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1 **Acute Stress Alters the Rates of Degradation of Cardiac Muscle Proteins**

2 Bethany Geary^a, Kieran Magee^b, Phillip Cash^c, Holger Husi^a, Iain S. Young^{b,1}, Phillip D. Whitfield^{a,1} Mary
3 K. Doherty^{a,*},¹

4

5 ^aDepartment of Diabetes and Cardiovascular Science, University of the Highlands and Islands,
6 Inverness, UK

7 ^bInstitute of Integrative Biology, University of Liverpool, Liverpool, UK

8 ^cDivision of Applied Medicine, University of Aberdeen, Aberdeen, UK

9

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11

12 *Corresponding Author: Dr. Mary K. Doherty
13 Department of Diabetes and Cardiovascular Science
14 University of the Highlands and Islands
15 Centre for Health Science
16 Old Perth Road
17 Inverness IV2 3JH

18

19 Email: mary.doherty@uhi.ac.uk

20 Fax: +44-1463-711-245

21

22 ¹Principal investigators of the study.

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26 Keywords: Protein degradation; stress; zebrafish, *Danio rerio*

27 **Abstract**

28 Stressful experiences can have detrimental effects on many aspects of health and wellbeing. The
29 zebrafish (*Danio rerio*) is a widely used model for stress research and a stress phenotype can be
30 induced by manipulating the environmental conditions and social interactions. In this study we have
31 combined a zebrafish stress model with the measurement of degradation rates of soluble cardiac
32 muscle proteins. The results showed that the greater the stress response in the zebrafish the lower
33 the level of overall protein degradation. On comparing the rates of degradation for individual proteins
34 it was found that four main pathways were altered in response to stress conditions with decreased
35 degradation for proteins involved in glucose metabolism, gluconeogenesis, the ubiquitin-proteasome
36 system (UPS) and peroxisomal proliferator-activated receptor (PPAR) signalling pathways. Taken
37 together, these data indicate that under stress conditions zebrafish preserve cardiac muscle proteins
38 required for the 'fight or flight' response together with proteins that play a role in stress mitigation.

39 **1. Introduction**

40 Stress is a composite, multidimensional concept that has complex physiological as well as behavioural
41 effects. A universal definition of stress remains elusive but can be considered as the biological
42 response elicited by an organism to a perceived threat to its homeostasis. The activation of stress
43 pathways is an initial adaptive response and whilst this can be beneficial, increasing survival chances,
44 intense acute or chronic stress may lead to maladaptive responses that are detrimental in the long
45 term [1]. Indeed, a normal stress response is required for healthy living but persistent stressful
46 experiences can manifest in a range of physical and psychological conditions [2, 3].

47

48 The zebrafish (*Danio rerio*) is an established model organism in biomedical research, its small size, low
49 maintenance and fast reproduction having the advantage of reducing both the time and cost of
50 experimental programmes. The zebrafish is increasingly being employed in studies that are focussed
51 on understanding the biological mechanisms of human stress and stress-related disorders [4-8].
52 However, like any model, the zebrafish has some limitations. There are distinct differences between
53 zebrafish and humans, for example, zebrafish live in an aquatic environment, have a duplicated
54 genome and certain areas of the brain are less developed [9]. Nonetheless, there are strong parallels
55 in terms of the organisation and function of the stress response systems. In particular the
56 hypothalamic-pituitary-adrenal (HPA) axis in humans and its analogue the hypothalamic-pituitary-
57 interrenal (HPI) axis in zebrafish are evolutionarily conserved and share extensive homologies [10,11].

58

59 Stress responses can be generated in zebrafish by a range of stimuli that include physical stressors
60 (e.g. capture and confinement), environmental stressors (e.g. changes in water temperature) and
61 psychological stressors (e.g. predator cues and overcrowding). The stress response in fish involves a
62 complex cascade of biochemical and physiological changes that can be broadly categorised into
63 primary, secondary and tertiary responses [12]. In the primary response the perception of an altered
64 state results in the release of catecholamines (adrenaline and noradrenaline) and corticosteroids

65 (cortisol) [13,14]. In the secondary response various metabolic pathways are activated leading to the
66 mobilization of glycogen reserves and an elevation of circulating glucose that serves as a substrate to
67 meet increased energy demands from tissues. Similarly, there are adjustments to haematological
68 factors and hydromineral balance [15]. The tertiary response represents the impact on whole animal
69 performance characteristics and modification of behaviour [16].

