anaesthesia And Intensive Care Medicine 20, 215-218.

Sloman K.A., Bouyoucos I.A., Brooks E.J. & Sneddon L.U. 2019 Ethical considerations in fish research. J. Fish Biol. 94, 556-577.

Sneddon, L. U. Clinical anesthesia and analgesia in fish. Journal Of Exotic Pet Medicine 21, 32-43, 2012.

Sneddon, L. U. Pain in aquatic animals. Journal of Experimental Biology 218, 967-976 (2015).

Speedie, N. and Gerlai, R. 2008. Alarm substance induced behavioral responses in zebrafish (*Danio rerio*). *Behavioural Brain Research,* **188**(1), 168–77.

Spence, R., Gerlach, G. Lawrence, C. & Smith C. The behaviour and ecology of the zebrafish, Danio rerio. Biol. Rev. Camb. Philos. Soc., 83 (2008), 13-34.

Taylor, J. C., Dewberry, L. S., Totsch, S. K., Yessick, L. R., DeBerry, J. J., Watts, S. A. and Sorge, R. E. A novel zebrafish-based model of nociception. Physiology & Behavior 174, 83-88 (2017).

UK Home Office 2018. https://www.gov.uk/government/statistics/statistics-of-scientific-procedures-on-living-animals-great-britain-2017.

Valentim, A.M., Felix, L.M., Carvalho, L., Diniz, E. and Antunes, L. M. 2016. A New Anaesthetic Protocol for Adult Zebrafish (*Danio rerio*): Propofol Combined with Lidocaine. *PLOS One.* **11**(1), 248-259.

Waldman, B. 1982. Quantitative and developmental analysis of the alarm reaction in the zebra Danio, *Brachydanio rerio*. *Copeia*, **1,** 1–9.

White, L. J., Thomson, J. S., Pounder, K. C., Coleman R. C. and. Sneddon, L. U. The impact of social context on behaviour and the recovery from welfare challenges in zebrafish, Danio rerio. Animal Behaviour 132, 189-199 (2017).

Wolkers, C. P. B., Barbosa, A., Menescal-de-Oliveira, L. and Hoffmann, A. 2013. Stress-Induced Antinociception in Fish Reversed by Naloxone. *Plos One*, **8**(7), e71175.

Figure Legends

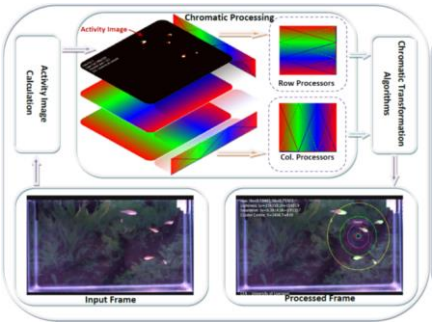

Fig. 1 Block diagram for zebrafish activity monitoring stages with sample input frame and its processing at each stage in the diagram.

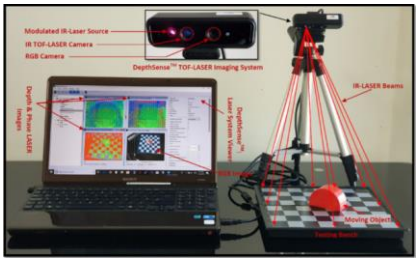

Fig. 2 Experimental setup used for evaluating the monitoring stags' performance.

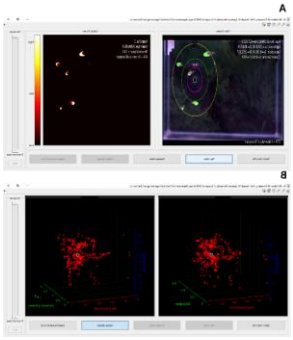

Fig. 3 CFA's Graphical User Interface and 3D representation for the chromatic parameters. (A) Graphical User Interface for the Chromatic Fish Analyser. (B) 3D representations for chromatic parameters and their average values.

Commented [TJ[3]: Reviewer #2 has made comments regarding the plot: "These values are plotted in a box and whisker (BW) plot, which first of all is not plotted correctly, and secondly is totally inadequate to plot this data. In Figure 4 for example, all 6 trials are shown as dots, apart from the box and whiskers. In a BW plot, one shows the median, certain quartiles, and percentiles and only the outliers as points. Furthermore, this is not the way this data should be presented. If the fish were to stay closer to the edges of the tank, as could be expected if they were in pain or stressed, the mean (and median) for this would be zero, just as if they were swimming uniformly throughout the tank."

My response:
The reviewer appears to have misinterpreted these figures. They are indeed box and whisker plots, but the group size of 6 reflects the number of fish within a social group, NOT the number of data within each experimental treatment. Sample size is indicated in figure legends. We have attempted to clarify the text where appropriate, including indication that the dots are outliers.
Furthermore, data represent change in average position or behaviour, as indicated in Section 2.4 – Statistical Tests. Thus mean/median of 0 would indicate that fish have not changed behaviour or position, on average, compared to the pre-treatment condition.

