Understanding how animals experience stress in a laboratory environment is crucial for improving their welfare. Increasing numbers of fish are being used in scientific studies, therefore, further research is required to ensure appropriate conditions are used to promote good conduct and correct housing as well as guaranteeing scientifically valid results. Zebrafish are a gregarious species thus social enrichment is particularly important, with individuals separated from a group experiencing isolation stress. The present study aimed to determine the effects of social context on recovery from common laboratory procedures. Additionally, this study investigated whether the non-invasive measure of water-borne cortisol can be utilised to gauge physiological stress by comparing it to an invasive measure, whole body cortisol concentration. Zebrafish (AB strain, male) were housed in differing social contexts and were randomly assigned to one of three treatment groups: undisturbed, anaesthetised and handled, or anaesthetised and fin clipped. Behavioural and physiological stress indicators were recorded before and after treatment. The data indicated social context, in the form of group housing, was important in enhancing recovery from welfare challenges in zebrafish, since group housed fish resumed normal behaviours more quickly than pairs or individuals. Moreover, the strong correlation between water-borne and whole body cortisol suggests that the non-invasive measure is an appropriate ethical alternative as an indicator of physiological stress. These findings represent an important refinement in reducing the severity of stress through housing zebrafish in their original groups and by adopting a non-invasive measure of cortisol which will act to reduce the numbers of individuals required for time series studies on physiological stress.

Key words: *Danio rerio,* fish welfare, recovery, social enrichment, stress

Stressors are known to have an effect on an animal’s behaviour and physiology; although a natural, adaptive response this can be exacerbated in laboratory animals when improper husbandry is applied. If chronically exposed, this can have a negative effect on the animal (e.g. Sneddon, Wolfenden, & Thomson, 2016). Animal models are important in laboratory research across broad areas of biology, biomedical science and toxicology (V Baumans, 2004). The incorporation of animals into scientific research has led to the production of legislation promoting good husbandry in experimentation aiming to ensure humane and ethical methods are applied (J. D. Clark, Gebhart, Gonder, Keeling, & Kohn, 1997). This extends to the housing of animals in laboratories with the purpose of improving welfare (e.g. for all adult vertebrates and cephalopods in the EU termed here as protected animals (European Directive, 2010)). It is understood that improving an animal’s welfare is important for its wellbeing as well as ensuring the reliability and validity of collected data (Van de Weerd et al., 2002). By reducing stress, interpretations of vertebrate responses within studies will not be confounded by variation in stress axis activation. Much research aimed at reducing stress in protected laboratory species has led to better standards that maintain health and welfare however, this is mainly restricted to rodent species (Vera Baumans, 2005; Gaskill, Karas, Garner, & Pritchett-Corning, 2013; Gaskill, Rohr, Pajor, Lucas, & Garner, 2011; Simpson & Kelly, 2011; Singhal, Jaehne, Corrigan, & Baune, 2014; Wells, 2009). With the number of non-rodent protected animals used in experimentation increasing, more must be done to ensure that housing standards continue to improve in all species used in laboratories settings (Goodman, Chandna, & Roe, 2015; Walker, Diez-Leon, & Mason, 2014) and that consistent housing conditions are employed to ensure reproducibility between laboratories (Richter, Garner, & Würbel, 2009).

Fish have become a predominant vertebrate model ranking second in terms of numbers used in experimental procedures in the UK (Home Office, 2015), Europe (Europa, 2015) and elsewhere (e.g. USA; USDA, 2013). Empirical evidence suggests that fish experience stress (Wendelaar Bonga, 1997) and respond during pain with underlying mechanisms comparable to mammals (Sneddon, 2015). Due to their similarities with the human genome, developmental mechanisms and a variety of other advantageous attributes including small size, rapid development and short generation time (K. Clark & Ekker, 2015; Lawrence, 2007), the zebrafish (*Danio rerio*), a model species in research, is often kept at relatively high density (Lawrence, 2007; RSPCA, 2012). Research on zebrafish husbandry has shown that anxiety (increased avoidance and decreased exploration) is seen to decrease when environmental enrichment is included within their tanks (Manuel et al., 2015). Physical enrichment such as substrates and artificial plants in the tank are preferred by zebrafish when given the choice between enriched and barren tanks (Schroeder, Jones, Young, & Sneddon, 2014). Providing enrichment to individually housed rainbow trout (*Oncorhynchus mykiss*) improved their welfare and they recovered more quickly from stressors (Pounder et al., 2016). Social enrichment is also crucial, especially in gregarious fish such as zebrafish(Spence, Gerlach, Lawrence, & Smith, 2008), where individuals separated from the group may experience isolation stress (Rosemberg et al., 2011). These stressful instances lead to a number of behavioural and physiological changes in fish that can be quantified (e.g. responses to social isolation, Shams, Chatterjee & Gerlai, 2015).

The fish stress response is directly comparable to humans with cortisol production coordinating biochemical and physiological changes during stress (Wendelaar Bonga, 1997). For example, both [corticotrophin-releasing factor](http://topics.sciencedirect.com/topics/page/Corticotropin-releasing_hormone) (CRF) and [proopiomelanocortin](http://topics.sciencedirect.com/topics/page/Proopiomelanocortin) ([POMC](http://topics.sciencedirect.com/topics/page/Proopiomelanocortin)) mediate the initial cascade of the hypothalamus-pituitary-interrenal axis in fish and both increase in transcript expression upon experiencing a stressor (Steenbergen, Richardson, & Champagne, 2011). As part of this axis they are directly responsible for the release of adrenocorticotropic hormone (ACTH) from the anterior-pituitary gland and subsequently the production and release of the stress hormone cortisol from the fish head-kidney into tissues and blood plasma (Wendelaar Bonga, 1997). Measurement of gene expression has demonstrated specific transcripts are also responsive under stressful instances (see SI for gene expression conducted in the present study). There are not only molecular and physiological alterations but there are often behavioural changes. Behaviours often noted in past literature as being good indicators of stress in zebrafish include increased number of erratic movements, increased time spent in the bottom of the tank (K. Wong et al., 2010), stronger shoal cohesion (Piato et al., 2011) and reduced aggression (Gerlai, Lahav, Guo, & Rosenthal, 2000).