70

71 Stressful conditions also elicit a generalised response at the level of the cell. A well-defined feature of
72 this response is the production of heat shock proteins (HSPs), a family of molecular chaperones that
73 play an important role in protein refolding and preventing protein aggregation [17]. Another key
74 process during cellular stress is protein degradation. One of the primary pathways of intracellular
75 protein degradation, the ubiquitin-proteasome system (UPS) has been implicated in sensing, signalling
76 and mediating the stress response [18]. By either promoting or inhibiting the degradation of proteins
77 the cell can rapidly maintain essential functions and limit non-critical activities.

78

79 Researchers are now beginning to utilise proteomics as a tool to investigate the molecular responses
80 to stress in zebrafish models. Magdeldin and colleagues [19] showed that elevated levels of anxiety in
81 zebrafish produces an up-regulation of HSPs, metabolic enzymes and cation transporters along with
82 structural proteins associated with locomotive activity. Further, differential expression of proteins
83 involved in mitochondrial function, hypoxia and oxidative stress has been observed in the brains of
84 zebrafish subjected to chronic unpredictable stress (CUS) [20]. The heart is also susceptible to stress
85 and recently it has been reported that exposure to short periods of stress can impair the natural
86 capacity of heart regeneration in the zebrafish [21]. We have previously developed an advanced
87 proteomics methodology to measure the absolute rates of protein turnover in the zebrafish heart,
88 which involves the dietary administration of a stable isotope-labelled amino acid and subsequent
89 analysis of protein populations by high resolution LC-MS/MS [22]. In the current study we have applied
90 this approach to determine the effect of acute stress on the degradation of zebrafish cardiac muscle

91 proteins. Our findings have enabled changes to pathways resulting from stress responses to be
92 mapped at the protein level.

93 **2. Materials and methods**

94 *2.1 Experimental conditions*

95 Zebrafish were maintained in the University of Liverpool aquarium at 28°C +/-1°C (at pH 7.6) on a 12h
96 light:12h dark photoperiod throughout the study. Zebrafish were housed in identical 25 cm x 11 cm x
97 15 cm zebrafish tanks (Pentair Aquatic Ecosystems, Manchester, UK). Daily checks were carried out
98 for pH, ammonia, nitrite and nitrate levels, with desired levels being pH 7.6, 0 mg/l, 0 mg/l and <20
99 mg/l respectively. Weekly water changes replaced at least 20% of the water of the system.

100

101 For stress studies, zebrafish were split into three study groups (n=10 per condition): barren and
102 grouped and enriched. The barren fish were kept in individual tanks where they could not see any
103 other fish and the tanks were otherwise empty. The grouped fish were all kept in a single tank with
104 gravel and plant life as a group. The enriched fish were kept in individual tanks where they had the
105 ability to see other fish. The tanks also contained synthetic gravel (>5 mm) and plant life. One plant
106 type ('submerged plant') simulated broad-leaved bright green submerged and rooted aquatic
107 vegetation with a black resin root base, while the other ('overhanging plant', green Supa Fern®) had
108 no root base, resembling overhanging ferns or similar unrooted fine-leaved vegetation to provide
109 overhead cover. The temperature, light cycle and feeding rate were kept constant throughout the
110 experiment. The fish were observed at least three times daily, at morning and afternoon feeds then
111 at approximately 5pm. Fish from all of the experimental groups were fed a diet in which 50% of the L-
112 leucine (that proportion added as crystalline amino acid) was replaced with [²H₇]-L-leucine (98%
113 purity) (Cambridge Isotope Laboratories, Andover, MA, USA) [22] for six weeks prior to the
114 commencement of the study. At the experimental start (t=0) the diet was changed to an unlabelled
115 diet and five fish immediately sacrificed weighed and dissected. After two weeks of stress exposure,
116 five additional fish from each study group were sacrificed. No fish sustained injuries during the
117 behavioural assays. Fish were killed in accordance with UK Home Office Schedule One regulations.
118 This research was approved by the Ethics Committee at the University of Liverpool.