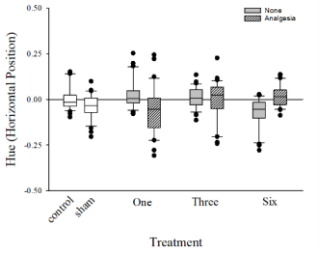

Fig. 4. Median change in average horizontal position (derived from hue obtained in video footage) observed in groups of six *D. rerio*. One, three or six members of the groups underwent a fin-clipping procedure and received either no analgesia (grey) or 5mg/L lidocaine as analgesia (hatched). Control groups were undisturbed; sham groups were removed from the tank and replaced without further procedure. N = 32 for all boxes. Boxes represent IQR, whiskers represent 10th and 90th percentiles, dots represent outliers.

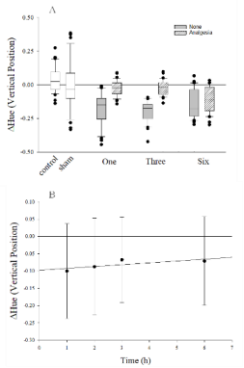

Fig. 5. Change in horizontal position. (A) Median change in average vertical position (derived from hue obtained in video footage) observed in groups of six D. rerio. One, three or six members of the groups underwent a fin-clipping procedure and received either no analgesia (grey) or 5mg/L lidocaine as analgesia (hatched). Control groups were undisturbed; sham groups were removed from the tank and replaced without further procedure. N = 32 for all boxes. Boxes represent IQR, whiskers represent 10th and 90th percentiles, dots represent outliers. (B) Mean changes in average vertical position (obtained as above) across all fish (N = 64 at each time point).

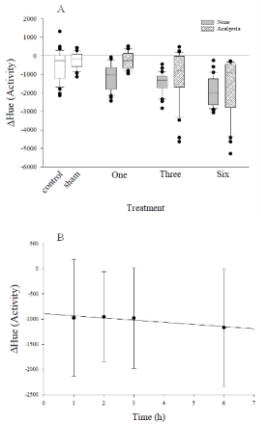

Fig. 6: Change in activity. (A) Median change in average activity (derived from lightness values obtained in video footage) observed in groups of six *D. rerio*. One, three or six members of the groups underwent a fin-clipping procedure and received either no analgesia (grey) or 5mg/L lidocaine as analgesia (hatched). Control groups were undisturbed; sham groups were removed from the tank and replaced without further procedure. N = 32 for all boxes. Boxes represent IQR, whiskers represent 10th and 90th percentiles, dots represent outliers. (B) Mean changes in average activity (obtained as above) across all fish (N = 64 at each time point).

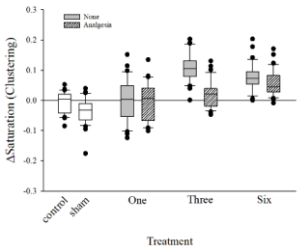

Fig. 7. Median change in average clustering (derived from saturation values obtained in video footage) observed in groups of six *D. rerio*. One, three or six members of the groups underwent a fin-clipping procedure and received either no analgesia (grey) or 5mg/L lidocaine as analgesia (hatched). Control groups were undisturbed; sham groups were removed from the tank and replaced without further procedure. N = 32 for all boxes. Boxes represent IQR, whiskers represent 10th and 90th percentiles, dots represent outliers.

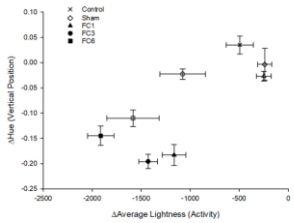

Fig. 8. Relationship between change in vertical position and activity. Mean (± SE) change in vertical position (derived from hues in video footage) against change in activity level (derived from lightness values in video footage) observed in groups of six *D. rerio*. One, three or six members of the groups underwent a fin-clipping procedure and received either no analgesia (black symbols) or 5mg/L lidocaine as analgesia (empty symbols). Control groups were undisturbed; sham groups were removed from the tank and replaced without further procedure. N = 32 for all groups.

Table 1

Treatments employed during the experiment where zebrafish were held in groups of six (n=6 replicates for each treatment; analgesic was lidocaine at 5mg/L tank water).

Abbreviation	**Treatment**
FC1	1 fish fin clipped (no lidocaine)
FC3	3 fish fin clipped (no lidocaine)
FC6	6 fish fin clipped (no lidocaine)
FC1+L	1 fish fin clipped + lidocaine
FC3+L	3 fish fin clipped + lidocaine
FC6+L	6 fish fin clipped + lidocaine
Control	Control (no treatment)
SHAM	Sham handled (1 fish anaesthetised only with no fin clip)

Table 2

Parameters measured by the chromatic fish analyser (CFA) from video images of zebrafish behaviour.

Measure	**Description**
Average Hue Values in Vertical Direction (Hv)	Average position of group across a vertical axis of the tank.
Average Hue Values in Horizontal Direction (Hh)	Average position of group across a horizontal axis of the tank.
Average Saturation Value (A.S)	Average distribution of group within tank. (The higher the value, the more clustered the group are).
Average Lightness Value (A.L)	Average activity level of group. (The higher the value, the more active the group are).