Stress responses are usually recorded as elevation of cortisol in plasma. For large fish this can be ascertained, though invasively, through taking a blood sample from the caudal vein. However, this procedure is problematic if fish are too small, rare or valuable to produce enough blood for sampling (Scott et al., 2008). Being a small species *D. rerio* must be euthanised and whole body homogenates produced to gain a large enough sample to quantify cortisol levels (Feist & Schreck, 2001; Ramsay et al., 2006). However, cortisol is freely available to diffuse across the gills (Mota, Martins, Eding, Canário, & Verreth, 2014). With this in mind recent research has focused on the use of water-borne cortisol, shown to be present in the holding tanks of zebrafish (Félix, Faustino, Cabral, & Oliveira, 2013). A common method to measure the cortisol present in water is the “beaker method”; here a single zebrafish is placed into a beaker of known volume and allowed to acclimatise for a given time. The water is then measured for the amount of cortisol present. Problems with this method causing increased levels of stress have been noted due to the novelty of the environment as well as the beaker eliciting confinement and isolation stress (S. C. Wong, Dykstra, Campbell, & Earley, 2008). Thus finding a suitable, non-invasive method for ascertaining stress levels in fish is an important goal.

Social enrichment for gregarious fish such as the zebrafish (Pickering & Pottinger, 1989) is generally accepted as good practice to maintain an optimal density in a given tank to reduce stress levels (Lawrence, 2007; Ramsay et al., 2006) . This husbandry practice however does not always apply to conditions during experimentation, where fish may be housed individually during and after a procedure or when breeding fish in pairs. Therefore, the present study aims to examine the impact of group size on behaviour and the recovery from fin clip in zebrafish, *D. rerio* and to observe changes in behaviour and physiology with exposure to both a stressful and painful stimulus*.* It was hypothesised that individuals housed socially in groups are in more favourable conditions so will recover more quickly to any stressors and show more natural behaviour. Furthermore, this study examined the correlation between whole body cortisol levels and water-borne cortisol siphoned from the home tanks of experimental fish to validate the use of such a non-invasive procedure as a measure of physiological stress.

METHODS

*Ethics Note*

Experiments were conducted with approval from the Home Office, U.K. (licence no. PPL 40/3534) and the University of Liverpool’s Ethics committee. Caudal (tail) fin clipping is routinely conducted to verify the genomics of zebrafish strains and transgenic or mutant lines and has rarely been subject to study for the welfare consequences of this procedure. To our knowledge only one study has been published demonstrating that fin clipping results in changes in behaviour and physiology indicative of pain in zebrafish that is ameliorated by pain-relieving drugs (Schroeder & Sneddon, 2017). Therefore, we conducted a power analysis prior to the experiment to ensure sample sizes were kept to an effective minimum due to the invasiveness of the procedures conducted (based upon previous results from zebrafish on rate of swimming taken from Reilly et al., 2008; Cohen’s d = 0.8; α = 0.05; effect size = 0.786; n = 6, Control mean = 14.9/min (SD = 3.0); Pain mean = 6.0 (SD = 3.9) calculations done in statistical software, R). Before the experiments fish were monitored at least twice daily for health and to ensure normal feeding of all fish prior to use. Water quality parameters were optimal for zebrafish (pH 7.2; ammonia <0.1mg/l; nitrite <0.1mg/l; nitrate <20 mg/l) during the entire experimental period. During the experiment fish were handled carefully and monitored continuously until the end of the experiment with a view of euthanasing any fish that showed an exaggerated response to anaesthesia or anaesthesia and fin clip. However, none did. Experiments were terminated at 3 h after fin clip when enough data had been collected to meet the objectives thus fish that were fin clipped experienced this for a relatively short amount of time (humane end point; pilot data and Schroeder and Sneddon (2016) suggest fin clipping affects behaviour for over 6h and recovery is seen at 24 h). For anaesthesia we used benzocaine which may be aversive to the fish (Readman, Owen, Murrell, & Knowles, 2013; D. Wong, von Keyserlingk, Richards, & Weary, 2014) and is known to cause a stress response (Sneddon, 2012). However, due to its local anaesthetic or pain relieving properties in mammals we adopted this to potentially ensure analgesia during clipping (Sneddon, 2012). Benzocaine is known to be eliminated from the body of the fish within 15 minutes and is unlikely to affect the results of our first behavioural observation at 1 h (Sneddon 2012). At the end of the experiment, fish were gently caught by netting and killed using concussion and removal of the brain with tissues harvested for use in this or other studies.