119 *2.2 Measurement of cortisol in tank water*

120 Water was removed from three of the tanks used for the barren and enriched fish and the single tank
121 for the group of fish. Particulate matter removed prior to loading onto a SepPak solid phase extraction
122 cartridge (Sep-Pak C18, 500 mg sorbent per cartridge, Waters, Manchester, UK) using a reservoir
123 attachment. The cortisol fraction was eluted with 2 ml methanol at a flow rate of 1 ml/min. The eluate
124 was then concentrated under vacuum using a MiVac Concentrator (Genevac, Ipswich, UK) to a volume
125 of approximately 300 µl.

126

127 The concentration of the cortisol was measured using an enzyme-linked immunosorbant assay (ELISA)
128 (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. Briefly, 100
129 µl of each sample were pipetted into individual wells of a 96-well plate and 50 µl of assay buffer (Tris-
130 buffered saline) added. Alkaline phosphatase conjugated to cortisol (50 µl) was aliquoted into each
131 well, followed by 50 µl of a mouse monoclonal anti-cortisol antibody. The plate was incubated at room
132 temperature for 2h after which the contents of were discarded and each well washed with 400 µl of
133 wash solution (Tris-buffered saline plus detergent). 200 µl of the substrate solution p-nitrophenyl
134 phosphate in buffer) were added and the plate incubated at room temperature for 1 h. 50 µl of
135 trisodium phosphate in water were then added to each well. The optical density was measured at 405
136 nm using a Varioskan plate reader (Thermo, Hemel Hempstead, UK) and the concentration of cortisol
137 determined from a standard curve. The statistical difference of cortisol concentrations between
138 barren and enriched groups was determined using a student's t-test.

139

140

141 *2.3 Zebrafish heart preparation*

142 Immediately after sacrifice, fish were weighed and the heart of each fish was dissected within
143 approximately 10 seconds. The heart was then rinsed in ice cold phosphate buffered saline then placed
144 in an Eppendorf tube and stored at -80°C until analysed. The heart samples (approximately 1 mg wet

145 weight of tissue) were mechanically homogenised in 500 µl of 1X phosphate buffered saline
146 (Invitrogen, Carlsbad, United States) containing Complete Protease Inhibitors (Roche, Lewes, UK). The
147 homogenate was centrifuged at 19,000 x *g* at 4°C for 45 min and the supernatant collected. The
148 protein concentration of the supernatant was determined using the Coomassie Plus Protein Assay
149 (Pierce Biotechnology, Rockford, IL, USA).

150

151 *2.4 1-D SDS-PAGE*

152 The soluble proteins (20 µg) from zebrafish heart were separated by 1-D SDS-PAGE using a Mini
153 Protean Tetra system (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Samples were
154 electrophoresed at a constant potential of 200 V through a 15% w/v polyacrylamide resolving gel with
155 a 4% w/v stacking gel. Samples were incubated at 95°C for 5 min in a reducing buffer (125 mM Tris-
156 HCl; 140 mM SDS; 20% v/v glycerol; 200 mM DTT and 30 mM bromophenol blue) prior to loading. Gels
157 were stained with Coomassie Blue (Bio-Rad).

158

159 *2.5 In-gel digestion*

160 Gel lanes were cut into 12 slices and each slice placed in distilled deionised water (50 µl). The water
161 was then removed and the gel piece was treated with destain solution (10 µl of ACN/100 mM
162 ammonium bicarbonate 1:1 v/v). The protein disulphide bonds were reduced by the addition of
163 dithiothreitol (20 µl of 10 mM for 30 min) and alkylated by iodoacetamide (20 µl of 55mM for 30 min
164 incubation in the dark). Each gel slice was dehydrated in acetonitrile. Trypsin (Roche) (0.2 µg/µl in 50
165 mM acetic acid) was added at a ratio of protein:trypsin 50:1 and the digestion allowed to proceed
166 overnight at 37°C. The peptides were then extracted from the gel by addition of acetonitrile and then
167 dried under vacuum in a MiVac concentrator (Genevac, Ipswich, United Kingdom) prior to
168 resuspension in 50% methanol.