*Experimental Procedure*

Experiments were carried out between October 2014 and February 2015. Single male zebrafish (AB strain, n=54, average weight 0.46 ±SD 0.1g; age 7-8 months) were selected as focal fish and taken at random from stocks from the University of Liverpool aquarium in-house breeding project. Stock zebrafish were maintained in a semi-closed recirculation system in 10 litre tanks (Aquatic Habitats, Florida USA) at 27 ± 1 oC, with constant aeration on a 12:12 h light: dark cycle. Individuals were selected at random, netted carefully from stock tanks and placed into observation tanks (5 L; Aquatic Habitats, Florida USA). These were randomly allocated to one of three treatments (Control, Anaesthetic and Fin Clip with anaesthetic) and either held individually, in a male/female pair or in a group of six, focal fish were always the largest for identification purposes (n = 6 replicates per treatment). Tanks were screened from each other to prevent visual disturbance. Fish were given two weeks to acclimatise before any experimentation was carried out. Controls were left undisturbed for the entirety of the experiment across all time points. Individuals in the Anaesthetic and Fin Clip with anaesthetic treatments were gently caught by netting, transferred to, and anaesthetised in a 1 litre beaker containing 500ml of aerated water dosed with benzocaine (0.033g L-1; Sigma-Aldrich Co. UK) until reaching deep plane anaesthesia. Fin Clip treatment involved the removal of 40% of caudal fin with sterilised surgical scissors whilst on a bed of wet blue roll with head covered as described in the Zebrafish handbook (Westerfield, 2000). Anaesthetic treated fish were handled for a similar time as if being subjected to the Fin Clip procedure but without undergoing surgery, before being returned to the tank. All fish recovered from anaesthesia in their home tank.

Fish were not fed on the day of experimentation to prevent the presence of food having an effect on behaviour and because cortisol levels are known to increase upon feeding (Boujard & Leatherland, 1992). All procedures were conducted at the same time of day to prevent any diel effects on physiology and behaviour (Dickmeis et al., 2007). Flow through of water into the tank was ceased for 30 minutes, since this is the duration required for cortisol excreted across the gill to be representative of a specific time point (Scott et al., 2008) and the fish allowed 10 minutes to habituate to the presence of the observer. Behaviours were measured by direct observation for 10 mins and the first water sample taken via a siphon tube to obtain normal values of behaviour and cortisol. The focal fish was then carefully removed by netting and subjected to treatment whereas the remaining individual/group members (where applicable) were left undisturbed. Likewise, control fish were left undisturbed. Behaviours were recorded at hourly intervals over a three-hour period following treatment and water samples taken at the one and three hour time points. Two behavioural scores were used in this instance. Firstly, the number of erratic movements exhibited by focal fish in a 10 minute interval, with an erratic movement defined as any sharp, rapid, unexpected change in direction during swimming (K. Wong et al., 2010). Secondly, the amount of time (s) the focal fish spent in the bottom third of the tank during the same 10 minute period; the bottom third was represented by a line drawn on each tank which precisely measured this area. Similarly to rodents, where anxiety or stress leads to spending greater amounts of time close to the walls for instinctive protection, zebrafish are seen to spend more time in the bottom third of the tank when stressed (Egan et al., 2009). After each water sample was taken the inflow was turned on to replenish the tank with water from the recirculating aquaculture system (RAS) to maintain a constant water level and to flush out the previous water (took 30 min). Blank water samples (n =3) were taken at each sampling point directly from empty tanks on the RAS to account for residual cortisol levels (ranged from 35 - 306 pg/cartridge and blank values were subtracted from the corresponding experimental sample taken at the same time) (Scott & Ellis, 2007). After experimentation and data collection, the focal fish were killed and weighed; the brains were removed and frozen at -80⁰c for gene expression analysis (see SI) whilst the bodies were flash frozen on dry ice before being stored at -20°C for cortisol extraction at a later date. Freezer storage of samples is not known to have any significant effect on steroid concentration (Ellis, James, Stewart, & Scott, 2004). Water cortisol levels at the end of the experiment were used to correlate whole body cortisol with water samples. The weight of the focal fish and all fish in a given tank were recorded to calculate the cortisol concentration in ng cortisol / g fish / h and body cortisol in ng cortisol / g fish.

*Water sampling*

Water samples were collected via siphons inserted into the tanks into two clean 120ml containers. Samples were taken before the treatment was implemented to record baseline concentrations of cortisol and then at the 1 and 3 h time points. Cortisol persists for up to 2h after excretion into the surrounding water thus breakdown of cortisol is unlikely to affect our samples (Scott & Ellis, 2007). Following collection samples were immediately frozen at -20°C and subsequently allowed to thaw for 24 hours before cortisol extraction. To remove particles that might obstruct cartridges, water samples were filtered with 0.45 micron nitrocellulose filters (10 cm diameter) (Whatman, UK) before undergoing solid phase extraction using Sep-pak cartridges C-18 fitted to the outlet tube of a mini-pulse machine set at 25ml min-1. Cartridges were first primed with 5ml of methanol and washed through with 5ml of distilled water. The water sample collected was then passed through the cartridge, before a final wash of 5 ml of distilled water to purge salts. All cartridges were labelled and allowed to dry before being frozen for future analysis (-20°C). Cortisol was assayed blind in 200 μl of eluate using a validated radioimmunoassay for both water and whole body samples (Pottinger & Carrick, 2001).

*Whole body cortisol*

Focal fish were taken directly from the freezer and their length measured (mm); they were then broken up in preparation for homogenisation with a single-edged blade in a glass petri dish. The tissue was added to Tris-HCL buffer solution (4:1, volume: weight) in a 12ml centrifuge tube and subsequently homogenised using an Ultra-Turrax T25 Basic homogeniser (IKA, Germany) whilst keeping the tube on ice. Aliquots were taken from the homogenate and transferred to capped 1.5ml centrifuge tubes for cortisol extraction. Samples were analysed blind for cortisol.

*Statistical analysis*

All analyses were performed in R (v. 3.2.3; R Core Team, 2015), unless otherwise stated. Water cortisol concentrations were corrected by removing the concentration of background cortisol (blanks) and all cortisol values were log transformed to meet the assumptions of the analysis.

A mixed model approach was used to analyse number of erratic movements, time spent in the bottom third of the tanks and water cortisol levels for the effects of group size, treatment and time, each considered as fixed factors, and any interactions thereof, with individual fish included as a random effect. In each case, for significant terms *post-hoc* pairwise comparisons were performed within each group size (single/paired/groups) to compare between treatment and control at each time point (to determine whether there was any effect of the treatment on behaviour or stress; two-sample *t*-tests) and within treatment comparisons of each time point to the pre-treatment condition (to determine how responses to these treatments changes over time and over what time scales recovery may occur; paired *t*-tests). Associated *p* values for multiple comparisons were corrected using a Holm-Bonferroni procedure; since this technique is considered conservative (i.e. may inflate the rate of Type II errors) all comparisons where *P* <0.05 are presented, with those still significant after correcting for multiple comparisons further highlighted. The Holm-Bonferroni procedure was applied separately for each such set of comparisons, rather than as a single experiment-wide correction. Cortisol concentrations, extracted from whole-body homogenates, were analysed by two-way ANOVA to explore the effects of group size and treatment. To test for correlation between the levels of cortisol found in whole body homogenates and the water borne cortisol concentrations a Pearson’s correlation coefficient was applied (using Minitab v17).

RESULTS

*Number of erratic movements*

The three-way interaction of group size, treatment and time had a significant influence on the number of erratic movements (*F*12,135 = 7.12, *P* < 0.0005). Control fish did not change their behaviour throughout the experiments regardless of group size. However, both anaesthetised and fin-clipped fish, when held in pairs or groups, immediately and significantly reduced the number of erratic movements which slowly increased with time after treatment; this change was more pronounced among fish held in pairs (Figure 1a) which did not recover over the time course of the experiment. Fish, Anaesthesia and Fin Clip with Anaesthesia, held singly exhibited a significant and temporary increase in the number of erratic movements at the 1 h time point (Figure 1a). However, the lowest number of erratic movements overall appeared to be in the fish held singly, whereas fish held in pairs appeared to exhibit the most (at least prior to treatment).

*Time spent in the bottom third of tank*

The three-way interaction of group size, treatment and time had a significant influence on the amount of time fish spent in the bottom third of the tank (*F*12,135 = 3.983, *P* < 0.0005). The overall lowest amount of time spent in the bottom third of the tank was exhibited among fish held in groups, with those in pairs seemingly spending the most time in the bottom third (Figure 1b). Regardless of group size control fish did not alter their behaviour over time (aside from a slight decrease in those held in pairs by the third hour). However, fish exposed to anaesthesia or to the fin clip under anaesthesia increased time spent in the bottom third of the tank compared to pre-treatment levels when held singly or in pairs but note Anaesthesia only fish had relatively low values pre-treatment and the increase in time spent in the bottom of the tank after anaesthesia is comparable with Control values (Figure 1b). Single fish recovered within the duration of the experiment, whilst those held in pairs appeared to show signs of recovery but did not return to pre-treatment levels within 3 hours. Anaesthetised fish also temporarily increased the time spent in the bottom third of the tank when held in groups, but returned to normal behaviour after three hours.

*Cortisol*

There was a significant interaction between treatment and time (*F*4,72= 42.21, *P* < 0.0005) as well as a main effect of group size (*F*2,72 = 11.21, *P* = 0.0002) on the amount of water-borne cortisol detected (Figure 2a). The fin clip treatment had the largest increase in water cortisol levels, with a smaller increase seen in the anaesthetic treatment, regardless of group size. Water cortisol concentrations plateaued one hour post treatment among fin-clipped fish, but appeared to return to approximately pre-treatment levels in anaesthetised fish after 3 hours since there was no statistical difference between these time points. Log-transformed water-borne cortisol levels did not differ between groups of fish and fish held singly, though cortisol was significantly higher among paired fish than either single or group-held fish (Figure 2b).

The two-way ANOVA testing the effects on whole body cortisol levels revealed a significant effect of group size (*F2* = 6.62, *P* = 0.003) and treatment (*F2* = 109.66, *P* < 0.001) on cortisol concentration (Figure 3). Fish held in groups exhibited the lowest cortisol response overall, paired fish the highest whilst fish held singly did not significantly differ to either according to *post-hoc* analysis. The fin clip treatment produced the largest increase in cortisol (Figure 3). There was a strong positive correlation between whole body cortisol and water-borne cortisol collected from tank water (r = 0.967, *P* < 0.001, *N* = 54) (Figure 4).

DISCUSSION

This study demonstrated the effects of social context on an individual’s behaviour and the recovery from stressors in zebrafish as well as the magnitude of indicators for stress and pain in individuals housed in different social conditions. Anaesthesia alone and anaesthesia plus Fin Clipping were seen to have significant impacts on behavioural and physiological stress responses in zebrafish held under differing social contexts. Individually housed zebrafish increased the amount of time spent in the bottom third of the tank and behaved more erratically, suggesting enhanced anxiety and stress. Zebrafish housed in pairs and groups also increased the amount of time spent in the bottom of the tank (except the group-housed fish exposed to the fin clip), but the number of erratic movements displayed decreased for both stressors with fish appearing to be less active. Thus social context modulates the behavioural responses to stress and pain. As expected both whole body and water-borne cortisol concentrations increased after a stressor, reflecting an enhanced physiological stress response to these procedures, and this was more pronounced for zebrafish held in a pair and for those exposed to the Fin-Clip. The strong correlation between these two parameters then supports the use of water-borne cortisol as an exact, non-invasive measure of stress.