169

170 *2.6 Peptide analysis by LC-MS/MS*

171 LC-MS/MS analysis of peptides was performed in positive ion mode using a Thermo LTQ-Orbitrap XL
172 LC-MSⁿ mass spectrometer equipped with a nanospray source and interfaced to a Waters nanoAcquity
173 ultra performance liquid chromatography (UPLC) system. The samples (5 µl) were initially desalted
174 and concentrated on a 5µm Symmetry C18 180 µm x 20 mm trapping column (Waters, Milford, MA,
175 USA). The peptides were then separated on a BEH C18 nanocolumn (1.7 µm, 75 µm x 250 mm,
176 Waters). Mobile phase A comprised 0.1% formic acid in water and mobile phase B comprised 0.1%
177 formic acid in acetonitrile (all Fisher Scientific, Loughborough, UK). A gradient of 10-40% acetonitrile
178 over 120 min was employed with a flow rate of 400 nl/min. Peptides were ionised using a PicoTip
179 emitter (New Objective, Woburn, MA, USA) at 3.5 kV source voltage. Acquisition was in data-
180 dependent mode over the range m/z 300-2000 with the top 10 ions being fragmented using the lock
181 mass setting for increased accuracy and comparability. Dynamic exclusion settings allowed a single
182 repeat with a duration of 30 s, keeping a list of 500 ions. Charge state screening was enabled, rejecting
183 unassigned and single positive charge states.

184

185 *2.7 Proteomic data analysis*

186 Proteomic data were analysed using MaxQUANT with the Andromeda search engine [23,24]. The
187 initial search parameters allowed for two trypsin missed cleavages, carbamidomethyl modification of
188 cysteine residues, oxidation of methionine and acetylation of N-terminal peptides and a false
189 discovery rate (FDR) of 0.01. A mass tolerance of 20 ppm for the precursor ion first search and a
190 tolerance of 6ppm for main search were allowed along with a fragment mass tolerance of ± 0.5 Da. A
191 maximum 1% false discovery rate was used for both protein and peptide identification. Protein
192 identifications were made from a minimum of two peptides per protein including at least one unique
193 peptide. Identified contaminants were removed. An additional parameter, coded as a pseudo post-
194 translational modification was included to search for peptides containing [²H₇] L-leucine.

195

196 *2.8 Calculation of protein degradation rates*

197 The relative isotope abundance (RIA) of the precursor pool of soluble cardiac muscle was determined
198 using peptides containing two leucine residues from a range of different proteins. These proteins were
199 taken from the list of identified proteins and covered a range of molecular weights. Our previous work
200 has shown good agreement between data derived from di- and tri-leucine peptides As the signal to
201 noise ratio (S/N) for the di-leucine peptides is in general more favourable, we focussed solely on these
202 peptides for determination of RIA. Once the precursor RIA was calculated it was used to deconvolute
203 the peptide ion intensity from mono-leucine peptides to pre-existing 'old' protein and newly
204 synthesised protein. This relative partition of intensity over time was calculated over time, allowing
205 the rates of degradation of each protein to be determined. The statistical differences in protein
206 degradation rates between the experimental groups was determined using non-parametric analysis
207 of the data (Mann Whitney for paired analysis, Kruskal-Wallis to compare all three groups).

208

209 *2.9 Gene ontology cluster analysis*

210 Ontologies from GO, KEGG and Reactome were clustered and statistically evaluated using ClueGO
211 (v2.3.3) and CluePedia (v1.3.3) within the Cytoscape (v3.5.0) environment. Input were UniProt
212 accession numbers, and the species reference database was Zebrafish (ClueGO mapped date
213 17.11.2016). Thresholding parameters were set to use GO term fusion (hierarchical combined
214 grouping of molecular function, biological process and cellular component), only to include pathways
215 with a statistical significance $p \leq 0.05$, and kappa score 0.3. Term enrichment used a two sided-
216 hypergeometric test including Bonferroni correction for multiple testing. CluePedia was used to
217 associate the genes from the input list to the specific terms. Group comparisons were performed using
218 either "barren" or "grouped" as input for group 1, and "enriched" for group 2.

219 **3. Results**

220 *3.1 The stressed phenotype in the zebrafish*

221 In this study three groups of zebrafish were subjected to barren, grouped or enriched conditions in
222 order to elicit a natural stressed phenotype. The response in the different groups was assessed by
223 measuring cortisol in the tank water, which is widely used as an indicator of stress in fish [25-27]. The
224 mean concentration of cortisol was significantly higher in the zebrafish maintained in a barren
225 environment (6938 pg/ml \pm 812) compared to the enriched environment (3227 pg/ml \pm 504, $p=0.005$).
226 In the single grouped tank the concentration of cortisol was 4663 pg/ml (Figure 1). These data indicate
227 that the zebrafish in the barren environment experienced greater stress than the other groups, a
228 finding entirely consistent with previous work that has demonstrated that zebrafish show a strong
229 preference for enriched conditions [28].

230

231 *3.2 Protein degradation in the zebrafish heart*

232 The zebrafish were maintained for six weeks on the experimental diet containing 50% of leucine in the
233 deuterated form. Throughout the trial all of the zebrafish fed well, remained active and alert. There
234 were no fatalities or evidence of abnormality in the fish. Following LC-MS/MS analysis, peptides
235 containing multiple leucine residues were identified and the RIA of the protein precursor pool
236 determined at each time-point using the equations derived in our previous studies [29, 30]. The RIA
237 was not found to vary between the different experimental conditions (Figure 2).

238

239 The degradation rates of 287 proteins were calculated from two time-points with at least two unique
240 peptides used in the identification (Supplemental Table 1). ClueGO analysis grouped the proteins into
241 fifteen functional families - actin filament depolymerisation, cellular response to oxidative stress,
242 energy derivation by oxidation of organic compounds, gas transport, generation of precursor
243 metabolites and energy, glutathione transferase activity, intermediate filament, one-carbon
244 metabolic process, oxidation-reduction process, oxidoreductase activity- acting on the aldehyde or

245 oxo group of donors, purine ribonucleoside triphosphate metabolic process, response to hypoxia,
246 single-organism catabolic process, small molecule metabolic process and threonine-type
247 endopeptidase activity (Supplemental Figure 1). This broad range of ontologies are representative of
248 the functions of the soluble cardiac muscle proteome.

249

250 The rates of protein degradation across all three conditions ranged from 0 week⁻¹ e.g.
251 phosphoenolpyruvate carboxykinase 2, heat shock protein 5 and parvalbumin-2 (barren); ATP
252 synthase subunit beta and phosphoenolpyruvate carboxykinase 2 (enriched) to 8.8 week⁻¹ for sterol
253 carrier protein 2B (enriched). The mean rate of protein degradation for the barren cohort was 0.52
254 week⁻¹ ± 0.03 (n = 135 proteins) compared to 0.47 week⁻¹ ± 0.02 for the grouped cohort (n = 188) and
255 0.70 ± 0.06 for the enriched cohort (n = 151). It was possible to calculate the degradation rates for 131
256 proteins in at least two of the experimental groups and for 57 proteins across all of the conditions
257 (Figure 4). For these 57 proteins the mean rates of degradation were significantly different (mean k_{deg}
258 barren = 0.54 week⁻¹ ± 0.05; mean k_{deg} grouped = 0.47 week⁻¹ ± 0.05; mean k_{deg} enriched = 0.68 week⁻¹
259 ± 0.04, n = 57 per group) as shown in Figure 3. Pairwise comparisons between the barren and
260 enriched conditions also revealed significant differences in the mean rate of protein degradation
261 (Mann Whitney test, $p = 0.0022$) as did the comparison between the grouped and enriched conditions
262 (Mann Whitney test, $p < 0.001$). There was no significant difference between the barren and grouped
263 conditions. This indicates that the rate of degradation is decreased in zebrafish exposed to stress
264 environments.