*Behavioural indicators of stress*

Zebrafish exposed to either Anaesthesia or Anaesthesia and Fin Clip treatments spent the majority of time at depth with increased use of the bottom of the tank possibly as an anti-predator strategy or anxiety behaviour, as observed in a variety of other species including zebrafish (e.g. rats: Katz et al., 1981; red-jungle fowl: Zidar and Løvlie, 2012; zebrafish: Champagne et al., 2010). The Fin Clip treatment in grouped fish, however, did not increase their use of depth and their response was consistent with the Control, undisturbed fish. Lack of behavioural responses to a painful treatment was recorded in group housed rainbow trout where exposure to a noxious substance did not result in the same responses that are shown in trout held individually (Ashley et al., 2009). One explanation may be related to stress induced analgesia occurring in group housed fish since they additionally had higher cortisol concentrations: the physiological stress response activates the hypothalamus-pituitary-interrenal axis which, as well as leading to the production of cortisol, stimulates the secretion of other molecules including the natural analgesic beta-endorphin (Charmandari, Tsigos, & Chrousos, 2005), allowing allocation of resources to defence strategies at a cost to exhibiting nociception (Maximino, 2011). Other animals may avoid acting abnormally after painful treatment when held in a group in an attempt to elude attracting attention to themselves either to maintain status within the dominance hierarchy (Paull et al., 2010) or avoid detection by a predator, as is seen in other prey animals (Mayer, 2007). Individually housed zebrafish exhibited an increase in the number of erratic movements upon experiencing the stressful treatments as is the case in other studies (Cachat et al., 2010; Egan et al., 2009), whereas pairs and groups in the present study exhibited a decrease in movement. Zebrafish are known to form dominance hierarchies in which larger individuals show aggression and increased dominance over smaller subordinate individuals (Paull et al., 2010) although, in the present study, similar sized individuals were used in the paired treatment and focal fish were always the largest in group housed fish. It is possible that stressed individuals, when housed with conspecifics, reduced their movement to avoid attracting increased aggressive interactions or reduce energy use to allow homeostasis to return. In pairs the dominance-subordinant relationship leads to increased aggression between individuals. If individuals experiencing stress or possible pain reduce their movements to avoid engaging with their tank mate then this is confirmed by the results of the present study since the greatest change in number of erratic movements was recorded in pairs. Of course, conservation of energy and prevention of further pain could also explain the reduction in movement. Further studies may be required to establish how group size effects social interactions, however, the present study indicates that behavioural indicators of stress are exaggerated in pairs and that housing in larger groups may be beneficial.

*Physiological stress response*

Measures of physiological stress through sampling of whole body and water-borne cortisol levels demonstrated that treatment and group size have an effect on the amount of cortisol produced. Anaesthesia and Fin Clipping resulted in enhanced cortisol concentrations in all social contexts. Whole body cortisol is known to increase during a stressful situation in fish and is also a graded response: the increase is proportional to the perceived severity of the situation (Barcellos et al., 2007). In the present study, fish within the Fin Clip treatment had the highest levels of cortisol after whole body analysis suggesting this was the most severe stressor. The enhanced stress response could be required for its analgesic properties via stress induced analgesia produced by the POMC derivative, beta-endorphin (Charmandari et al., 2005). This would have to be confirmed by further molecular analysis to determine the levels of beta-endorphin produced to confirm whether individuals exposed to painful treatment have higher concentrations of endorphins. Water-borne cortisol was seen to increase after Anaesthesia and handling but was highest after Fin Clipping. Since cortisol is known to freely diffuse across the gills (Mota et al., 2014) it is no surprise that concentrations rose concordant with whole body cortisol in response to stress and pain. An alternate explanation could be that increased cortisol emanates from the severed blood vessels of the tail fin, however, bleeding ceases on average within an hour in zebrafish subject to tail fin clip (Huang et al. 2003) making it unlikely that this route was solely responsible for cortisol concentrations after 3 hours. The present study has shown that water-borne cortisol collected from the home tank can too be considered a valuable non-invasive measure (Barcellos et al., 2007). The present study also showed interesting differences in the expression of genes in the brain of zebrafish which are believed to be important for the stress response. These findings somewhat reflect the results from the cortisol analysis (see SI).

*Impact of social context upon recovery*

An individual’s social context differentially affected the time course of physiological and behavioural stress responses in the Anaesthetic and Fin Clip treatments for both behavioural scores and for water cortisol concentrations. Trends towards recovery are seen in the behavioural scores for individuals and group housed fish, however, when comparing pre-treatment performance of these behaviours they were seen to be considerably higher in individual fish. This was as expected due to the increased stress of social isolation in this gregarious species. Water-borne and whole body cortisol concentrations are known to be elevated in socially isolated zebrafish (Ziv et al., 2013). Therefore, any recovery towards normal levels in these individuals is confounded by the fact that their stress levels exceed those of fish allowed to exhibit more natural shoaling behaviours in groups. Paired fish on the other hand exhibited no recovery over the length of the experiment for behavioural responses, tending to show a more exaggerated reaction. The absence of any return to normal levels could be explained by the aforementioned dominance hierarchy; through increased instances of social defeat, driven by focused attention of a single opponent, acting as an additional stressor thus recovery could be delayed if the fish in a pair is already experiencing chronic social stress or if a more dominant fish is expending greater energy to maintain high status (Larson, O’Malley, & Melloni, 2006). Whole body cortisol was affected by group size, whereby group housed fish had a lower response. Control and Anaesthetic treatments were highest suggesting lower levels of stress. Social context in the form of group housing may enhance the recovery from welfare challenges in zebrafish indicating that being returned to a familiar group may be beneficial after experimental surgical procedures. Whereas housing in pairs should be avoided since detrimental effects were observed in the present study.