265

266 *3.3 Comparison of rates of protein degradation of individual cardiac muscle proteins*

267 The degradation rates of individual proteins were compared across the study group (Figure 5). There
268 were 125 proteins common to both the barren and enriched groups of which 38 showed a decrease
269 in protein degradation in the barren **group compared to the enriched**. These proteins are involved in
270 ATP metabolic processes, threonine-type endopeptidase activity (proteasome activity) and the

271 peroxisomal proliferator-activated receptor (PPAR) signalling pathway were specifically enriched in
272 those proteins where there was (Figure 6A). Conversely, 13 proteins showed an increased rate of
273 degradation in the barren cohort compared to the enriched group. GO analysis indicated there was
274 no clustering of these proteins. There were 61 proteins common to both the grouped and enriched
275 conditions with 27 proteins showing a decrease in protein degradation and 5 an increase in protein
276 degradation in the grouped condition compared to the enriched. Those proteins with a decrease in
277 degradation rate were clustered by GO analysis to proteins related to response to hypoxia and
278 nicotinamide nucleotide metabolic processes including glucose metabolism, glycolysis and
279 gluconeogenesis (Figure 6B). There were 59 proteins common to the barren and grouped cohorts with
280 24 showing significant changes in protein degradation rate between the groups (13 decreased rates
281 of degradation in the barren group with 11 increased). GO analysis showed no conclusive changes
282 between the cohorts in terms of function of these proteins.

283

284 The data were checked manually to determine whether there were changes in the degradation rates
285 of proteins known to be involved in cellular stress responses. There were no significant changes in rate
286 of degradation between the experimental conditions of oxidative enzymes and transport complexes
287 in mitochondria. We also examined HSPs. The rates of protein degradation for nine HSPs were
288 calculated, four of which were determined in each experimental condition. There was only a
289 significant change in the rate of degradation of HSP5, which had a reduced rate of degradation in both
290 the grouped and barren conditions. Indeed, there was no detectable degradation of this protein under
291 the barren condition at all, which is perhaps reflective of the increase in protein concentration
292 observed in a previous study [19].

293 **4. Discussion**

294 Changing the environment of zebrafish has previously been shown to alter their behaviour and induce
295 a stress response [31]. Fish tend to prefer living in groups and with an enriched environment e.g.
296 access to plant life. Fish housed individually with no enrichment show increased anxiety-like
297 behaviours [32]. Fish housed in groups also show preference for specific types of enrichment [28]. In
298 this study we have utilised well-established methods of isolation and decreased stimulation to model
299 stress in the zebrafish. The measurement of cortisol in the tank water revealed that the zebrafish
300 housed in a barren environment were more stressed than those fish maintained in the enriched
301 environments. In addition, it is known from previous studies that fish maintained in a group but with
302 no access to plant and gravel (the enrichment) will migrate to an enriched environment when possible
303 [33]. Although the cortisol value for our grouped cohort could not be included in the statistical
304 evaluation (n=1), it did indicate that these fish were more stressed than those maintained in an
305 enriched environment and less stressed than those kept individually in the barren environment.

306
307 We subsequently measured the rates of degradation of soluble cardiac muscle proteins from each
308 cohort of zebrafish. At a global level, protein degradation was found to decline as the stress response
309 increased. This indicates that the more stressed zebrafish were degrading proteins at a slower rate
310 than the less stressed fish. At an individual protein level however, both increased and decreased
311 protein degradation was observed between the different experimental groups. Four pathways were
312 highlighted as having decreased degradation in the stressed phenotype compared to the non-stressed
313 zebrafish. In both the barren and grouped conditions, there was a decrease in degradation of proteins
314 involved in metabolic processes including glucose metabolism, glycogenolysis and gluconeogenesis
315 and nicotinamide nucleotide metabolic processes. In the immediate stress response there is an
316 activation of the neuroendocrine system. The subsequent release of stress hormones results in
317 elevated blood glucose concentrations and stimulates the catabolic processes involved in the
318 liberation of amino acids and other substrates for gluconeogenesis [15]. An increase in glucose

319 concentration would be useful for the anticipated increase in energy demand, required by cardiac
320 muscle as part of a fight or flight response. However it should be noted that not all proteins involved
321 in energy production show a decreased degradation under stressed conditions. This includes enoyl
322 CoA hydratase, which catalyses the second step of β -oxidation showed an increased rate of protein
323 degradation in fish maintained under barren conditions. This may reflect the utilisation of different
324 pathways for energy generation under stressed conditions, with a preference for glucose as a resource
325 over fatty acids.