*Non-invasive vs invasive measures of cortisol*

Invasive techniques in measuring stress in fish are often detrimental (Scott et al., 2008). By establishing if there is a correlation between water-borne cortisol collected from tank water and whole body cortisol concentrations evidence may be gained for the adoption of non-invasive measures of stress over the potentially fatal invasive measures. Additionally, by utilising the siphon method of tank water collection over the “beaker method” (S. C. Wong et al., 2008), where confinement stress may become problematic, more reliable results may be achieved. Indeed the collection of water cortisol using the beaker method is exceptionally stressful with values 9-14 fold higher for the siphon method in zebrafish (Sneddon et al., unpub. data). The strong correlation between water-borne cortisol concentration and whole body sampling indicates that this non-invasive technique will be valuable in detecting physiological stress in the future. Previous studies comparing water-borne cortisol to whole body homogenates have been undertaken for zebrafish, determining that the time lag between increases in plasma cortisol and a detectable increase in the tank water is around 30 minutes (Pavlidis et al., 2013). In the present study, by using the siphon method any stress possibly induced by collecting water samples was avoided. Additionally, cortisol is known to accumulate in the RAS (Mota et al., 2014), therefore, it is necessary to account for background cortisol levels to gain a more accurate reading of concentration changes over time and hence water samples were taken directly from the RAS. Taking these factors into consideration, water-borne cortisol is shown to strongly correlate to the physiological stress response in the body, validating its use, as is the case for a number of other fish species (e.g. sea bass: Fanouraki et al., 2008 ; rainbow trout: Ellis et al., 2004) . This acts as an important refinement reducing the severity of measures of stress and also reducing the numbers of fish in time series studies.

*Conclusions*

Behavioural responses to anaesthesia and fin clipping after anaesthesia were seen across all social housing conditions, however, there were differences in the extent and direction of behavioural and physiological stress indicators. Moreover, there were clear differences observed over time between group sizes suggesting that an individual’s social context influences the magnitude of response and how quickly those individuals are able to recover. The present study has shown that AB strain male zebrafish appear to be less affected when housed in groups. Thus using the behavioural and physiological parameters in the present study, social enrichment may aid recovery from stressors. By housing gregarious experimental animals in social groups, as is natural, their welfare and hence well-being may be improved. However, as we cannot enter the animal mind and truly know what an animal experiences there may be alternative explanations for the enhanced recovery of group housed animals. They may experience the same pain as individually or pair housed fish but are unwilling to show it to either maintain their dominance status (e.g. in rainbow trout, Ashley et al 2009) or as an anti-predator response so that their behaviour conforms to the group norm (e.g. cats, rodents rabbits, Lockhead 2013). Future studies could investigate this by assessing behaviour in zebrafish returned to an unfamiliar group or by measuring behavioural responses of group held fin clipped fish in isolation rather than returning them to a group. Furthermore, evidence for stress induced analgesia is growing in fish (Alves, Júnior, & Hoffmann, 2013) and given the group fish had the highest cortisol concentrations it may be that endorphins are acting as natural pain-relief resulting in group fish displaying less signs of pain: this could be explored by using the opioid antagonist naloxone which would block the action of endorphins and this approach has been demonstrated in another species of fish (Wolkers, Junior, Menescal-de-Oliveira, & Hoffmann, 2013). This study has validated the use of non-invasive measures of cortisol providing an important refinement to current protocols and allows us to reduce the numbers of zebrafish used in such studies since time series data can be collected on the same individuals without terminal sampling for whole body or plasma. Improving the husbandry of laboratory species will potentially ensure validity of scientific outputs and although the present study provides valuable information on recovery from stress and pain further research on females, mixed sex and other strains of zebrafish are needed to confirm these findings apply to all zebrafish.

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Appendix:

Gene expression analysis

Brains taken from zebrafish in the main study were subject to RT-PCR for four candidate genes: CRF, POMC, GABAa receptor and ependymin. Both corticotrophin-releasing factor (CRF) and proopiomelanocortin (POMC) mediate the initial cascade of the hypothalamus-pituitary-interrenal axis in fish and both increase in transcript expression upon experiencing a stressor (Steenbergen et al., 2011). As part of this axis they are directly responsible for the release of adrenocorticotropic hormone (ACTH) from the anterior-pituitary gland and subsequently the production and release of the stress hormone cortisol from the fish head-kidney into tissues and blood plasma (Wendelaar Bonga, 1997). Ependymin has a known role in social dominance and hierarchy formation in zebrafish and, when inactivated, clear deviations in behaviour from normal aggression are observed (Sneddon, Schmidt, Fang, & Cossins, 2011). Finally, the GABAA receptor has known effects on the release of ACTH with an inverse correlation between its presence in the brain and levels of stress and aggression (Makara & Stark, 1974; Miczek, Fish, & De Bold, 2003). However, due to low yield of RNA the brains had to be pooled resulting in a low sample size and low power. The editor and anonymous reviewers believed this information may be relevant to future selection of candidates in other studies, therefore, we present this data with the caveat that sample size does need to be increased.