326

327 A further functional grouping that showed statistically significant changes in protein degradation in
328 response to stress were proteins associated with PPAR signalling. Again, the rate of protein
329 degradation was found to be lower in zebrafish with the most stressed phenotype. PPAR proteins are
330 a nuclear hormone receptor superfamily of ligand-activated transcription factors [34,35]. Previous
331 studies have implicated both PPAR α and PPAR γ as having a potential role in regulating the stress
332 response [36]. Exposure to stress has been found to increase expression and activity of PPAR γ in the
333 brain that may affect acute neuronal responses [37,38]. PPAR α regulates genes involved in lipid
334 metabolism with a defined role in normal cardiac metabolic homeostasis. It controls the expression of
335 enzymes involved in fatty acid β -oxidation and has been identified as participating in cardiac p38
336 kinase-dependent stress-activated signalling [39]. PPAR α is proposed to act as a link between
337 extracellular stressors and alterations in energy metabolism [40]. This would reflect the required shift
338 in metabolism under stressed conditions.

339

340 There was also a decrease in degradation rate of proteins related to the hypoxic responses. A
341 tolerance to hypoxia is a well-defined stress indicator and by maintaining these proteins, the zebrafish
342 would be in a more favourable position to respond to stressor such as predators. The UPS has also
343 been implicated in the response to hypoxia and may act as a co-ordinator of the stress response [18].
344 In the zebrafish that exhibited the most extreme stress phenotype, components of the UPS showed a

345 decreased level of protein degradation suggesting that in a stressed state the cell acts to maintain
346 control and integrity of the system. Proteolysis by the UPS plays an important role in stress response
347 pathways by rapidly degrading any damaged or unwanted proteins and recycling amino acids for new
348 protein synthesis. Once the stress has been detected, cells transduce the signal to ensure that the
349 appropriate countermeasures are switched-on. The stress signal ultimately reaches transcription
350 factors to activate a stress response by modulation of gene expression. The subsequent increase in
351 repair and defence capacities may be sufficient for adaptation to the stress condition.

352 **5. Conclusions**

353 Stressful events elicit a multitude of biochemical changes, which makes it possible to describe the
354 stress reaction in molecular terms. Protein degradation is a major tool that an organism can use to
355 maintain cellular homeostasis during times of stress. In this study we induced a stress phenotype in
356 zebrafish by manipulating their local environment and ability to interact with other fish. The rate of
357 degradation of soluble cardiac muscle proteins under each stress condition was measured using stable
358 isotope labelling in conjunction with high resolution mass spectrometric analysis. It was observed that
359 the greater the stress response in the zebrafish the lower the level of overall protein degradation. This
360 suggests that during stress the cell may reduce the basal degradation of proteins and protect specific
361 pathways important to survival. In doing so, the organism conserves both energy and resource to use
362 in a 'danger' situation.

363

364 Detailed GO analysis revealed four primary pathways with a decrease in individual protein degradation
365 during the stressed phenotype. These have all been previously correlated with the stress response be
366 that as part of a 'fight or flight' response or in the regulation pathways linked to stress amelioration.
367 By focussing specifically on the heart, an organ that is often linked to stress-induced dysfunction we
368 have provided additional information into the cardiac stress response at a molecular level. In future
369 work, we intend to further explore these pathways in cellular systems and higher organisms to
370 determine the specific role that protein turnover has to play in mediating the stress response.

371 **Supplementary material**

372 Supporting Figure 1 details the GO analysis of the total protein cohort analysed in this study

373 Supporting Table 1 contains the calculated rates of synthesis of the proteins

374 **Acknowledgement**

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378 the European Social Fund.

379 **Figure Legends**

380

381 **Figure 1. Measurement of cortisol concentrations.** Cortisol was directly measured in water collected
382 from tanks that housed each of the experimental groups of zebrafish. Blue = barren zebrafish; Black =
383 grouped zebrafish; Red = enriched zebrafish. Concentrations in expressed as the mean \pm SEM where
384 appropriate.

385

386 **Figure 2. Calculation of RIA from multi-leucine peptides.** The relative abundance of the dietary-
387 supplemented amino acid [$^2\text{H}_7$] L-leucine in the precursor pool for protein degradation was
388 determined experimentally. Data were obtained from dileucine containing peptides from multiple
389 proteins across the time-course of the experiment. The relative isotope abundance of labelled leucine
390 in cardiac muscle calculated as determined as 0.46 ± 0.01 decreasing to 0.25 ± 0.01 .

391

392 **Figure 3. Comparison of rates of degradation of soluble cardiac proteins in zebrafish maintained in**
393 **enriched, grouped and barren environments.** The degradation rates of 287 proteins were calculated
394 from two time-points with at least two unique peptides used in the identification (A). Proteins shown
395 are Histone H4 (red), heat shock protein 11 (green), proteasome sub-unit β -4 (blue), α -2-HS
396 glycoprotein 2 (pink) and malic enzyme 3 (black). Degradation rates for 57 proteins common to all
397 conditions were compared (B). The intersecting line indicates the median values (k_{deg} barren = 0.47
398 week^{-1} ; k_{deg} grouped = 0.42 week^{-1} ; k_{deg} enriched = 0.71 week^{-1} , $n = 57$ per group). Statistical analysis
399 indicated that there was a difference between the mean rate of protein degradation between the
400 enhanced ('non-stressed') condition and both the barren and grouped ('stressed') conditions.
401 (Kruskal-Wallis test $p = 0.0001$; mean k_{deg} barren = $0.54 \text{ week}^{-1} \pm 0.05$; mean k_{deg} grouped = 0.47 week^{-1}
402 ± 0.05 ; mean k_{deg} enriched = $0.68 \text{ week}^{-1} \pm 0.04$, $n = 57$ per group).

403

404 **Figure 4: Overlap of degradation rate constants between experimental groups.** The degradation
405 rates of proteins from each experimental condition was determined and compared across the sample
406 set. The commonality between experimental groups is shown.

407

408 **Figure 5. Effect of different stress conditions on the absolute rates of degradation of soluble cardiac**
409 **proteins in zebrafish.** The rates of degradation for individual proteins were compared across the
410 experimental cohort. Exemplar plots of four proteins are shown. The significance of the difference
411 between each condition is indicated. For heterogeneous nuclear ribonucleoprotein the significance
412 was – all conditions $p = 0.0029$; barren v grouped $p = 0.0286$; barren v enriched $p = 0.0286$; grouped v
413 enriched $p = 0.2$, for myoglobin - all conditions $p < 0.0001$; barren v grouped $p = 0.0265$; barren v
414 enriched $p < 0.0001$; grouped v enriched $p < 0.0001$, for enoyl CoA hydratase - all conditions $p = 0.0002$;
415 barren v grouped $p = 0.0286$; barren v enriched $p = 0.0286$; grouped v enriched $p = 0.0286$, for malate
416 dehydrogenase - all conditions $p < 0.0001$; barren v grouped $p = 0.6684$; barren v enriched $p < 0.0001$;
417 grouped v enriched $p < 0.0001$.

418

419 **Figure 6. Gene ontology mapping of soluble cardiac protein pathways.** (A) Proteins with an increase
420 in protein degradation in the enriched cohort were compared to the proteins increased in the barren
421 group. GO analysis showed that proteins with an increase in protein degradation in the enriched group
422 compared to the barren one clustered into three main functional groups. (B) Proteins from the
423 enriched cohort with an increase in protein degradation were compared to the grouped zebrafish
424 cohort. GO analysis revealed that molecules with an increase in protein degradation in the enriched
425 group compared to the grouped zebrafish cohort could be clustered into two main functional groups.
426 All proteins shown were specifically up-regulated in the enriched group. Statistical significance for all
427 groups shown is ≤ 0.05 after correction for multiple testing.

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