Methods

*RNA extraction and qRT-PCR*

RNA was extracted blind from pooled brains (three brains per sample, therefore each treatment/group-size combination was reduced to n=2; pooling was required due to the small quantity of tissue available per individual) using a phenol-chloroform method. Brains were homogenised in TRIzol reagent (Invitrogen Life Science, UK) using an Ultra-Turrax T25 Basic homogeniser (IKA, Germany). RNA was subsequently isolated using columns (RNEasy Minikit; Qiagen, Germany) with an on-column DNase (Invitrogen Life Science, UK) step, as per manufacturer instructions. RNA was subsequently quantified, and absorbance ratios inspected for purity, using NanoDrop (Thermo Scientific). For each sample ~1µg RNA was reverse-transcribed into cDNA using SuperScript III Reverse-Transcriptase (Invitrogen Life Science, UK) following the manufacturer’s protocol and using random primers (Invitrogen Life Science, UK). Relative quantitation of 0.06µg cDNA was accomplished using FAST RT-qPCR (Applied Biosystems 7500 FAST RT-qPCR) with Fast SYBR Green dye (Invitrogen Life Science, UK). Samples were combined with Fast SYBR Green dye and 2pmol each of forward and reverse primer (Table A1) in a 10µl reaction volume in MicroAmp Fast Optical 96-well reaction plates (Thermo Scientific) sealed with MicroAmp optical adhesive film (Thermo Scientific). RT-qPCR consisted of a holding stage [10min at 95°C], followed by 44x [3s at 95°C, 45s at 60°C] and a final denaturation step [15s at 95°C, 60s at 60°C, 15s at 95°C, 15s at 60°C]. All plates were run with NTCs, and all samples run in triplicate. For quantitation Cq was determined using 7500 Software (Applied Biosystems, v.2.0.6). Sample Cq was inspected, and outliers removed to improve precision; subsequently, mean coefficient of variation was calculated on 2-Cq for all samples as a measure of overall precision for the study.

*Statistical analysis*

Expression of genes was compared between group size and treatment, and their interaction, using ANOVA. Gene expression was taken as ΔCt (Cttarget – Ctreference) where Ctreference was calculated as the geometric mean of the Ct of both reference genes (elf1α and rpl13). *Post-hoc* comparisons were made with Tukey HSD tests using the agricolae package (De Mendiburu, 2015) in R (v.3.2.3; (R Core Team, 2015)). The low sample size for this means the power of this analysis is low however, gene differences may be indicative of changes in stress level.

Results

*Gene Expression*

Mean CV across all samples was 12.86%, indicating very low intra-assay variability. There were no significant differences in the expression of CRF either between group size or treatment, or their interaction (Table A2). For POMC, there was a weak trend for group size on expression but neither treatment nor the interaction of group size and treatment were significant (Table A2). Expression of ependymin significantly differed between group sizes (Table A2): expression did not differ between single and paired fish, but there was a mean Ct difference of 1.96 (equivalent to a mean fold change of 10.62) between single fish and fish held in a group (Figure A1). Finally, there was a significant interaction of treatment and group size influencing the expression of GABAA (Table A2). Expression was greatest among Control and Anaesthetised treated fish held in a group and lowest among the same treatments when held singly or in pairs; fish exposed to a Fin Clip had intermediate expression levels of GABAA in comparison (Figure A2).

**Tentative Discussion**

Expression of both GABAA and ependymin was greatest in group-held fish; for ependymin expression there was no effect of treatment, but intermediate expression of GABAA was observed among fish exposed to the fin clip. In contrast, CRF and POMC were seen to have no strong significant differences between group sizes or treatments.

Two of the genes under investigation in this study, CRF and POMC, have well established roles in the hypothalamus-pituitary-interrenal axis (Flik, Klaren, Van den Burg, Metz, & Huising, 2006) but did not differ significantly between treatments or group sizes. CRF initiates the stress hormone cascade upon experiencing a stressful stimulus and POMC is a polypeptide precursor to other hormones within this cascade, as such they ultimately result in the production and release of cortisol from the fish head kidney (Wendelaar Bonga, 1997). To prevent over expression cortisol is known to suppress production via a negative feedback loop with further control occurring in the pituitary gland. A cleavage product of POMC, ACTH, is thought to be a primary candidate, expressed early in the stress axis, in regulating cortisol expression (Wendelaar Bonga, 1997). With this being the case it is likely that the negative feedback and self-suppression of cortisol production within the hypothalamus-pituitary-interrenal axis has resulted in diminished expression of these genes upon sampling, three hours after experiencing the stressor. Perhaps if sampling was conducted sooner or if sample size was increased we may see small but important differences in expression of CRF and POMC but as these are the initial mediators of the stress response this may only be seen at an early time point. Control and Anaesthetised fish held singly or in pairs had the lowest expression levels of GABAA, these same treatments showed the highest expression in group held individuals. GABAA has inhibitory effects on the production of ACTH therefore there is an inverse relationship between its presence in the brain and levels of stress and stressful behaviours (Makara & Stark, 1974). The increased expression witnessed in group housed fish would suggest that these individuals are experiencing lower levels of stress in these treatments than their counterparts held singly or in pairs. Intermediate levels of GABAA were recorded for individuals in all group sizes exposed to the Fin Clip treatment where they may have been experiencing stress and pain. The differences witnessed between instances of stress and pain can be explained by the fact that the physiological response to these stimuli elicit actions in different areas of the brain in teleost fish; stress activates the hypothalamus-pituitary-interrenal axis whereas pain stimulates areas of the fore-, mid- and hind-brain (Sneddon, 2015). Finally, levels of ependymin were not seen to be effected by treatment suggesting that stress is not affecting its production; however, gene expression was seen to differ between group sizes. Additionally to having a role in coping with environmental stress (Tang et al., 1999) ependymin is believed to be important in establishing dominance hierarchies (Sneddon et al., 2011), hence it is not surprising that increased levels are found in group housed fish where social status will be dependent upon maintaining rank within a hierarchy. These results should be accepted with caution since we had low biological replication, however, samples were run in triplicate and the variation is very low. Future studies should repeat these analyses with a higher sample size, however, the results do point to useful candidates for fuelling further research on stress responses in zebrafish.

**Table A1:** Genes, accession numbers and primer sequences utilised in the study for *Danio rerio*.

|  |  |  |  |
| --- | --- | --- | --- |
| **Gene** | **Accession #** |  | **Primer** |
| 1Ependymin | NM\_131005 |  | F: | CAG TGG AAC GAT GAA AGT GG |
|  |  |  | R: | TGG TGT CCT CAA CAA AAC GA |
|  |  |  |  |  |
| 2GABAA | FJ915065 |  | F: | TCA GGC AGA GCT GGA AGG AT |
|  |  |  | R: | TGC CGT TGT GGA AGA ACG T |
|  |  |  |  |  |
| 3POMC | AY125332 |  | F: | CAG AGA TGG TGA GGG GAG TGA GGA TGT TGT GT |
|  |  |  | R: | CTT AAA GCC ACT ACA TCA TCC TTC CTC GGT TG |
|  |  |  |  |  |
| 4CRF | BC164878 |  | F: | CGA GAC ATC CCA GTA TCC AAA AAG |
|  |  |  | R: | TCC AAC AGA CGC TGC GTT AA |
|  |  |  |  |  |
| 4elf1α \* | AY422992 |  | F: | CTG GAG GCC AGC TCA AAC AT |
|  |  |  | R: | TCA AGA AGA GTA GTA CCG CTA GCA TTA C |
|  |  |  |  |  |
| 4rpl13 \* | NM\_212784 |  | F: | TCT GGA GGA CTG TAA GAG GTA TGC |
|  |  |  | R: | AGA CGC ACA ATC TTG AGA GCA G |

\* indicates reference genes. Where indicated with a number in superscript primers were obtained from previous studies. 1(Huang & Huang, 2011); 2(Hortopan, Dinday, & Baraban, 2010); 3 (Hansen et al., 2003); 4(Manuel et al., 2015).

**Table A2:** Results from ANOVA of comparisons in relative gene expression for CRF, POMC, ependymin and GABAA.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Gene** | **Comparison** | ***F*** | ***P*** | ***df*** |
| **CRF** | Group Size | 1.355 | 0.306 | 2 |
|  | Treatment | 0.434 | 0.661 | 2 |
|  | Group Size × Treatment | 0.574 | 0.689 | 4 |
|  |  |  |  |  |
| **POMC** | Group Size | 3.830 | 0.063 | 2 |
|  | Treatment | 0.488 | 0.629 | 2 |
|  | Group Size × Treatment | 0.497 | 0.739 | 4 |
|  |  |  |  |  |
| **Ependymin** | Group Size | **4.438** | **0.046** | 2 |
|  | Treatment | 0.061 | 0.942 | 2 |
|  | Group Size × Treatment | 1.362 | 0.321 | 4 |
|  |  |  |  |  |
| **GABAA** | Group Size | **8.184** | **0.009** | 2 |
|  | Treatment | 0.437 | 0.659 | 2 |
|  | Group Size × Treatment | **6.069** | **0.012** | 4 |

For each gene, error degrees of freedom = 9. Significant effects are in bold.

**Figure 1**: Mean ± SD (a) number of erratic movements and (b) time spent in the bottom third of the tank by zebrafish at pre-treatment (time = 0 hours) and 1-3 hours post-treatment (control: empty circle, solid line; anaesthesia, black triangle, dashed line; fin clip under anaesthesia: black square, dotted line). Fish were held either individually (left column), in pairs (middle column) or in a group (right column). *N* = 6 per group size per treatment. Symbols above a set of points indicate at least one of the anaesthetic or fin clip treatments resulted in behaviour significantly different to control fish (*post hoc* two sample *t* test), and symbols next to a point indicates behaviour significantly different to time = 0 for that treatment (*post hoc* paired *t* test). In both cases, † indicates *p* < 0.05, \* indicates a significant difference after correction for multiple tests using the Holm-Bonferroni procedure.

**Figure 2**: a) Change in mean ± SD log-transformed cortisol concentrations (ng g-1 hr-1 ) in water of tanks containing zebrafish over time following exposure to one of three treatments (control, anaesthesia or fin clip under anaesthesia ). \* above a set of points indicate at least one of the anaesthetic or fin clip treatments resulted in behaviour significantly different to control fish (*p* < 0.05, two-sample *t* tests with Holm-Bonferroni correction), and \* next to a point indicates behaviour significantly different to time = 0 for that treatment (*P* < 0.05, paired *t* tests with Holm-Bonferroni correction). b) Median log-transformed cortisol concentrations (ng g-1 hr-1) for fish held either as individuals, in pairs or in groups (6 fish). Boxes represent interquartile range, whiskers indicate 10th and 90th percentiles, dots indicate outliers. Boxes which do not share a letter are significantly different (*p* < 0.05).

**Figure 3:** Median log-transformed concentration of whole body cortisol (ng g-1) in zebrafish (a) held either individually, in pairs or in groups (*N*=6 fish) or (b) and having undergone one of three treatments: Control, Anaesthetic or Fin Clip (*N* = 6 per treatment). Boxes indicate interquartile range, whiskers indicate 10th and 90th percentiles, dots indicate outliers. Boxes that share a letter were not significantly different (Tukey HSD test).

**Figure 4:** Correlation between zebrafish whole body cortisol (ng/g) and water-borne cortisol (ng/g/h) collected from tank water using the siphon method. Each point indicates an individual fish for which both whole body and water-borne cortisol was measured. Dashed lines represent 95% prediction intervals. (Pearson’s coefficient = 0.967).

**Figure A1:** Median relative expression of ependymin (calculated as ΔCt: Cttarget - Ctreference) in zebrafish, *Danio rerio* held either singly, pairs or in groups (6 fish). Boxes indicate interquartile range, whiskers indicate 10thand 90th percentiles. Boxes that share a letter were not significantly different (Tukey HSD test).

**Figure A2:** Median relative expression of GABAA (calculated as ΔCt: Cttarget - Ctreference) in zebrafish, *Danio rerio*, under control (white), anaesthetic (light grey) and fin clip (dark grey) treatments. Fish were held either singly, pairs or in groups (6 fish). Boxes that share a letter were not significantly different (Tukey HSD test).